ICAR Short Course on

Application of Advanced Molecular Methods in Marine Fishery Resource Management, Conservation and Sustainable Mariculture

24th October - 2nd November 2018

Sponsored by

Indian Council of Agricultural Research, New Delhi

Training Manual

Organised by

ICAR-Central Marine Fisheries Research Institute

Post Box No. 1603, Ernakulam North P.O., Kochi-682 018

Kerala, India

Training Manual

ICAR Short course on

Application of advanced molecular methods in marine fishery resource management, conservation and sustainable mariculture

24/10/2018 – 02/11/2018

CMFRI Training Manual Series No. 19/2018
ICAR-Central Marine Fisheries Research Institute

Publisher : **A. Gopalakrishnan**
Director
ICAR-Central Marine Fisheries Research Institute
Ernakulam North P.O., Pin – 682018, Kochi, Kerala

Editors : **Sandhya Sukumaran**
Sumithra T.G.

Editorial Assistance : **Wilson Sebastian**
Neenu Raj
Anjaly Jose
Lakshmi P. Mukundan
Nisha K.

Course Director : **Sandhya Sukumaran**
Senior Scientist
Marine Biotechnology Division

Course Co-Director : **Sumithra T.G.**
Scientist
Marine Biotechnology Division

Cover Design : **Abhilash P.R.,**
Wilson Sebastian

For citing this book
Sukumaran, S. and Sumithra T. G. (eds) (2018). Application of advanced molecular methods in marine fishery resource management, conservation and sustainable mariculture. Training manual. ICAR-Central Marine Fisheries Research Institute, Kochi, INDIA

For citing a chapter
Vijayagopal P. (2018). Nutritional challenges in aquaculture and mariculture In: Sukumaran, S. and Sumithra T. G. (eds) *Application of advanced molecular methods in fishery resource management, conservation and sustainable mariculture*" ICAR-Central Marine Fisheries Research Institute, Kochi, INDIA

ISBN: 978-93-82263-28-9

FOREWORD

Molecular Biology and Biotechnology has undergone incredible progress in this decade mainly due to the rapid advancements in DNA sequencing technologies. Marine biology and fishery science also reaped the fruits of these modern inventions improving our understanding regarding complex adaptations in aquatic organisms. Fish Genetics have evolved into genomics incorporating knowledge about neutral and non-neutral markers. A project called Genome 10k was started by the international community of scientists for sequencing the genome of 10000 vertebrates. Whole genomes of many marine organisms are now available which provided insights into the evolution of many important traits. Transcriptome sequencing provides insights into expressed genes and metagenome sequencing provides information regarding the microbes present in environment. All these technologies are rapid and cost effective. Over years, these technologies provided exciting opportunities for understanding ecology and evolution. Genomic information can also be sustainably utilized to enhance productivity of mariculture activities by selective breeding, genetic improvement and manipulation of economically important traits.

ICAR-Central Marine Fisheries Research Institute has contributed significantly to marine biotechnology research in the country and played a pivotal role in development of marine fisheries sector. The short course on "Application of advanced molecular methods in marine fisheries resource management, conservation and sustainable mariculture" conducted in ICAR-CMFRI from 24th October, 2018 to 2nd November, 2018 is specially designed to provide exposure to various applications of molecular tools in fisheries resource management, conservation of biodiversity and mariculture. I hope this compendium of lectures and protocols will be extremely useful for the participants to effectively utilize the knowledge in their own area of research. Simultaneously, on behalf of ICAR-CMFRI, I warmly welcome all the participants from various institutions and wish them all success in their future endeavors. I am sure that this training will result in new knowledge, collaborations and friendships.

Dr. A. Gopalakrishnan
Director
ICAR-CMFRI

PREFACE

Knowledge about DNA, the building block of life has contributed significantly to the progress of society as a whole. Understanding biological processes at molecular levels has made possible many interventions by us to alter, sustain or regulate the functions of DNA which contributed substantially to the progress of scientific disciplines like medicine, agriculture, veterinary, fishery and many other interdisciplinary sciences. Even though the knowledge about the genetic code has been deciphered in early 1950s the rapid progress of DNA sequencing technologies occurred during this decade. DNA sequencing methods have advanced from first to second, second to third and third to fourth generations. Molecular information has become very vital to any successful fishery management programmes or aquaculture ventures and with this in view a training was organized on " Application of advanced molecular methods in marine fishery resource management, conservation and sustainable mariculture" at ICAR-CMFRI, Kochi from 24th October, 2018 to 2nd November, 2018 on receiving funding support from Indian Council of Agricultural Research.

Most of the important aspects of genetics and genomics that are relevant to marine biology and fisheries are included in this course manual. The themes which are given focus in this course manual are population genetics, population genomics, functional genomics, next generation sequencing methods, transcriptomics, eDNA based estimation of marine biodiversity and metagenomics. In addition to these, topics that are of general interest to the fisheries sector mainly, mainstreaming marine biodiversity, impact of plastic pollution in the ocean to fisheries sector, evolution and interrelationships of teleost fishes and challenges in mariculture are also included in this manual. Laboratory protocols of many advanced techniques and use of software packages are also illustrated. I hope that this compendium of lectures and protocols will be very valuable to those who wish to apply the latest technologies in their respective areas of research.

Sandhya Sukumaran

Course Director

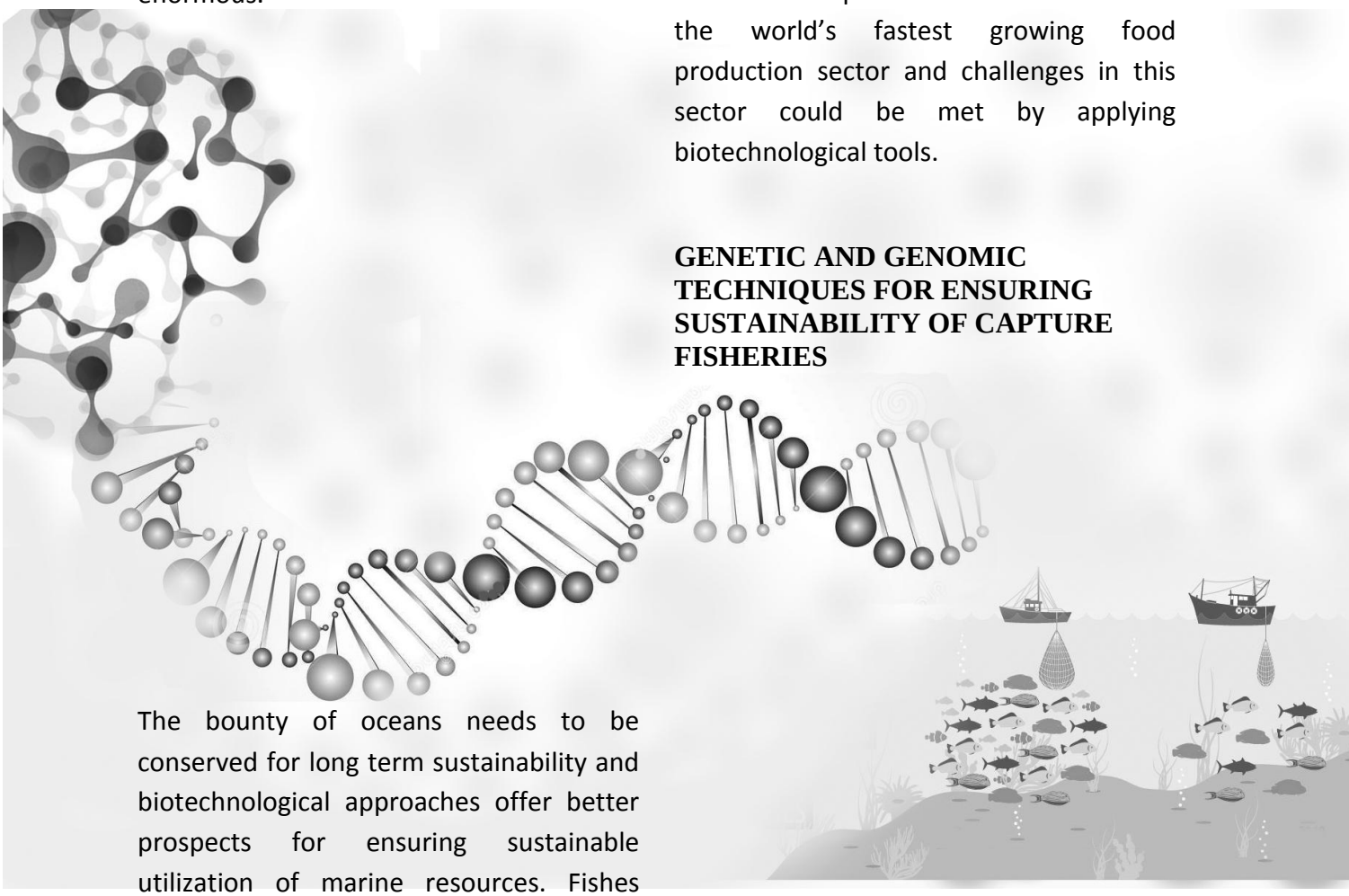
Contents

Importance of biotechnological approaches in marine fishery resource management, conservation and sustainable aquaculture

Sandhya Sukumaran, P. Vijayagopal, A. Gopalakrishnan
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The scientific discipline "Biotechnology" has undergone tremendous advances over the last few decades and the scope of application of biotechnological tools in fisheries and aquaculture sector is enormous.



The bounty of oceans needs to be conserved for long term sustainability and biotechnological approaches offer better prospects for ensuring sustainable utilization of marine resources. Fishes constitute a major source of protein for the millions and ensuring responsible fisherymanagement is the need of the hour to prevent erosion of genetic diversity over time. Aquaculture contributes as equally as capture fisheries to the country's fish production and biotechnological innovations in both capture and culture fisheries will enhance this share. Aquaculture is considered as the world's fastest growing food production sector and challenges in this sector could be met by applying biotechnological tools.

GENETIC AND GENOMIC TECHNIQUES FOR ENSURING SUSTAINABILITY OF CAPTURE FISHERIES

Genetic stock structure assessment

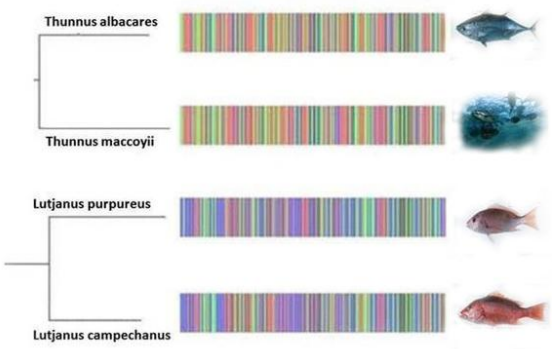
Fish stock structure information is very vital for fishery management plans for ensuring sustainable management of fish resources. Stocks are temporally or spatially discrete units possessing distinct biological characteristics. If distinct subpopulations or stocks occur in a population, they have to be managed separately. Sustainability of stocks also should be assessed separately for devising sustainable fishery management strategies. If a stock is overfished, strategies for fishing or harvesting should be carefully considered and regulatory measures taken. Genetic and genomic technologies are being increasingly used now to understand stock structure and stock boundaries. Traditional morphological methods are often cumbersome and inaccurate.

Mitochondrial genes like cytochrome c oxidase 1, control region (D-loop), cytochrome b, ATPase 6/8 and NADH genes have been widely utilized for understanding stock structure. Mitochondrial genome is haploid, double stranded and maternally inherited which reduces the effective population size of mitochondrial DNA. When effective population size is reduced, genetic differentiation between gene pools is enhanced which makes mitochondrial DNA an attractive marker for studying subpopulation structure. Effective amplification can be carried out using a small amount of tissue as it is present in several copies inside the cell depending on cell type. Nuclear DNA markers like RAPD or Random Amplified Polymorphic DNA make use of a random primer for amplification of genomic DNA which producing several bands which could be compared across populations. Due to the lack of repeatability and reproducibility it is no longer being used now. Microsatellite markers, the regions of genome with repeat units of 1-6base pairs in length have become the marker of choice for population genetic studies due its abundance in the genome, ease of detection and amplification along with specificity. Due to their co-dominant nature, they are inherited in a Mendelian fashion and hence homozygous and heterozygous individuals can be distinguished effectively. They exhibit higher evolutionary rate which is ideal for detecting low genetic stock structure and their non-coding nature assures that variations are independent of natural selection. Single Nucleotide Polymorphisms (SNPs) are point mutations which are found abundantly in the genome. SNPs are the latest markers of choice for many investigations aimed at detecting population level variations.

Molecular taxonomy & DNA Barcoding

Tropical seas are rich in biodiversity and it is very important to ensure that this diversity is protected. Documentation of diversity is the first step to ensure effective protection. Information about spatio-temporal distribution patterns of marine biodiversity and community structure is also important to implement conservation. Cataloguing threatened or endangered species also should be carried

out accurately for which molecular techniques like barcoding can be used. Strains, stocks and hybrids could be identified and characterized using molecular tools effectively.



Mitochondrial Cytochrome C Oxidase 1 gene is used as the universal barcode for species identification and this method has become very popular. The sequences of thousands of organisms are available in NCBI, GenBank data repository and the identity to the particular species sequence can be checked using NCBI, BLAST search. Another online interface which allows researchers to share sequences is Barcode of Life Data System (BoLD). Barcode database of many important fin and shell fishes like tunas, whale sharks, sardines, oysters, mussels and cuttlefishes have been generated by CMFRI. Diagnosing the presence of invasive species and their spread in the ecosystem also can be carried out by DNA barcoding which will help in efficient quarantine and eradication efforts. DNA barcodes could be effectively utilized for identifying predator-prey interactions by tracking prey species, identifying the presence of pest or pathogen species present in the ecosystem, development of species specific markers and a number of other related research works. Presence of eggs and larvae of many species could be identified in advance using DNA barcodes. In mussel mariculture, timing and location of spat fall could be identified using molecular markers.

Aquaculture biotechnology

Aquaculture is very vital to supplement marine fishery production and long term sustainable fishery management can only be ensured with the help of aquaculture activities. Marine fisheries is facing many challenges due to climate change and overfishing and improving the productivity of aquaculture activities by the use of advanced genomic tools is the need of the hour. Aquaculture activities should be carried out sustainably without disturbing the delicate balance of the ecosystem. The performance of aquaculture sector can be improved by the use of superior germ plasm resources and aquaculture biotechnology play a major role in ensuring this. Biotechnological innovations can be sustainably utilized for induced breeding, disease diagnosis and prevention, aquaculture nutrition through application of nutrigenomic tools and genetic improvement of farmed stock through selective breeding.

Quality of the germ plasm can be improved by the use of quantitative genetic tools. Better broodstock of many commercially important fishes can be produced by selective breeding. A proper selective breeding strategy can be selected with adequate knowledge regarding heritability of the trait under

selection, correlations between genotypic and phenotypic characteristics, heterosis and genotype-environment interaction. CMFRI has developed improved strains of Artemia franciscana with altered naupliar size using principles of quantitative genetics. Selective breeding of Rohu, Labeo rohita has been carried out at Central Institute of Freshwater Aquaculture, Bhubaneswar and the variety Jayanti Rohu with accelerated growth has been produced. Marine farming or mariculture is gaining momentum in India with a few species like Cobia, Rachycentron canadum and Pompano, Trachinotus blochii. Selective breeding and genetic improvement can be taken up as a next step to make the production sustainable.

Improvement of crops or livestock can be made by choosing proper inbreeding or cross breeding strategies. Superior offspring with hybrid vigor can be produced by combining inbreeding with crossbreeding. Hybrid vigor can be obtained by many inbreeding and crossbreeding trials.

Chromosomal engineering can be effectively utilized for production of superior quality individuals which show higher growth and reproduction. Methods like androgenesis, gynogenesis and ploidy manipulation have been practiced for production of individuals with better growth and vigor in aquaculture. In androgenesis, reproductive process is manipulated in such a way that only paternal genetic material is inherited. Thus viable YY supermales could be produced when male is heterogametic and successful production of YY supermales has been carried out in cyprinids, cichlids and salmonids. In gynogenesis, only maternal genetic material will be inherited by the progenies so that all female populations can be produced. Androgenesis or gynogenesis will be useful when males or females exhibit superior growth rate and performance.

Ploidy manipulation is another alternative to enhance growth rate where techniques like triploidy or polyploidy can be employed and this will be more useful in shellfishes due to ease of maintenance. Triploidy has been successfully induced in *C. gigas*, *C. virginica*, *Saccostrea glomerata* and *Ostrea edulis*.

Production of all male or female populations can be carried out by hormonal manipulation of sex and reproduction. Synthetically produced analogues of hormones are available in the market which can be used for hormonal manipulation. Induced breeding of freshwater carps was carried out by introduction of hormonal extract containing GnRH which is the key regulator and trigger of reproductive cascade in vertebrates. But, now synthetic hormones like ovaprim and ovatide are available for induced breeding.

Cryopreservation of gametes and embryos in the cold environment to overcome seasonal barriers in reproduction is another major intervention in aquaculture. Cryopreservation offers a better technique for preservation of fish

gametes which can be used for controlled reproduction which will reduce dependence on wild collected seeds. Cryopreservation will also be beneficial in the case of sequential hermaphrodites like seabass or grouper (protandrous/protogynous) as getting males or females from wild for controlled hatchery production is very difficult. Sperm cryopreservation is standardized and widely practiced in fishes whereas ova or embryo cryopreservation is yet to be standardized due to some inherent problems associated with fish eggs and embryo.

Superior genetic stock can be produced with the help of genetic engineering tools. Transgenic tools can be efficiently employed for the production of fishes with faster growth rate, improve environmental tolerance and disease resistance. Antifreeze protein genes, growth hormone genes or fluorescent protein genes can be inserted into the genome of the desired species of fish which subsequently get expressed in the progeny. Preliminary success has been reported in India in developing gene transfer technology in zebra fish, medaka and Indian catfish. Production of genetically modified zebra fish (Glofish) which can produce fluorescent pigments red, green and yellow has been successful and it is a very popular household aquarium pet.

In marker assisted selection (MAS) technique, prospective breeders are chosen based on genotypes with the help of molecular markers. Marker assisted selection programmes can be carried out using molecular markers like allozymes, RFLP, RAPD, AFLP, microsatellite, SNPs, ESTs and mitochondrial DNA. Marker assisted selection is useful for identification of genetic releatednes, diversity, determination of pedigree, genetic tagging, tracking of family and population lines and identification of strains. Identifying markers linked to quantitative trait loci or QTL also could be done with Marker Assisted Selection techniques.

Feed biotechnology and nutrition

Any aquaculture venture will be successful by the incorporation of proper feeds and hence feed biotechnology and nutrition is of utmost importance. The performance of candidate fishes can be improved by fruitful biotechnological interventions in nutritional research. The availability of nutrients in formulated feeds can be enhanced by incorporating enzymes into feeds. These enzymes should have the capacity to withstand variations in physico-chemical parameters like increased temperature conditions and have a long shelf life. Phytase is an enzyme which when incorporated into feeds will help in breaking down of indigestible phytic acid in plant based nutrient sources and thus help in release of digestible phosphorous.

Probiotic bacteria can be incorporated into formulated feeds to improve disease resistance in cultivable fishes. Probiotics consist of live microorganisms that can be incorporated into diets which confer some kind of a health benefit to the host due to

competitive exclusion of pathogenic bacteria. These bacteria are also capable of releasing enzymes that accelerate the digestion of food. Probiotic products are commonly enriched with Aspergillus oryzae, Lactobacillus acidophilus, L. bulgaricus, L. planetarium, Saccharomyces cerevisiae and Bifidobacterium bifidium.

Prebiotics consist of feed for probiotic organisms which are resistant to attack by endogenous enzymes and thus they allow the proliferation of gut microflora. Prebiotics are able to withstand high pelletizing temperatures in the feed and have a long shelf life. The quality of feed can also be enhanced by supplementing dietary amino acids using genetically improved microorganisms. Feed quality can also be improved by incorporating essential amino acids like lysine and methionine into feed. Incorporation of nucleotides into feed as feed additives which will increase the expression of desired traits like growth or disease resistance is also a recent innovation in feed biotechnology. Functional genomics principles can be incorporated into nutrition research by studying the influence of nutrition on an organism at molecular levels.

Fish Health

Health management is very important to ensure sustainability and economic viability of aquaculture ventures. Conventional methods in disease diagnosis are time consuming with limited specificity, sensitivity and speed. Accurate and efficient disease diagnosis is very pertinent to make valuable decisions regarding optimal management strategies. A number of vital, bacterial and fungal diseases occur in finfish and shellfish aquaculture of which white spot viral disease of shrimp P. monodon has been considered as a major threat. Research institutions like CMFRI and CIBA have developed kits for early detection of white spot virus for early detection and prevention of white spot virus.

DNA vaccine administration is another biotechnological intervention which will be helpful in disease prevention and management. DNA vaccines consist of DNA of and infectious organism introduced into a host which gets expressed subsequently in the host.

Phage therapy is used to treat pathogenic bacterial infections by introducing lytic bacteriophages. Bacteria are killed with the help of phage virus and phage must not interact with surrounding tissue or with other beneficial bacteria. The virus is able to replicate quickly and hence a single, small dose should be introduced. Phage therapy is yet to find momentum with its application in aquatic ecosystems.

RNA interference (RNAi) consists of RNA guided regulation of gene expression patterns in eukaryotic cells. Short chains of double stranded ribonucleic acid (dsRNA) which are present in the cell may interfere with the expression of genes which have complementary sequences to this dsRNA. RNA interference is a type of silencing of genes post transcriptional when dsRNA binds to specific mRNA inducing

degradation of the homologous endogenous transcript thus reducing gene activity. RNAi can be considered as a promising and important tool for the management of viral diseases in culture conditions.

Recombinant technology is beneficial to produce genetically modified organisms with altered genetic material. Several research efforts are underway to produce transgenic fishes with disease resistance.

Marine Bioprospecting

Marine bioprospecting involves exploring new sources of chemical compounds, microorganisms, genes and other valuable products from the sea. These biological resources can be exploited in a sustainable way by using biotechnological tools so as to ensure socio-economic development of local communities. Marine organisms can be considered as potential sources of pharmaceuticals, cryoprotectants, cosmaceuticals and neutraceuticals. Many novel drugs can be extracted from marine organisms which may be substitutes to antibiotics. Secondary metabolites of marine bacteria and invertebrates can be a source of anti-inflammatory and anti-cancer agents.

CONTRIBUTIONS OF CMFRI TO MARINE BIOTECHNOLOGICAL RESEARCH

Application of biotechnology in fisheries and aquaculture sector has enormous potential which is in a developmental phase in India. Research in CMFRI has immensely contributed to application of biotechnology in fisheries resource management and aquaculture. CMFRI has carried our pioneering research in understanding genetic variability and stock structure of Indian oil sardine, *Sardinella longiceps*, Indian mackerel, *Rastrelliger kanagurta*, Indian anchovy, *Stolephorus indicus* using mitochondrial and microsatellite markers. Whole mitogenome of several fishes like Indian oil sardine *Sardinella longiceps*, Goldstripe Sardinella, *Sardinella gibbosa*, pearl spot, *Etroplus suratensis* and ribbon fish, *Trichiurus lepturus* has been characterized. Climatic adaptations of Indian oil sardine studied using whole mitogenome scans which revealed the presence of locally adapted populations. Further research is underway to understand genomic adaptations to climate change in Indian oil sardine, *Sardinella longiceps*. Biotechnological interventions in aquaculture led to the development formulated feeds like Varna for ornamental fish rearing and commercialization of several nutraceuticals from marine organisms. Several kits have been developed by CMFRI to detect various diseases of farmed marine fin and shell fishes. Triploid oysters are another biotechnological innovation by CMFRI. Several probiotics have also been developed by CMFRI.

CONCLUSION

India with a coastline of about 7500km, Exclusive Economic Zone of about 2 million square kilometer and vast areas of fresh and brackishwater resources has immense potential to harness the valuable

aquatic resources for a sustainable blue economy. Blue economy aims to tap ocean resources sustainably and biotechnological innovations play a major role in contributing to blue economy. Biotechnology is a multi disciplinary science which requires intergration of biological chemical, engineering and material sciences. A holistic approach involving government and private sector for application of biotechnological tools in emerging areas of fisheries science will bring about rapid advancements which will be beneficial for the society as a whole.

SUGGESTED READINGS

Askari, Gh., Shabani, A., Miandare, H.K. 2013. Application of molecular markers in fisheries and aquaculture. Scientific Journal of Animal Science 2(4): 82-88.

Davis, G.P. and Hetzel, D.J.S.2000. Integrating molecular genetic technology with traditional approaches for genetic improvement in aquaculture species. *Aquaculture Research*, 31, 3-10.

Hallerman, E.M. 2006. Use of molecular tools for research and improvement of aquaculture stocks. *The Israeli Journal of Aquaculture – Bamidgeh* 58(4): 286-296.

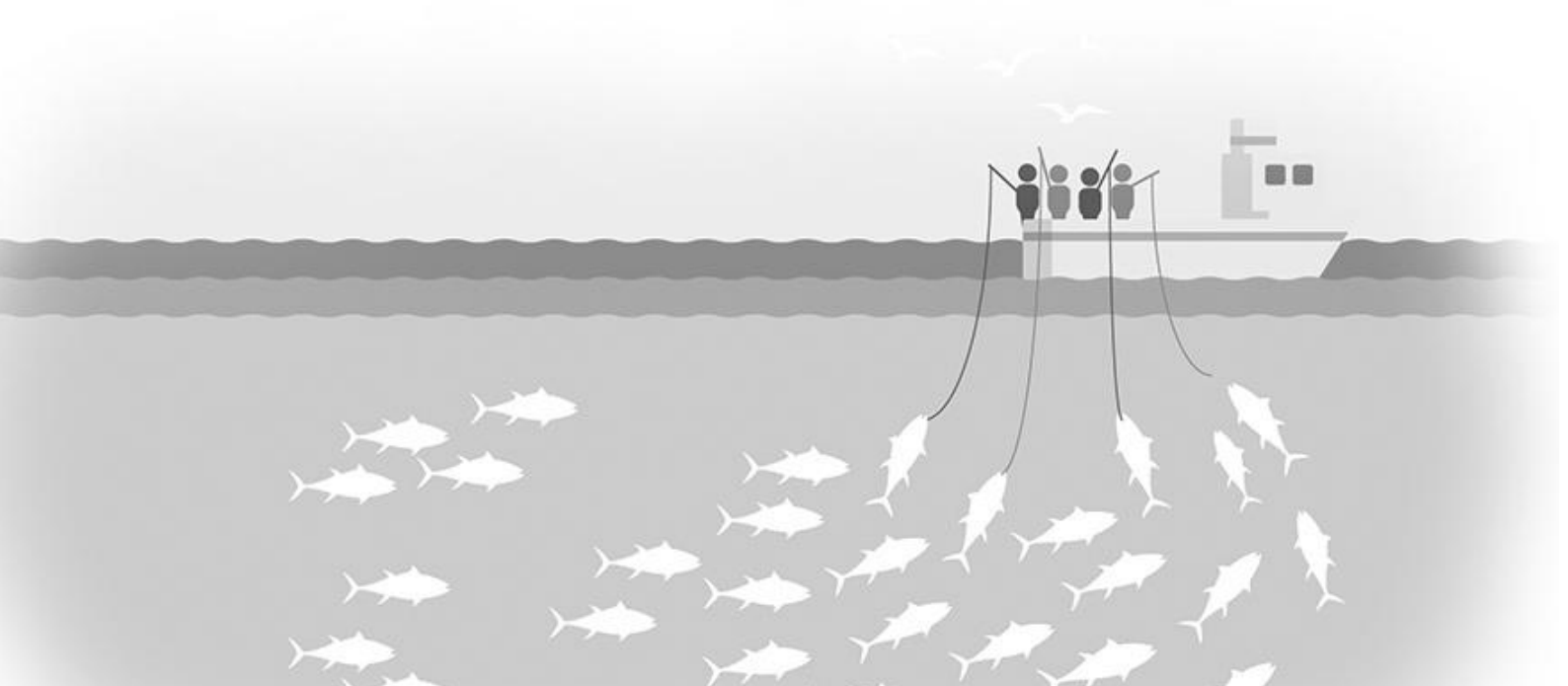
Hernandez-Urcera, J., Vera, M., Magadan, S., Pino-Querido, A., Cal, R.M. and Martinez, P. 2012. Development and validation of a molecular tool for assessing triploidy in turbot (*Scophthalmus maximus*). Aquaculture 330-333: 179-184.

Ken Overturf. 2009. Molecular research in Aquaculture. Wiley-Blackwell .

Okumus, I. and Ciftci, Y. 2003. Fish population genetics and molecular markers:II-Molecular markers and their applications in fisheries and aquaculture. *Turkish Journal of Fisheries and Aquaculture* 3: 51-79.

Presti, R.L., Lisa, C. and Di Stasio, L.2009. Molecular genetics in aquaculture. *Italian Journal of Animal Sciences*. 8: 299-313.

Zheng, J.Y., Zhuang, W., Yi, Y.T., Wu, G., Gong, J. and Shao, H.B. 2013. Developmentally utilizing molecular biological techniques into aquaculture. *Reviews in fisheries science*. http://dx.doi.org./10.1080/10641260903477499.



Mainstreaming Biodiversity: Approaches and Programmes

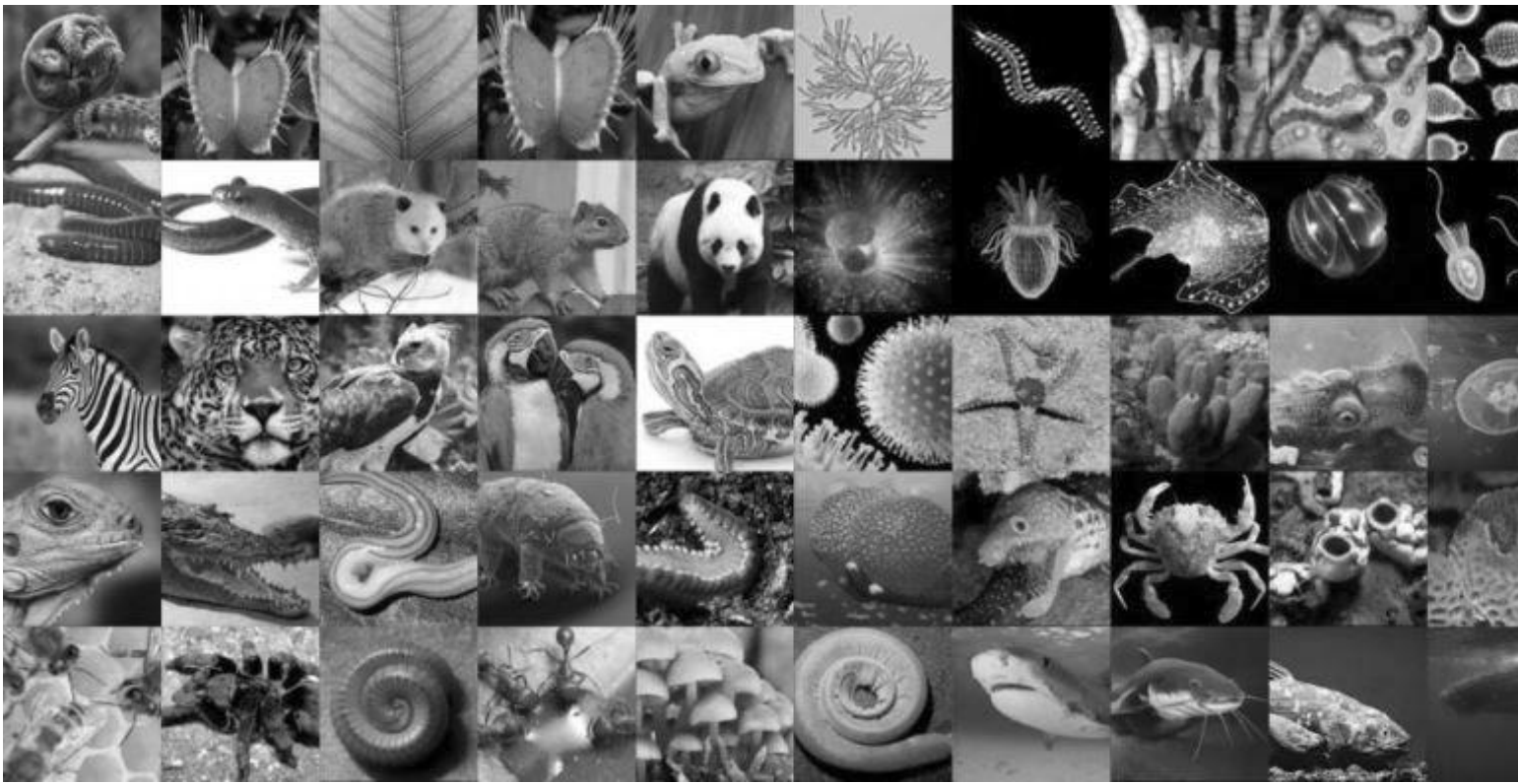
K.K. Joshi, M.S. Varsha, P.A. Tobias
Marine Biodiversity Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The world population is 7.7 billion growing at a rate of 1.09 % per year. It has doubled in 40 years from 1959 (3 billion) to 1999 (6 billion) and it will take another 39 years to increase another 50% to become 9 billion by 2038. Out of the 7.5 billion population, 29.8% people are in the below poverty line. At the same time, the obesity rate increased to 4.9% from 2010-2014. World Obesity data show that in 2014 more than 1.9 billion (39%) adults are overweight and 600 million (13%) was obese. The percentage of urban population has increased to 32.7% of the total population during the last 10 years. The major reasons for poverty in the world are the low family income, the non-availability of cultivable agriculture land per household, lack of financial support, negligence of small scale farming practices and non-availability of nutritious food to the starving population. The fundamental cause of obesity is the food intake, energy metabolism, calories consumed and calories expended.

INFORMATION USED FOR MAINSTREAMING

Mainstreaming biodiversity was developed as an approach for addressing the biodiversity conservation goals which are sometimes contradictory to the goals of development and economic growth. The mainstreaming has been often referred to as integrating biodiversity into the development and it has the meaning of changing the focus of development policies and interventions towards incorporating the values of biodiversity.



The mainstreaming biodiversity has no single agreed definition and most of the definitions are similar to that of Peterson and Huntley; “to internalize the goals of biodiversity conservation and the sustainable use of biological resources into economic sectors and development models, policies and programs, and therefore into all human behavior”. The STAP/GEF definition of mainstreaming biodiversity: *The process of embedding biodiversity considerations into policies, strategies and practices of key public and private actors that impact or rely on biodiversity, so that biodiversity is conserved, and sustainably used, both locally and globally.* The real success of the Convention on Biological Diversity and Millennium Development Goals are not achieved to its fullest degree, so far and it seems difficult due to the single disciplinary approaches and solutions at the expense of other developmental problems.

New approaches to tackle the issues lies in the solutions arising from the product of multidisciplinary collaborations focused on integrated solutions. In this context the new interdisciplinary fields like Eco-nutrition, Eco-agriculture, Ecosystem services and Eco-health becomes relevant. Eco-nutrition includes the core disciplines, nutrition, agronomy, ecology and economics with an objective of integrating nutrition and human health, agriculture and food production, environmental health, and economic development to jointly reduce hunger and malnutrition, increase agricultural productivity, protect the environment and promote economic development. Eco-agriculture includes disciplines like ecology, agriculture, economics, development practitioners and community groups which aim at management of resources by rural communities to enhance rural livelihoods, protect biodiversity and ecosystem services; and development of more sustainable and productive agricultural systems. Ecosystem services include ecology, biodiversity, economics, physiology, nutrition, sociology aimed to recognize the contribution of natural and managed ecosystems, human well-being and livelihood. In the broadest sense, these include services such as provisional services, regulating services, supporting services and Cultural services. It provides food, clean air, and clean water through primary production and habitat provisions. Eco-health includes ecology and health sciences to understand the connections between nature, society and health and how drivers of social and ecosystem change ultimately influence human health and well-being.

APPROACHES AND TOOLS FOR MAINSTREAMING

There are several new approaches developed for the mainstreaming by international organisations. The major approaches for mainstreaming includes ecosystem assessment approach, strategic environmental assessment (SEA)/EIA, the CBD ecosystem approach, and spatial planning, while the major tools for mainstreaming are legal instruments, economic and financial tools, sectoral standards, codes of conduct, Guidelines,

Certification schemes and good practices. We can examine the details of some of the approaches and tools for the mainstreaming of biodiversity.

Approaches for Mainstreaming

1. 1. Ecosystem Service Approach

This approach is based on the Millennium Assessment Ecosystem Services framework and done by a five step process for assessing the risks and opportunities. It includes scenario planning to explore possible alternative future and decisions. Finally, at the end, this approach provides guidance on choosing and implementing policies to sustain the ecosystem services. ESA approach is being widely followed around the world due to its methodological advantage and stakeholder acceptance.

2. Environmental Input Assessment (EIA) / Strategic Environmental Assessment (SEA)

Integrating EIA requirements into development planning is a powerful approach to mainstream biodiversity. The EIA results will be translated into biodiversity management plans and it will help in the sustainability of the ecosystem in the long run. SEA identifies and assesses the possible outcomes of policies, plans or programs before they are implemented to balance economic, social and environmental priorities. The recent modifications in the EIA and SEA enhance the possibilities thereby decreasing the negative impacts of big development projects otherwise less bothered about the biodiversity loss. The development project should include biodiversity management plans as a part of their project proposals in order to reduce negative impacts on the biodiversity.

3. The CBD Ecosystem Approach

The CBD Ecosystem approach provides a framework of 12 principles used to guide the planning process at national and sub-national levels in order to ensure that conservation plans consider biodiversity along with economic and social objectives. The ecosystem Approach is extensively used in area- based management plans such as Integrated Marine and Coastal Area Management, Wetland Ecosystem Management and Integrated Watershed Management. Several countries have adopted this approach and developed regional biodiversity management plans for their development projects like infrastructure deployment and construction of ports and mining activities.

2. 4. Spatial planning

Spatial plans give an opportunity for mainstreaming biodiversity into sectoral and cross- sectoral plans as they determine where economic activities and infrastructure developments are established. Spatial planning provides the coordination of different sectors of government. It takes inputs from an expert, local people and stakeholders. Characteristics of effective marine spatial planning include the balancing of ecological, economic, and social goals and objectives towards sustainable

development through different government sectors and agencies in an area-based approach mainly focused on the long-term goals and experience gained from the stakeholders.

Tools for Mainstreaming

The major tools for mainstreaming biodiversity are

1. Ecosystem services

Ecosystem service indicators are valuable in mainstreaming as they facilitate the understanding and appreciation of the complex relationship between biodiversity and human- well-being. An example of the Ecosystem service indicators is Provisioning of Food, Provisioning of Raw materials, Regulation of Air quality, Recreation and Eco-tourism. The Millennium Ecosystem Assessment (MEA) classifies ecosystem services into four broad categories; provisioning, regulating, cultural and supporting services. Human nutrition is a function of provisioning services which provide us with raw materials of nutrients, clean air and clean water. The recipes and traditional foods are prevalent in most civilizations which are the result of long term interactions between human societies and the raw materials obtained from the agricultural fields of our ancestors.

2. Legal Instruments

Laws, rules and regulations regarding the use of natural resources are important for the protection and sustainable use of biodiversity. For example, in India we had several legislations such as Indian Forest Act, 1927, Forest (Conservation) Act, 1980, Wildlife (Protection) Act, 1972, Air (Prevention and Control of Pollution) Act, 1974, Water (Prevention and Control of Pollution) Act, 1974, Water Cess Act, 1977, Environmental (Protection) Act, 1986, Biological Diversity Act, 2002 and Coastal Regulation Zone Notification, 1991. It is customary to examine the pre- existing laws and management institutions already in use and the new laws should be complementary to these which promote sustainable and equitable use of resources.

3. Standard Codes of conduct, Guidelines and Certificates

Production sectors use a number of tools for attaining environmentally and socially sustainable resource management practices. Several tools have developed and established at international level and countries are accepted it on a voluntary basis, such as FAO code of Conduct for Responsible Fisheries (FAOCCRF), Marine Stewardship Council (MSC). Countries usually adopt the guidelines and standard practices for biodiversity mainstreaming or develop national standards where international standards are not applicable to the country.

a) FAO code of Conduct for Responsible Fisheries

FAO code of Conduct for Responsible Fisheries was adopted on 31 October 1995. Code is voluntary and global in scope for the members and non-members

of FAO, fishing entities, sub regional, regional and global organizations and all persons concerned with the conservation of fishery resource management and development. Article 7 Fisheries Management, Article 9 Aquaculture development and Article 10 Integration of fisheries into coastal area management deals with many important biodiversity related issues like overcapacity, sustainability of small scale fisheries, conservation of habitats, aquaculture, by-catch reduction, conservation of species diversity and genetic diversity and coastal zone management.

b) Marine Stewardship Council (MSC)

The Marine Stewardship Council (MSC) is a global non-profit organization set up to promote the sustainable fishing by harnessing the market process. The MSC developed environmental criteria for sustainable and healthy managed fisheries. MSC is a product label to get environmentally responsible fishery management and practices. Consumers can choose a product which has not contributed to the ecological problem of over exploitation of fishes. The MSC principles and criteria stipulate the fishing operations should allow for the maintenance of the structure, productivity, function and diversity of the ecosystem on which the fishery depends. Ashtamudi's clam fishery joins a growing number of other MSC-engaged fisheries in the developing world, which represent 7% of fisheries in the program. The MSC have developed new tools and practices to increase the accessibility for fisheries that are data-deficient, such as the Risk-based Framework, which was used in the Ashtamudi clam fishery.

MAINSTREAMING BIODIVERSITY FOR HUMAN WELL BEING

Biodiversity is affected by multiple drivers and pressures that negatively impact on the production of ecosystem services to people. The major drivers include demographic, economic, socio- political, scientific and technological which increases the pressure on biodiversity, resulting in the further decline, degradation and loss of biodiversity. The major pressures on biodiversity are habitat loss, degradation, overexploitation, alien species, climate change and pollution. It is the integration of the conservation and sustainable use of biodiversity in both cross-sectoral plans like sustainable development, poverty alleviation, climate change mitigation, track and international cooperation and sector oriented plans like Agriculture, fisheries, mangroves, transport, tourism, mining and others. It proposes major changes in the development models, strategies and paradigm. It is not about creating a new system, but integrating biodiversity into existing and or new sectoral and cross-sectoral structures, process and system. It helps to re-organize value of biodiversity and ecosystem services and act to maximize the positive and minimize the negative impacts of human activities on biodiversity. Human well-being and poverty reduction are closely related and major biodiversity indicators are the availability of basic

material for a comfortable life, health, security from disasters, stable societies, freedom of choice and action and enhancement of science and art.

Agriculture faces with problem of feeding nine billion global populations in 2050, with the decreasing environmental qualities. Earlier the agriculture met the challenge of more production through Green revolution, but the tremendous environmental degradation occurred. The proposed blue revolution was also viewed in this way to produce more fish without much cost to the environment. Agricultural systems are vulnerable to climate change, globalization and increased cost of inputs and the degradation of the natural resource base. Hence the agricultural landscapes should be an ideal net producer of an ecosystem rather than a consumer service. The issues of hunger have been the domain of nutrition, crop production- the domain of agronomy and conservation -the domain of ecology. It is well known that the nutritive value of fish/rice foods/ vegetables is ultimately the result of dynamic interaction between crops and their environment. For example, the role of micro flora and fauna in the process of mineralization and nutrient regeneration is the vital step in the crop production systems. Now it is clear that the production of food/fish in the farmer's field is attributed to the food production and nutrition are tied to the ecosystem services. Finally, it is clear that human nutrition is the important component of human well-being and is ultimately dependent on the numerous ecosystem services operated in the field.

Farmers should recognize that agricultural landscapes must be multifunctional producing water, sequestration of carbon, supporting pollinators, providing corridors for wild biodiversity. The production of food in a primary provisioning service maintains soil fertility or the inter-annual productivity of cropping system as regulating services. Soil micro fauna can convert organic matter to nutrients as supporting services. Most past cultures identify with a traditional food prepared from the locally available resources. Combinations of more species provide more nutrition than the single species. The mainstreaming aimed at integrating biodiversity consideration throughout the government and society and it may start at different levels of government/specific sectors such as national level, regional levels, state level plans, programs, strategies can be executed.

Different plans and programs proposed for the mainstreaming biodiversity for human well-being and nutritional security are;

1. 1). Reduce the negative impacts and enhance the positive impacts on biodiversity

In agriculture, the strategies to minimize/optimize the use and application of chemicals and pesticides to reduce the negative impact on soil, water and air. In fisheries, plans to reduce the catch of young ones, reduce the damage occurring

during the bottom trawling, improvement in the management strategies to optimize the return from fisheries. In the case of Aquaculture, reduced use of chemicals reduces the pollution of the environment; avoid the proliferation / escape of alien species to natural water bodies.

2). Restore biodiversity and ecosystem services

This may be achieved through fishing ban, seasonal closure, area closure, establishment of protected areas. Restocking for the enhancement of endangered and vulnerable species through hatchery produced seeds. It may also involve replanting or reintroduction of native plant or animal species in areas where they are depleted. It also involves in-situ conservation of areas of wild varieties of organisms.

3). Access and benefit sharing from the use of biodiversity by local communities

In forestry and fisheries, plans should include reserving certain areas for exclusive use by local communities and traditional people. Local communities and traditional people are empowered to manage their resources sustainably and such plans should help in resulting in poverty alleviation and human well-being and nutritional security.

4). Establishing Marine Protected Areas

IUCN defines a protected area as an area of land or sea dedicated by law or tradition and manages the protection of biodiversity and associated natural and cultural resources. Protected area may be established by an act of local or regional Government, by private individuals and conservation organisations, action of indigenous people and traditional societies, by the action of Universities and other research organisations. Marine protected areas are less compared to terrestrial areas due to the difficulties in establishing and managing. Here also our priority should be the protection of freshwater ecosystem which declines at a faster rate than marine ecosystem. The prioritization of protection can be used in three criteria viz. Endemism, Endangerment and Utility. Based on these criteria, several approaches are developed to prioritize the conservation of species by ecosystem approach and the hotspot approach.

5). Restoration Ecology

Ecological restoration is the practice of reestablishing population and whole ecosystem from degraded, damaged or even destroyed habitat. Restoration ecology is the scientific study of such restorations. There are four main approaches available at restoring biological communities and ecosystems, i.e., no action, rehabilitation, partial restoration and complete restoration. In the case of no action, it allows the ecosystem to recover on its own when restoration seems to be expensive when provision attempts shows that ecosystem will recover on its own. Replacing the degraded ecosystem with another

productive type using just a few species is called Rehabilitation. In partial restorations, some of the ecosystem functions and few dominant species are restored. Complete restoration refers to the restoration of the area into the original species composition and structure of active programs of area modification and introduction of the original species.

ECOSYSTEM SERVICES

Importance of ecosystem services to the human welfare has been already well known. Millennium Ecosystem Assessment (MEA) defines the ecosystem services and is classified into four major categories like provisional services which include food and water, regulating services like regulation of floods, drought, habitat degradation and disease, supporting services such as soil formation, nutrient cycling and cultural service like recreational, spiritual and religious. Although there are several investigations of the biology and ecology of marine flora and fauna, studies on the ecosystem services are very few. The qualitative and quantitative information about the ecosystem services are the prerequisite for the biodiversity valuation of the ecosystems.

Water regulation

The major source of water in the coastal area is from the South West monsoon and North East monsoon and runoff from 44 rivers all along the coast. Kerala is the land of rivers and backwaters. Its rivers criss-cross the state physique like network of veins. They fertilize the land; turn the waste into the wealth of the rich, black, alluvial soil. The lowlands of the coastal area made up of river deltas, backwaters, lagoons, canals and the Arabian coast is essentially a land of coconuts and rice. These rivers which are small and monsoon fed turnout to be rivulets in summer. The different services provided by the water could be primary production, upwelling, mud bank formation, migration of species, breeding and larval rearing and nutrient cycling. Degradation of water quality possesses a serious threat to the population living in the coastal and marine areas. Mangroves play an important role in trapping silts and sediment and in physically, biologically and chemically treating the waters of coastal and marine areas. Ecosystem water regulation services are of immense benefit to the households, industries that are located in the coastal zone.

Shore line protection

Soil/shoreline protection is another important service provided by the coastal ecosystems. Mangroves and rocky shores provide shoreline stabilization and erosion control services which are of great biodiversity value of the coastal and marine communities. The coastal population is protected against storms, cyclones, tidal surges, erosion and other natural hazards by these ecosystem services. Several estimates have been done mostly on the basis of avoiding coastal reclamation expenditures expressed in terms of area and length.

Gas regulation

Marine plants include seaweeds, sea grass, true mangroves and phytoplankton play an important role in the gas regulation in the marine and coastal ecosystems. The coconut trees, pokkali rice and other plants near the coastal area help in the gas regulation process. The climate of Kerala helps in the good proliferation of plants throughout the year. The monsoon provides heavy rains which helps in the remineralization and replenishment of the nutrients in the wetland areas of Kerala. Whereas the post monsoon season gets an ample supply of sunlight which results in the very high primary productivity in the wetland areas. The system provides important services like primary production and gas regulation.

Nutrient cycling

Marine plants and microorganisms help in nutrient cycling by absorption and degradation process. The nutrients in the coastal and marine ecosystem regenerates through different biotic and abiotic components like plants, animals, microorganisms and different habitats. The peat land formed in different parts of Kerala provides ecosystem services like nutrient cycling, waste removal and prevention of floods.

Measures for mainstreaming biodiversity

Measures for mainstreaming ecosystem services in fisheries involves water conservation, management of marine fisheries, species conservation, fishing ban and area closures, ban of disastrous fishing methods, conservation of young ones and breeding population, control of pollution, traditional knowledge and innovations, value chain approach and national policies and legislation for ecosystem services.

- The fisheries sector should have a plan of action for the fisheries development and management containing broader activities of the sector developed with the participation of a wide range of stakeholders.

- The plan of action should incorporate biodiversity relevant issues to sustainable development strategies and poverty alleviation programs, human well-being and nutritional security.

- Detailed consideration should be given to economics of biodiversity in the planning process of development of fisheries and related projects.

- An economic valuation provides a convincing justification for the biodiversity conservation.

- Fisheries biodiversity management requires participation of all sectors including fisheries, agriculture, transport, water supply, port, shipping, environment and forest, pollution control, inland navigation, commerce, traditional custodians and public. A strong coordination mechanism is required to bring all the

stakeholders to decision on management.

- Biodiversity and legal framework should take each other into considerationwithout neglecting the traditional rights and knowledge.

- Ecosystem approaches to fisheries can be a very effective tool for mainstreaming biodiversity concerns of the different sections of the society.

We can use the ways mentioned to mainstream biodiversity concerns to human well-being and nutritional security in any sector in formulating relevant sectoral strategies, plans and programs. This can be demonstrated through integrating biodiversity concerns into the operating aspects of concerned sectors. Ultimately the mainstreaming can be further integrated to the biodiversity related sectors of state, region and the country.

SUGGESTED READING

Devaraj, M, V. Sriramachandra Murthy, R. Sathiadhas and K. K. Joshi. 1998. The new Economic policy and perspective for marine fisheries research and development in India . Fishing Chimes, 18 (5):18-29.

Joshi, K. K., P. A. Thobias and M. S. Varsha. 2017. Present status of Ichthyofaunal diversity of Indian seas. In: Course Manual Summer School on Advanced Methods for Fish Stock Assessment and Fisheries Management. Lecture Note Series No. 2/2017. CMFRI; Kochi, Kochi, pp. 1-22.

Rao, D. S., C. P. Ramamritham , A.V. S. Murthy , S. Muthusamy, N. P. K. Unnikrishnan and L. R. Khambadkar , 1992. Oceanography of the Arabian sea with particular reference to the south west monsoon. Bull. Central Marine Fisheries Research Institute, p.45- 48.

Sathianandan, T .V.,J. Jayasankar, Somy Kuriakose, K. G. Mini and Wilson T. Mathew. 2011. Indian marine fishery resources: optimistic present, challenging future. Indian Journal of Fisheries, 58 (4): 1-15.

Singh, H.S. 2003. Marine protected areas in India. Indian Journal of Marine Sciences, 32 (3): 226-233.

Plastics in Marine Environment: The need for Environmental Monitoring and Management

V.Kripa
Fisheries Environment and Management Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Marine debris which is defined as any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment is one of the most pervasive, yet potentially solvable, pollution affecting the world's oceans, coastal ecosystems and rivers. Whereas impacts of most anthropogenic activities are usually found near the point source, marine debris has been found to impact even distant locations, often affecting uninhabited areas also. According to United Nations Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP), 60 to 80%, of the global litter found in the coastal and marine ecosystems has originated from land and only the rest from sea based activities.

The slow degradable nature of marine litter and the potential to pollute all spheres of oceans irrespective of point source has raised the alarm bells. The UNEP has recently initiated a special program ***'Global Initiative on Marine Litter'***. Three main industries which are affected by marine debris are fisheries, shipping and tourism and the estimated damage to these sectors in APEC region is US$1.265 million annually.

One of the major threats faced by fishermen operating their fishing gears in coastal waters of India is the alarming influx of litter in the fishing area. There are about 4 million fishermen spread across 3288 fishing villages. Of the 1.9 lakh fishing crafts, 36.7% are motorised and 26% are non-motorised.

Though the Indian EEZ is spread to an area of 1,629,607 km2, major fishing takes place in the inshore waters (total area-225,029 km2) and in the continental shelf (total area-393,527 km2). There are different types of fishing craft and gear combinations and among these the most affected by marine debris is the bag type of fishing gears and the trawlers.

Marine debris is a fairly recent problem which has been found to affect the ecosystems. The problem is becoming worse day by day and the impacts on the ecosystem and livelihoods which depend on the coastal and marine ecosystems cannot be ignored anymore. A brief account of the issues related to marine litter is given below.

WHAT IS THE IMPACT OF PLASTIC WASTE ON THE ECOSYSTEM?

Most coastal villages and urban cities do not have well planned solid waste management programs. With the increasing coastal population and lack of proper solid waste management protocols, the quantity of solid waste entering the coastal waters through rivers, estuaries and canals is enormous. Studies indicate that if the plastic waste is not managed properly there will be more plastics in the sea than fish by 2050. It is essential to find out more about the impacts of litter on the ecosystems and assess the damages already made. Studies conducted in CMFRI indicate that this has impacted the benthic ecosystem and there are areas which are hypoxic with low benthic biomass. However, there is a need for extensive assessment of the impacts. From this we should make a move to control further degradation and then plan for a restoration of the impacted marine/coastal habitats.

HOW MUCH PLASTIC GOES TO THE SEA EVERY YEAR?

CMFRI has made assessments of quantity of beach litter and has also made a GIS map on this information. However, there is a big lacunae on the quantity of litter present in open waters. Targeted surveys have to be conducted in the continental shelf waters to understand the level of pollution by marine debris, so that action can be taken to clean up these and prevent plastic gyre formation in Arabian Sea and Bay of Bengal.

WHAT IS THE IMPACT OF LITTER ON FISHERIES?

The interlinking canals of major estuarine systems along the Indian coast carry the domestic waste to the coastal waters which often get collected in the several bag type of fishing gears like the stake net and dol net along the Indian coast. The high silt in our coastal waters reduces the buoyancy of drifting articles, making them sink down quite close to their place of origin. Monsoon waters from the coastal areas aggravate the situation by washing off the land based litter to the open waters. It has been observed that during spring tides, the quantity of litter in the stake nets close to the coast have almost doubled the quantity of litter observed during low tide.

Studies have been conducted along Kerala, Maharashtra and Gujarat coast. However, more information is required about the impact on fisheries. The quantity of litter in the fishing nets gives an idea on the abundance and density of litter in the particular area.

WHAT IS THE LEVEL OF MICRO AND MACRO PLASTICS IN THE MARINE FOOD CHAIN?

The type of plastic entering the ecosystem is also diverse and depending on the size and spread, they either settle at the site of origin or drift away, only to sink down and spread in a distant location. Similarly, smaller the size, easier it is for the particle to enter the food chain. The marine food web starts with the plankton and observations in the plankton samples collected regularly in some of the major fishing areas have indicated the presence of micro-plastics.

These tiny particles have been found to enter the stomach of filter feeding organisms like mussels, clams and oysters and also higher bony fishes like the sardines. Though the percentage occurrence of micro-plastics of size 50micron to 5mm is less than 5%, it still is a matter of concern. These particles can go up the food chain. The number of reports on micro and macro-plastics in the gut of fishes has increased over the years. Now Indian researchers have observed plastic ingestion in sardine, mackerel, anchovy, ribbon fishes, dolphin-fish, tunas and several other fishes caught from almost all states along the southwest and southeast coasts. Apart from these, plastic pieces were observed in the carcasses of sea bird and stranded whales.

IS THERE ANY BIOACCUMULATION AND TOXIC IMPACTS FROM MICRO PLASTICS?

Plastics are known to contain toxic substances like Bisphenol A. However, only very little information is available on the chemical aspects of micro-plastics and macro-plastics. Considerable research has to be done on this theme so that Public health advisories can be proposed.

HOW HAS MARINE LITTER AFFECTED OUR CRITICAL ECOSYSTEMS?

Littering is rampant in beaches and these have been found to affect the vulnerable ecosystems like the sea grass, coral reef and the mangroves. Only very few beaches have regular cleaning programs. Though the coral reefs of India are just 0.660 % of world coral reefs, they support the livelihood of several thousand of fishers. The proximity of Indian coral reefs to the mainland makes them more vulnerable to anthropogenic impacts. Based on observations by researchers across the Indian coasts, it can be authentically stated that different types of plastic litter is affecting the biological functioning of critical habitats.

The impacts of litter on the functioning of ecosystems should be evaluated. Since most critical habitats are near to tourists spots, the chances of more litter entering the ecosystem are high. Targeted studies

should be conducted to understand how this has affected the ecosystem services of critical habitats and evolve methods to rectify these.

HOW MANY ANIMALS ARE IMPACTED BY ENTANGLEMENT?

Another major issue is the abandoned or lostderelict gear. Ghost fishing issue was first brought to the world's attention at the 16th session of FAO Committee on fisheries in April 1985. According to the UN, 640 000 tones of fishing gear are lost or discarded in our oceans annually. Several sightings of ghost net entanglement of turtles from the Bay of Bengal and Arabian Sea have been observed. All these observations are messages from the deep that derelict fishing gears are swaying like ghosts threatening the benthic habitat and the marine biota. Recent reports have shown that unexpected natural disasters like cyclones along the coast lead to loss of fishing nets. These cannot be retrieved easily and the Indian fishing community is not aware about the impacts of these on the fauna.

There are no assessments of the impacts of ghost nets on the marine fauna and the quantity that enters the ecosystem every year. Moreover, hotspots of abandoned nets should be mapped through surveys so that large scale clean-ups can be organized.

It is strongly recommended that activities by NGOs and fishing communities be encouraged to remove these derelict gear, create awareness among the fishing community on the need to bring back the damaged gear and dispose the same on land. The recycling industry can also try to find ways to effectively utilize these.

NEED FOR AWARENESS PROGRAMS

Lack of awareness about the impacts of litter on the coastal and marine ecosystem is one of the reasons for the increasing litter in marine habitats. However, we have to go beyond all these research and awareness programs and work towards a permanent solution to this problem.

REDUCE USE AND ALSO DEVELOP FACILITIES IN PUBLIC PLACES

Reduction in use of plastics and proper solid waste management programs are required. Moreover, there should be facilities to deposit non-degradable litter in public places. Along with these programs, we should also plan for extensive village level coastal clean- up programs to remove already accumulated litter. Marine debris is not something which can be neglected; if ignored, it can completely destroy the resources and the livelihoods depending on aquatic ecosystems.

SUCCESS STORIES IN MARINE DEBRIS MANAGEMENT

The marine debris problem has affected the developed nations during last century itself and they have implemented some programs which have been found to effectively reduce marine debris, few such cases are given below.

Waste to energy: In Hawaii there is a Nets-to-Energy Program, and every yeartrained NOAA divers remove nets carefully from PMNM reefs and other underwater habitats. The nets are transported to the island of Oahu, where a scrap metal recycling company (Schnitzer Steel) uses its powerful shredder to chop the nets into fragments. The fragments are then taken to a waste-to-energy facility, which burns the material to produce electricity for the island. Since the program's launch in 2002, it has processed more than 700 metric tons of nets, line, and rope-producing enough energy to nearly 350 of Oahu's homes for an entire year.

Deposit Scheme: South Australia container deposit scheme which provides AUS$0.10 refundable deposit to beverage containers, resulted in a 3-fold reduction in the number of beverage containers lost to beaches.

Litter reduction and increase in tourism: A study of thirty-one beaches in Orange County, California, USA (Leggett et al. 2014) found that a 75% reduction in marine litter at six popular beaches generated over €40 million in benefits to residents over three months.

Incentives to fisher boats for marine litter collection: In the Republic of Korea, fishing boats are provided with large bags to collect litter and an economic incentive of US$10 per 100litre bag is provided to fishermen.

NEED FOR NATIONAL MARINE DEBRIS MANAGEMENT STRATEGY

Though the UNEP was founded in 1972, a targeted program for marine debris control was initiated only since 2003. The problem of marine litter was recognized by the U.N General Assembly (UNGA), which in its Resolution A/60/L.22 (Nov. 2005) calls for national, regional and global actions to address the problem of marine litter. Now apart from Regional Seas programs, each nation is implementing its own Strategy for marine debris reduction and control. ICAR- CMFRI has been organizing stakeholders meeting every year in all maritime states. Marine litter is one of the major problems identified by fishermen and they have demanded a solution for this. Fishermen of Kerala and Maharashtra have actually demanded an urgent action for reducing marine debris in fishing area. Considering the growing threat to resources sustainability and reduction in ecosystem functional services leading to loss of livelihood in fisheries sector, we strongly recommend that there should be a National Marine Debris Strategy with specific goals for Prevention and Control of debris accumulating, spreading and in coastal and marine ecosystems affecting the fish production.

The Ministry of Environment, Forest and Climate Change (MoEFCC) has issued MSW management and handling rules for scientific MSWM –but this has not targeted marine debris. Considering the

global importance of plastics, we have to develop a responsible method of disposing used plastics instead of making it a “litter”. The long term solution lies in proper development and utilization of waste management facilities in all villages, municipalities and corporation so that it does not become a regional and global issue. Few suggestions for reduction marine debris are given below.

1. Prevent/reduce generation of waste that contributes to the marine litter (First identification of major component in the litter then measure to reduce).
2. Prevent/reduce litter reaching the marine environment: Proper segregation of litter which can be recycled and reused (Eg Korea, US).
3. Collect litter from the marine environment through incentives.
4. Provide Incentives to fisher for marine litter collection (n the Republic of Korea, fishing boats are provided with large bags to collect litter and an economic incentive of US$10 per 100litre bag is provided to fishermen).
5. Development of Environmentally-friendly design in packaging (Eg Japan).
6. Extended producer responsibility (EPR) makes a producer financially and/or logistically responsible for the post-consumer (i.e. waste) stage of a product’s life cycle (Eg EU states).
7. Provision of adequate low-cost or free and easy-to-use collection facilities in ports
8. Incentive schemes to promote proper disposal of discarded gear.
9. Improvements in waste management infrastructure in tourist areas (e.g. placing suitable bins on beaches, followed by regular clearing).

There is a need for greater producer responsibility and more widespread application of the polluter pays principle.

Marine litter clean-ups - costly but necessary downstream actions.

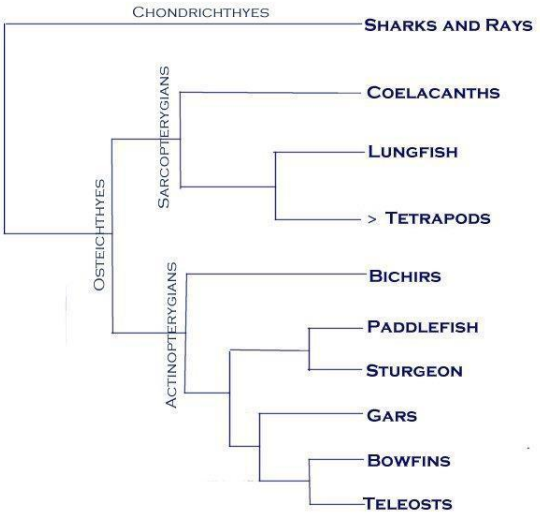
The development of new recycled plastic products can create additional demand and support the development of a circular economy.

Evolution and Interrelationships of Teleosts

J. Rajasekharan Nair
Kerala University of Fisheries and Ocean Studies, Kochi

INTRODUCTION

The following cladogram gives a perspective on the relationship of teleosts with the other extant group of fishes, and the teleostean derived status.



The evolution of teleosts can be traced to the Mesozoic Era (225 – 65 million years ago) from the advanced extinct holostean (now neopterygian) order †Pholidophoriformes. The generalized fossil group, the order †Leptolepidiformes (*Leptolepiscoryphaenoides*) may be the first teleostean fish, so placed because of the homocercaltail condition.

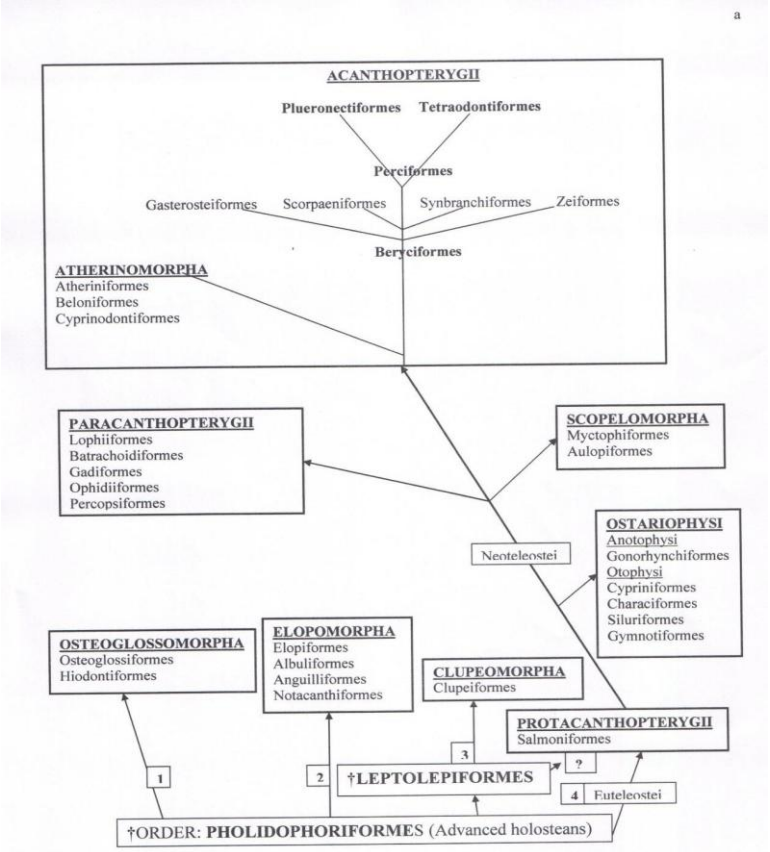
Initial divergence occurred during the late trassic and jurassic periods among the generalised type of herring-like and salmon-like fishes which constitute the bulk of ancestoral soft-rayed teleosts (lower teleosts or sometimes referred to as Malacopterygii). Many families of marine and freshwater fishes have evolved in this group. They occupied all continents by the late jurassic about 150-145 million years ago (Arratia, 2004). During the Cretaceous period the berycoids provided a transitionto the spiny-rayed teleosts (higher teleosts or Acanthopterygians). This led to another flowering of large number of families resulting in the perch and perch-like fishes. The teleostean evolution has thus resulted in fishes which are capable of rapid and complex locomotion; have very efficient ventilation and thereby respiratory efficiency; use a wide variety of food materials (autochthonous and allochthonous); and have an array of complex reproductive styles and strategies. Thus these fishes came to occupy the microhabitats and niches in the aquatic environment up to then unavailable or unoccupied by the ancestral fishes.

In 1966, four of world's leading ichthyologists, Peter Humphry Greenwood of the British Museum of Natural History, London; Donn Eric Rosen, American Museum of Natural History, New York; Stanley Howard Weitzman, National Museum of Natural History, Washington D.C.; and George Sprague Myers, Stanford

University, California produced what is arguably the single most important publication on fishes of the second half of the 20th century: “Phyletic studies of Teleostean Fishes, with a Provisional Classification of Living Forms” (Pietsch, 2011). As S.H. Weitzman later wrote to T.W. Pietsch “The greatest contribution of Greenwood *et al*. (1966) is not its classification; it is its challenge to ichthyologists”. Although published before cladistics was adopted in Ichthyological publications, the arrangement is that of a vertical classification and as a consequence resembles a cladistic interpretation (Nelson, 2010). To a large extent, the picture of teleost relationships that is generally accepted today dates to this paper written just prior to the advent of the cladistic revolution (Pietisch, 2011).

Here an attempt is made to give a basic simplified version of the interrelationships, so that a student of taxonomy can get a picture of where each group stands and why. So much research has gone into fish systematics since 1966, too many to even list, that is changing the tenets of the phyletic tree every other day.

First lineage: The first lineage of teleostean evolution culminated in the superorder (or subdidvision) **Osteoglossomorpha**, a very distinctive group of fishes considered to be the most primitive group of teleosts, with around 220 extant species. The group includes the freshwater orders Osteoglossiformes (bony tongues, featherbacks and mormyrids) and Hiodontiformes (moon-eyes). The osteoglossids are mainly seen in South America, the hiodontids in North America, the notopterids in Asia and the mormyrids in Africa. The extant species are relics of a once much more abundant group. In fact these fishes were the prime inhabitants of the freshwaters before the advent of the otophysean fishes. The African mormyrids or elephant fishes (Mormyridae) is established with around 201 species constituting the bulk (90%) of all osteoglossomorphs.



The Figure gives a modified version of the phylogenetic tree as given by Moyle and Cech (1982)

Second lineage: The second line of evolution is the superorder (or subdidvision) **Elopomorpha** which includes some disparate orders namely, Elopiformes(tarpons, giant herrings), Albuliformes (lady fishes, spiny eels),Anguilliformes (eels) and Saccopharyngiformes (guper and viper

eels) with around 850 marine species. These orders are united by the presence of a distinctive larval form- the leptocephalus larva and distinct larval metamorphosis. There is even a reduction in length from the larval to the juvenile life-history stage in many forms. These planktonic larvae are thin, transparent and ribbon-like, mostly drifting passively with the currents. The eel larvae are so unlike the adults that for a long time they were identified as different species belonging to the genus, *Leptocephalus*. The elopomorph fishes are scattered in coastal marine and deep seas.The anguillid eels are catadromous. The elopiforms still carry with them strong ancestral characters like the presence of gular plate and the toothed parasphenoid.

Third lineage: The third line of evolution led to highly specialized group of filter feeders seen mainly in the continental shelves of the world oceans, mainly inshore, shoaling small fishes,namely the superorder **Clupeomorpha** (around 364 species).Consists of a single order of extant fishes, the Clupeiformes (herrings, pilchards, sardines, shads, anchovies, sprats, gizzard shads, menhaden, denticle herrings, wolf herrings). This is one of the most well defined teleostean orders. The clupeiform fishes are adapted for living in well lighted surface waters, where most species school and feed on plankton. They include some of the most abundant fish species in numbers (the Peruvian anchovy, Atlantic herring etc.).

The first three lineages are considered to be relict (ancestral) or specialized dead-end off-shoots of the main line of teleostean evolution. There are clearly many ways to be a successful teleost. The osteoglossomorphs can be regarded as early successes that faded as other teleost groups especially the series otophysi evolved. The African mormyrids adapted to the unstable freshwaters and hence diversified. The eels have taken one particular morphological pattern (similar body shape) and larval form to diversify in astonishing ways. They have come to occupy a wide array of habitats from streams and lakes to the estuaries, to the coastal waters, to the coral reefs and to the deep sea. Like-wise the clupeiforms developed into specialized plankton feeders with relatively fewer species but extraordinarily high numbers, in shallow coastal waters especially the west coast upwelling areas. One key to the success of both eels and clupeiforms is their ability to make long migrations, including migration into freshwater, that separate life-history stages.

But there is a major difference of opinion in what is the most primitive extant teleost taxon under the 'Teleostean origins, phylogeny and classification': The lineages and their relationships are:

Patterson and Rosen (1977)	**Arratia (1997)**
1.Osteoglossomorpha	1.Elopomorpha
2.Elopomorpha	2. Osteoglossomorpha
3.Clupeomorpha	3. Ostarioclupeomorpha
4.Euteleostei (including Ostariophysi)	4.Euteleostei(excluding Ostariophysi)

Arratia(1991) challenged the view that osteoglossomorphs are more primitive than elopomorphs on the grounds that the caudal skeleton of *Elops* is more primitive

than that of the osteoglossomorphs. Further studies of Gloria Arratia support the view that elopomorphs are the living sister group of all other living teleosts (Nelson, 2006). Debates continue based on the later works of Colin Patterson and Gloria Arratia and the recent molecular data of Inoue *et al.* (2003).

There has been growing evidence that the sister-group of the clupeomorphs is the ostariophysans as per the Arratia's (1997) grouping. Arratia's Ostarioclupeomorpha (a taxon-based name) includes the Clupeomorpha + Ostariophysi. Strong phyletic evidence exists for a sister-group relationship between Clupeomorpha and Ostariophysi, based on both morphological and molecular evidence (Nelson, 2006). Several features were thought to demonstrate an affinity between the Gonorynchiformes (milkfish) of Ostariophysi (Anotophysi) and the Clupeiformes. Components of the Weberian apparatus (the binding character of the ostariophysans) are found in clupeoids. Gonorynchiformes were thought by some, before cladistic analysis, to represent an evolutionary link between clupeiformes (where many earlier works placed them) and other ostariophysans. The removal of Ostariophysi from Euteleostei is a major change; however as Arratia notes, the highly diversified Euteleostei is still a poorly supported group with many characters that are homoplasious and with little knowledge about basal members (Nelson, 2010).

Fourth lineage: The fourth line (the euteleostean line) is the major line of teleost evolution with more than 25000 species (including the debated ostariophysans). The base of this evolutionary line is the superorder

Protacanthopterygii. This is considered the ancestoral or generalized euteleost containing the order Salmoniformes (pikes, salmons, trouts, chars) and others. Many of the fishes in this group show anadromous spawning migrations. From this basal Euteleosts has evolved the most advanced or derived teleostean superorder Acanthopterygii (spiny-rayed teleosts).

The **Acanthopterygii** (around 14,800 species): The Order Beryciformes (squirrel fishes, soldier fishes) is the basal order from which the perch and perch-like fishes have evolved. The major order is the Perciformes. This is the most diversified of all fish orders with 20 suborders, 160 families, around 1539 genera and about 10,033 species (Nelson, 2006). This is the largest order of vertebrates. The perciforms dominate in the ocean continental shelf, especially in the structured rocky and coral reefs. They are also dominant in the tropical and subtropical freshwaters, example, the cichlids of the African great lakes. This order includes major suborders like Percoidei (seabass, groupers, trevallies, grunts, seabreams, croakers, snappers, ponyfishes, goatfishes, butterflyfishes, cichlids, damsels); Labroidei (wrasses, parrotfishes); Acanthuroidei (rabbitfishes, surgeonfishes); (?) Mugiloidei (mullets, barracudas); Polynemoidei (threadfins); Blennioidei (blennies, clingfishes);

Scombroidei (ribbonfishes, mackerels, Spanish mackerels, bonitos, tunas and billfishes); Stromateoidei (pomfrets, driftfishes); Gobioidei (gobies, sleepers, mudskippers), Anabantoidei (climbing perch, gouramies, fighters); Channoidei (snakeheads) etc.(Nelson, 1994). The extraordinary success of perciform fishes is related to the adaptive characters that have allowed them to take advantage of complex shallow water habitats like the coral reefs. Complex social behaviour patterns including strong inter and intra-specific communication, symbiotic relationships, and colouration are notable additions.

From the Perciformes have evolved the most derived teleost orders namely the Pleuronectiformes (Flatfishes – halibuts, flounders, soles – 678 species) and the Tetraodontiformes (puffers, filefishes, boxfishes, sunfishes – 357 species). They have secondarily lost many acanthopterygian characters and have evolved many specializations to occupy new niches. The migration of the eye across the head in flatfishes has got to be regarded as one of the developmental wonders of animal evolution. They came to dominate the benthic habitat previously occupied by the rays and nobody else. The puffers and their kin have reinvented the concept of armour and spines and strong biting jaws to occupy their own niche in the tropical shallow waters and reefs.

The base of the acanthopterygian box is occupied by the superorder **Atherinomorpha**, the pelagic surface dwelling marine and freshwater fishes with their own adaptations in locomotion like body shape, mouth structure, fin positions etc. It includes the orders Atheriniformes (silversides, hardyheads – 312 species); Beloniformes (fullbeaks, sauries, halfbeaks, flyingfishes – 227 species); and Cyprinodontiformes (killifishes, guppies, mollies, platys, swordtails – 1013 species).

The pre-perciform orders include the Scorpaeniformes (lionfishes, stonefishes, flatheads- 1477 species); Gasterosteiformes (sticklebacks, pipefishes, seahorses – 278 species); Synbranchiformes (swampeels, spinyeels – 99 species) and Zeiformes (dories – 32 species).

Mid-way to acantopterygian evolution is the successful offshoot – the **Paracanthopterygii**. This is a major evolutionary line of predominantly marine fishes(36 families, 270 genera and around 1340 species). They have a mix of soft-rayed (ancestoral) and spiny-rayed (derived) characters. There is doubt that they represent a single unified evolutionary line. The superorder includes orders like Gadiformes (cods, Pollack, hake, haddock, saithe, pout – 555 species); Lophiiformes (anglerfishes, goosefishes, frogfishes – 313 species); Batrachoidiformes (toadfishes – 78 species); Ophidiiformes (cusk-eels, brotulas – 385 species) etc. This superorder has produced fishes which live in the littoral (continental shelf), continental slope, and deep-sea regions and dominate these benthic habitats.

The superorder **Ostariophysi**: One of the most distinctive, diverse and successful

offshoot (?) of euteleostean evolution is the ostariophysans. (Note the Ostarioclupeomorpha lineage of Arratia, 1997, given previously in the text). They are characterised by specializations like the weberian apparatus, and alarm substance and fright reaction. The presence of unicellular horny projections (unculi) and multicellular horny tubercles with keratinous caps (nuptial or breeding tubercles) around the snout and ventral surface of paired fins are also unique characters of the group. The upper jaw is protractile in many species, but the sequence of jaw evolution is different fromthat of the acanthopterygian evolution. The superorder includes five orders, 68 families, 1075 genera and about 7931 species.

The Ostariohysi includes the series Anotophysi and Otophysi. The Anotophysi is characterized by the modification of anterior vertebrae (proto-weberian apparatus). It includes the order Gonorynchiformes (milkfish).

The series Otophysi includes the four orders Cypriniformes (carps, barbs, suckers, loaches – 3268 species); Characiformes (characins, tetras, piranhas – 1674 species); Siluriformes (catfishes – 2867 species) and Gymnotiformes (knife eels, electric eels – 173 species).Nearly three-fourths of all freshwater fish species belong to the ostariophysans. They are the dominant freshwater fish in all continents except in Australia and Antarctica. The fact that this group contains some of the most abundant species and some of the most economically important capture and culture species is a tribute to their adaptability. They demonstrate how the right combination of behavioural, physiological, morphological and life-history traits allowed one major evolutionary line of fish to dominate the turbulent, turbid, fluctuating (temperature and water chemistry) freshwater habitats of the world. Some of these adaptations are the acute sense of hearing (weberian apparatus), alarm substance and fright reaction (predator-avoidance), acute sense of taste, double-pump suction feeding, accessory respiration in catfishes, electric organs in gymnotids etc. To conclude, although a distinct evolutionary group, the ostariophysans are tied phylogenetically to the herrings and their relatives (clupeomorphs), as a separate lineage from the rest of the modern teleosts.

Superorder **Scopelomorpha**: Another successful offshoot of neoeleostean evolution with fishes inhabiting the continental shelf and slope floors and the mesopelagic waters. This includes the orders Aulopiformes (lizardfishes, Bombay duck, green eyes, barracudinas - 236 species) and Myctophiformes (lanternfishes – 246 species). The order Stomiiformes (hatchetfishes, bristlemouths – 391 species) is probably the sister-group to all the neoteleosts including the scopelomorphs. Many of these deep-seagroups are characterized by the presence of bioluminescent organs. They form an important food source to the large epipelagic fishes in the oligotrophic open oceans.

The evolutionary history of fishes is a dynamic field of study because the fishes arenot only diverse today, but they were diverse in the past also. Major groups have developed, flourished and then died out (ostracoderms, placoderms, major lineages of chondrichthyes and osteichthyes). It is curious, for example that teleost fishes, the dominant group today, are apparently monophyletic when there is good evidence of many potential ancestoral groups among the ancient chondrosteans. The evolutionary processes have resulted in a steady improvement of teleostean fish design with increased specializations and thus species richness.

(I feel that Pietsch (2011), in which he tells the story behind the publication of the Greenwood *et al*. (1966) classic paper through a series of correspondence with the authors, is a must read for all budding ichthyologists).

SUGGESTED READINGS:

Greenwood, P.H., Rosen, D.E., Weitzman, S.H. and Myers, G.S. 1966. Phyletic studies of teleostean fishes, with a provisional classification of living forms. *Bull. Amer. Mus. Nat.Hist*. 131: 339-456

Greenwood, P.H., Miles, R.S., and Patterson, C. (eds.) 1973. Interrelationships of fishes. *J. Linn. Soc. (Zool.). 53 (Suppl. 1)*: 233-305

Nelson, J.S., Schutze, H.P., and Wilson, M.V.H. 2010. *Origin and phylogeneticinterrelationships of teleosts. Honoring Gloria Arratia.* Verlag Dr Friedrich Pfiel.Munchen, Germany.

ietsch,T.W. 2011. Greenwood, Rosen, Weitzman, and Myers: Circumstances surrounding the publication of "Phyletic studies of teleostean fishes, with a provisional classification of living forms" 1966. *Copeia* 2011, N0 4, 590-598

Arratia, G. 2004. Mesozoic halecostomes and the early radiation of teleosts. In: *Mesozoic fishes. 3.Sysyematics, palaeoenvironments and biodiversity* (Arratia, G. andTintori, A. eds.), 279-315. Verlag Dr Friedrich Pfiel. Munchen, Germany.

Teleostean Fishes: Why are they so species-diverse and successful?

J. Rajasekharan Nair
Kerala University of Fisheries and Ocean Studies, Kochi

INTRODUCTION

Class: Actinopterygii
Subclass: Neopterygii
Division: Teleostei

Teleosts are by far the most abundant (in species) and diversified group of all the vertebrates, constituting around 50% of all vertebrates. They dominate in the world's rivers, lakes, and oceans. About 26,840 extant species, about 96% of all extant fishes, are placed in 40 orders, 448 families, and 4,278 genera (Nelson, 2006). The teleost fishes now dominate both marine and freshwaters. Of the extant teleosts about 58% are marine (cover 70% of earth's surface), 41.5% are exclusively freshwater (cover 1% of earth's surface) and around 0.5% is diadromous, migrating between the fresh and salt waters. Teleosts live in almost every conceivable type of aquatic habitat. They are found in high altitude lakes, in hot springs, deepest lakes (1000m),fast torrential streams, subterranean caves and streams, flood-plains, swamps and marshes, rock pools, structured communities like tropical shelf coral reefs, deep oceanic zones including thermal vents, being exposed to wide ranges of temperature, salinity and dissolved oxygen (Nelson, 2006).

The teleostean fishes have been said to have evolved from the advanced holostean order Pholidophoriformes during the Triassic period and proliferated during the Jurassic and Cretaceous periods of the Mesozoic era (225 – 65 million years ago). Despite the presence of distinct evolutionary lines within the teleosts, the modern teleosts are a fairly compact monophyletic (?) group with a number of distinct characters which helped them to proliferate into such a large speciose group occupying the diverse aquatic environment.

Bony endoskeleton: 1). The vertebrae are completely ossified, amphicoelous (flexible vertebral column) and reduced in number. The ossification is by means osteocyte cells forming osteoblast tissue and growth by means of calcification and mineralization. Their structure makes them lighter and stronger. The major bones are of two types-

a). Cartilage bones (replacing bones) - these are homologous with the cartilage of elasmobranchs having origin from the sclerotome of the dorsal mesoderm. E.g. the endoskeleton and majority of the other bones.

b). Dermal bones (membrane bones) - these are of origin from the dermatome of the dorsal mesoderm being similar to the scales. E.g. premaxilla, maxilla, opercular

bones, branchiostegals etc. These bones are newly derived in the teleosts and play very important roles in the evolution of feeding types and of ventilatory efficiency.

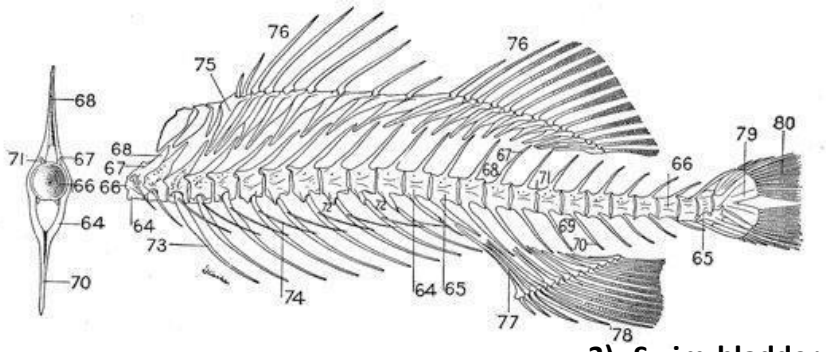


Fig.1. Vertebral column and caudal skeleton (www.australianmuseum.net.au)

2). **Homocercal tail:** The tail is variously homocercal and symmetrical. The vertebral column ends anterior to the caudal fin skeleton (hypural complex), to which the caudal fin rays are attached (Fig.1). This has led to the development of variously shaped caudal fins (lunate, forked, truncate, emarginate, rounded, elongate) and distinct caudal peduncles (narrow, broad, keeled), depending on the habit and habitat of the species (e.g. narrow and keeled caudal peduncle and highly forked caudal fin in fast swimming scombroids, carangids; broad caudal peduncle and rounded caudal fin in ambushing serranids; broad and strong caudal
peduncle and mildly forked caudal fin in migrating salmons).

The modified neural arch of the ural vertebra (uroneural bone) is the 'end-bone' giving the fishes the title of 'teleostei'. The major factor responsible for the evolution of the homocercal tail is the development of the swim bladder and neutral buoyancy, leading to the redundancy of the heterocercal tail.

3). Swim bladder/Air bladder: Also called the gas bladder. The primary function is hydrostatic leading to neutral buoyancy.

The evolution of swim bladder helped the fishes to maintain or change their depths of occurrence with the least expenditure of energy. The other functions like sound reception or production (resonating chamber) are secondarily derived. The swim bladder has become so specialized for non-respiratory functions that accessory respiration in teleosts is by other means than air bladder.

The swim bladder may be 'physostomous' with a pneumatic duct, mainly seen among the lower teleosts or 'physoclistous' without the duct, seen among the higher teleosts. The duct functions primarily as a means for regulating the volume of gas. The 'rete mirabile' the capillary net work of arteries and veins takes up the function in the derived physoclistous swim bladders (Fig.2).

4). Bony operculum: The bony operculum consisting of the opercle, subopercle, preopercle and interopercle covers the

fully filamentary gills. The newly derived dermal branchiostegal rays connect the bony operculum to the hyoid arches, helping in the flexing of the branchial chamber for increased

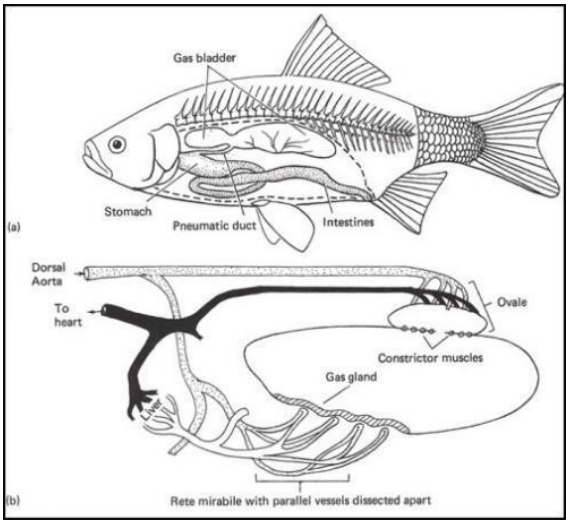


Fig.2. Physostomous and physoclistous swim bladders (www.cssforum.com.pk)

ventilation and greater respiratory efficiency (active pumping). Another off-shoot of this sequence of evolution is the double-pump suction feeding leading to different specialized feeding habits in structured communities (e.g. coral reef fishes, lake cichlids). The interopercle is a modification of the branchiostegal ray and a definite feature of modern teleosts.

The evolution of branchiostegal rays has been another important factor in teleostean diversity. Among the lower teleosts the membrane-connected rays are large in number and variable and almost equal sized (broom-like) and less efficient, while among the higher teleoststhey are reduced (often 4 – 7) and arranged in an increasing series, being highly efficient bellows, leading to greater ventilatory efficiency

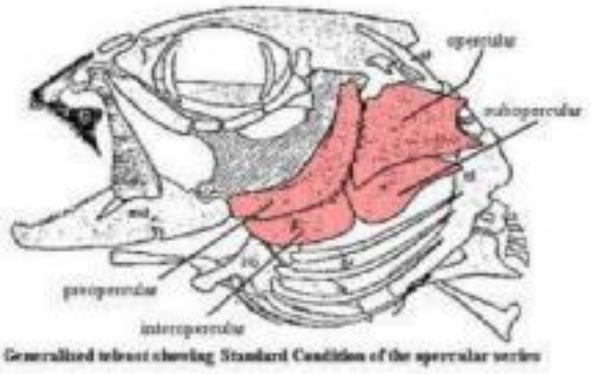


Fig.3. The opercular bones, branchiostegal rays and the jaw bones (www.palaeos.com)

5). Jaws: The jaw suspension is of the 'hyostylic' type, the upper and lower jaw bones being connected to the neurocranium via the hyomandibular bone, especially freeing the upper jaw elements from the neurocranium. The upper jaw is constituted by the new dermal bones, the premaxilla and maxilla. Among the lower teleosts the premaxilla and maxilla are included in the gape of the mouth. The maxilla is the main tooth-bearing bone and the premxilla is small.

The evolution of the jaw bones is very dramatic among the higher teleosts. The premaxilla is the tooth-bearing gape bone with the corresponding ascending, articular and maxillary processes, while the maxilla has become the supporting bone with the prominent maxillary head and rostral cartilage (Fig.3). This sequence of evolution has led to the protrusibility of the mouth leading to a wide range of new feeding adaptations and habits. The lower jaw elements are reduced to the main dentary, the angular and articular. The jaws thus changed from being rigid,

toothed structures adapted for biting and grabbing to much more flexible structures, often without teeth, adapted for feeding by a wide variety of methods (e.g. Cichlids of the great lakes of Africa).

6. Scales: Bony ridge scales with two thin layers of unmineralised bone lacking ganoine, making them lighter and more flexible, sometimes referred to as 'elasmoid' scales. Even there is elimination of scales in many forms. By structure they may be cycloid (lower teleosts) or ctenoid (higher teleosts)

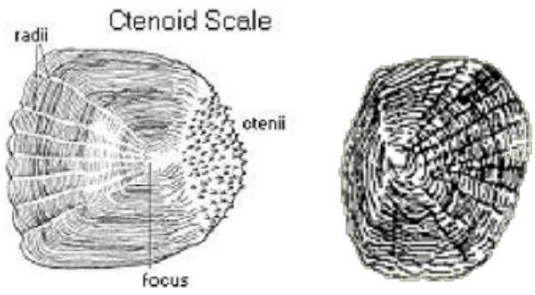


Fig.4. Ctenoid and cycloid scales (www.eoearth.org)

Modified scales like lateral line scutes (carangids) and ventral scutes (clupeids), bony plates (armoured catfishes), bony case (box fishes), and spines (porcupine puffers) are seen as adaptations.

7. Fins and fin-rays: The fins with soft rays (segmented, often branched) and spiny rays (unsegmented, unbranched, feeble or hard), which are mobile and are connected by membranous web. The fins can be folded or erected except for the caudal fin. In general the pelvic fins are backwardly placed (abdominal) and pectoral fins are ventral with horizontal base in lower teleosts, which are generally elongated, sub-cylindrical pelagic fishes. The pelvic fins are more thoracic and the pectoral fins are laterally placed with vertical fin base in higher teleosts many of whom are deep-bodied, living in structured communities.

During the evolution from the lower teleosts to the higher teleosts the outer fin ray elements are reduced and the inner bony elements (pterygiophores of dorsal and anal fins; radials of pectoral; hypural elements of caudal) are increased in number to have greater control over the fin rays and thereby on maneuverability, especially in fishes living in structured communities.

8. Size and shape: Majority of the teleostean fishes are small in size (< 30 cm) e.g., cyprinids, gobies, characins, cyprinodontids, clupeids, percoids. They come in all varieties of body shapes, from elongated rounded, elongated compressed, to deep laterally compressed, to globular, to box-shaped, to needle-like. Many are sleek and fusiform, while some are grotesque and ugly, mimicking the surroundings. These characters made them capable of occupying microhabitats and niches not previously occupied by fishes

.

9. Colour: The lower teleosts in general being pelagic fishes show counter shading with dark dorsal surface and light silvery ventral half. Majority of the higher teleosts occupying the structured colourful communities like the planted freshwaters and the rich coral reefs are brilliantly coloured (disruptive, cryptic, warning, camouflage) helping them to

communicate, or merge with the surroundings. In communities the fishes show a lot of social interaction through communication (visual, sound, and chemical).

10. Specialisations: In addition to these generalized characters the teleosts show specializations depending on their habitats and behavior. Some of the better examples are i) fine tuned hearing (weberian apparatus) and alarm substance and fright reaction in the primary freshwater otophysean fishes ii) bioluminescence in the deep-sea fishes iii) poison and body toxins iv) electric organs v) accessory respiration vi) hermaphroditism vii) gynogenesis viii) internal bearing ix) schooling and shoaling x) migration and homing and xi) symbiotic relationships The teleostean evolution has thus resulted in fishes which are capable of rapid and complex locomotion; have very efficient ventilation and thereby respiratory efficiency; use a wide variety of food materials (autochthonous and allochthonous); and have an array of complex reproductive styles and strategies. Thus these fishes came to occupy the microhabitats and niches in the aquatic environment up to then unavailable or unoccupied by the ancestral fishes.

Finally, it is worth noting that fish did not evolve independently of their environment, but both were shaped by them and shaped through their activities. For example, the structure of coral reefs is very much the result of evolutionary interactions of teleost fishes and reef-building invertebrates, where the invertebrates are continually evolving defenses against fish predation, while the fish evolve mechanisms to overcome these defenses, literally an ‘arms race’.

SUGGESTED READINGS

Moyle, P.B. and J.J. Cech. 1982. *Fishes: An introduction to Ichthyology*. Prentice Hall, Inc., New Jersey. 593 pp.

Nelson, J. S. 2006. *Fishes of the World*. Fourth Edition, John Wiley and Sons, Inc., New Jersey. 601 pp

Principles of Population Genetics

N.S. Jeena
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Population genetics is the study of genetic composition of populations, including distributions and changes in genotype and phenotype frequency as a result of operation of evolutionary forces of natural selection, genetic drift, mutation and gene flow. This branch of evolutionary biology focus on the variationin alleles and genotypes within the gene pool and investigates how this variation changes from one generation to the next. **Gene pool** is the collection of all the alleles of all of the genes found within a freely interbreeding population. Each member of the population receives its alleles from gene pool of its parents and passes them on to the gene pool of offspring. Population genetics was developed as a theoretical discipline by R. A. Fisher, J. B. S. Haldane and Sewall Wright.The fundamental importance of this branch is the basic insights it provides into the mechanisms of evolution.

Main ideas of population genetics

Gene is the segment of a DNA molecule that codes for the synthesis of a single polypeptide. Genetic variation describes differences between the DNA sequences of individual genomes. Each individual has two nuclear genomes (a paternal genome and a maternal genome). Allele is alternative form of a gene that occurs at the same locus on homologous

chromosomes. A locus is any location on a chromosome, or any region of genomic DNA that is considered to be a discrete genetic unit for the purpose of linkage analysis or molecular genetic studies. A locus is monomorphic if there is only one allele in the population and polymorphic if there are two or more alleles in the population at appreciable frequencies. At any genetic locus the maternal and paternal alleles normally have identical or slightly different DNA sequences. If the two alleles are identical, they are homozygotes and if different, termed as heterozygotes.

Every individual has both a phenotype and a genotype. The **genotype** is the specific set of genes carried by the individual. The **phenotype** is the set of characteristics (e.g., morphological, physiological, behavioral) expressed by the individual. The phenotype is produced by the genotype in interaction with environment. **Genotypes** are formed due to pairing of alleles during union of gametes for zygote formation. It is the set of alleles an organism carries at one or more loci. If the number of alternative alleles is `n`, there will be n (n+1)/2 possible genotypes. The

genotypes of the parents are broken down in the gametes to form a new set of genotypes in the progeny. Consequently, only alleles have continuity over time while the genotypes do not, and the gene pool evolves through changes in the frequencies of alleles.

The fundamental quantities in population genetics are frequencies of genes (alleles) and genotypes by which the genetic structure of populations is expressed. A **frequency** is a proportion or a percent. It always ranges between 0 and 1. **Genotypic frequency** is the number of individuals with one particular genotype divided by the total number of individuals in the population. The sum of the genotypic frequencies should be 1. The distribution of genes in different individuals of the population is the **allelic (gene)frequencies**. When allelic frequencies are used, gene pool can be described with fewerparameters. Allelic frequencies may be calculated from observed numbers of different genotypes at a particular locus, or from the genotypic frequencies. The frequencies of two alleles are commonly symbolized as *p* and *q* where *q= 1–p*. A third allele is symbolized as *r.*

There are several basic evolutionary processes that act on a population and cause genetic variation. The first and the most fundamental is **mutation** which is defined as any heritable change in the genetic material. Mutation is the ultimate source of all genetic variation without which evolution cannot be materialized. **Recombination** is the secondary source of genetic variation which can create new combination of alleles, but not new alleles. New recombination of alleles can lead to new phenotypes upon which natural selection can act. **Natural selection** means that individuals with heritable favorable variations survive and reproduce at a higher rate than other individuals in the population. It operates through differences in the fertility of parents that is controlled by genes. **Fitness** is a concept related to selection. It is the lifetime reproductive output of the individual. The average fitness of all individuals in the population is called **population fitness** or mean fitness.

Another important evolutionary process is the **genetic drift**. Random change in allelic frequency from one generation to the next due to repeated random sampling of gametes from a population is called genetic drift or simply drift. It is also called the *Sewall Wright Effect*. Genetic drift will cause isolated populations to diverge from oneanother.

Gene flow is a source of genetic variation which introduces new alleles to thepopulation and spreads unique alleles to other populations, long-term effect of which is the opposite of genetic drift. Through exchange of genes, different populations remain similar, and thus **migration** is a homogenizing force that tends to prevent populations form accumulating genetic differences among them.

Panmixia means ***random mating*** of individuals within a population with no

tendency to choose partners with particular traits.**Non-random mating** occurs when mating individuals are genetically related to one another or are phenotypically similar to each other than two individuals chosen at random.

Population genetics deals with how the evolutionary forces distribute DNA polymorphism in biological populations. For evolution of a species to occur, the gene frequencies of that population must undergo change and the evolutionary processes described above play a major role in it.

The Hardy-Weinberg law

Hardy-Weinberg law, formulated in 1908, is a mathematical model and is the fundamental principle in population genetics because itoffers a simple explanation for how the Mendelian principles that result from meiosis and sexual reproduction influence allelic and genotypic frequencies of a population. The assumptions or conditions for the law to apply in a population are that, it should be (1) infinitely largerandomly mating (3) free from mutation (4) migration and (5) natural selection. The law states that "In a large random-mating population with no selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation". When the genotypes are in the proportions, the population is said to be in Hardy-Weinberg equilibrium. Genotypic frequencies after one generation of random mating are given by terms in the expansion of (p + q) 2 = p2 + 2pq + q2 where *p* and *q* represent allele frequencies. An important use of the H-W law is that it provides a mechanism for determining the genotypic frequencies from the allelic frequencies when the population is in genetic equilibrium.

Testing for Hardy-Weinberg proportions

The H-W law can be used as null model to which the genetic structure of any particular population can be compared. From allelic frequencies, we can calculate the expected genotypic frequencies and compare these frequencies with the actual observed frequencies of the genotypes using a chi-square test.

Factors affecting genetic structure of populations

In contrast to idealized populations at Hardy-Weinberg equilibrium, real stocks and populations experience changes in allelic and genotypic frequencies. Two types of processes cause these changes: dispersive processes (inbreeding and genetic drift) and systematic processes (mutation, migration and selection). Dispersive processes cause changes in allelic and genotypic frequencies that are random in direction and amount. Systematic processes cause changes that are consistent and predictable. The allelic and genotypic frequencies of any population are due to the combined effects of inbreeding, genetic drift, mutation, migration, and selection.

Mutation converts one allelic form of a gene to another. A1to A2is called a

forwardmutation; A2 to A1 is called a reverse mutation. The rate of mutation is generally low, but varies among loci and among species. Mutations can change the frequencies ofalleles. Mutation provides the raw genetic material for evolution. Most mutations will be detrimental and will be eliminated from the population.

Migration has the potential to disrupt H-W equilibrium and may influence theevolution of allelic frequencies within populations. Migration among populations tends toincrease the effective population size (Ne) of the populations and divergence among populations.

Natural selection is the dominant force in the evolution of many traits and has shapedmuch of the phenotypic variation observed in nature. Selection in natural populations can be *a)* Directional selection *b)* Stabilizing selection or *c)* Disruptive selection

Fitness and the coefficient of selection.

We measure natural selection by assessing reproduction. It is measured in terms of **fitness** which is defined as the relative reproductive ability of a genotype orcontribution of offspring of an individual to the next generation. Often symbolized as ***W,*** and is also called the adaptive value or selective value of a genotype**. Selectioncoefficient**,***s,***is a measure of the relative intensity of selection against a genotype and***s*** = 1-*W*. The coefficient measures the selective advantage of the fitter genotype, or theintensity of selection against the less fit genotype.

Genetic drift occurs due to changes in allelic frequency in small populations due tochance or sampling error. When the sample is small, the sampling error can be large. Based on population size we can make predictions about the magnitude of drift, direction of which is unpredictable. If the sexes are equal and all individuals have an equal probability of producing offspring, the effective population size is the effective number of breeding individuals.

When males and females are not present in equal numbers the effective population size (Ne) is

Ne= 4 x Nf x Nm / Nf + Nm

Other factors such as differential production of offspring, fluctuating population size, and overlapping generations can further reduce the effective population size. The amount of variation among populations resulting from genetic drift is measured by the variance of allelic frequency: sp2= *pq*/2Ne

There are several ways in which genetic drift via sampling error occurs in natural populations.

1). Small population size over many generations

2). Founder effect - It is the loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population. Although the population may subsequently grow in size, the gene pool

of the population is derived from the genes present in the original founders. This has a profound effect on the gene pool in subsequent generations.

1). Bottleneck effect is a form of genetic drift that occurs when a population size is drastically reduced. Some genes may be lost from the gene pool due to chance events. This can be considered as a form of founder effect, since the population is refounded by those few individuals that survive the reduction.

The genetic drift causes *a)* change in allelic frequencies of a population over time and cause fixation and extinction alleles *b)* Reduction in genetic variation within populations *c)* Differentiation between sub-populations and *d)* Increased homozygosity.

Nonrandom mating- In order for the H-W principle to hold, individuals in apopulation must mate at random. But many populations do not mate randomly for some traits, and when nonrandom mating occurs, the genotypes will not exist in H-W equilibrium.

In the broad sense, there are three kinds of nonrandom mating. **Assortative mating** occurs when two mating individuals are phenotypically more alike than two individuals chosen at random. **Disassortative mating** occurs when mates arephenotypically less alike than two individuals chosen at random. Neither affects the allelic frequencies, but both may affect the genotypic frequencies if the phenotypes are genetically determined.

Inbreeding is the mating of consanguineous individuals (individuals having a commonancestor) which may have alleles that are identical by descent. Two alleles are identical by descent if they have originated from the replication of one single gene in the previous generation. Inbreeding is always defined relative to some reference population in which all individuals are assumed to be unrelated. It is often measured as the **coefficient of inbreeding (*F*)**which is the probability that two alleles in an individual are identical by descent and can be calculated from pedigrees. It can also be defined as the proportionate reduction in heterozygosity compared to a reference population.

F = Expected heterozygosity - Observed heterozygosity / Expected heterozygosity

In a finite population, the average inbreeding coefficient increases each generation, the rate of which depends on population size. Self-fertilization decreases heterozygosity by half each generation and in random mating, *F*=0

Inbreeding depression occurs when inbred individuals have lower fitness than non-inbred individuals because of the increased homozygosity with harmful alleles in double doses and lower frequencies of heterozygotes. Outbreeding is the opposite of inbreeding; *i.e.* mating between individuals less related than the average in the population. The outbred population can have higher fitness than any of the

involved inbred populations because of "hybrid vigour", but **outbreeding depression** can also occur if mating occur between substantially diverged populations.

Population subdivision

If subpopulations are completely isolated and differ in allele frequencies, but are sampled without distinguishing between them, there will be a perceived deficiency of heterozygotes in the sample, even though there is no such deficiency in any subpopulation. This is called **Wahlund effect**.

In a subdivided population, the overall deviation from H-W expected heterozygosityhas two components: the deviation due to factors acting within subpopulations, andthe deviation due to subdivision (Wahlund effect). Wright (1951) defined three *F*coefficients that describe these divisions *viz. FIS,FIT& FSTwhich* are related by theexpression

(1- *FIS*) (1- *FST*) = 1- *FIT*

FIS is a statistic used to estimate the deviations of genotype frequencies from the H-Wmodel within subpopulations which make up the total population. *FIT* is a statistic used to estimate deviations from the H-W model within the total population. *FST* is an index genetic differentiation used to describe the degree to which a total population is genetically divided into subpopulations and can vary between 0-1. Nei (1973) developed a similar statistic to *FST* called the coefficient of genetic differentiation *GST* which is based on the heterozygosity values across local and total population. *FST* and *GST* are valuable measures of population subdivision.

Genetic variation in natural populations

One of the most significant questions addressed in population genetics is how much genetic variation exists within natural populations. Phenotypic values of quantitative traits are almost jointly determined by the effects of many genes and environmental effects.

Genetic variation is important for several reasons:

It determines the potential for evolutionary change and adaptation.

1). The amount of variation provide us with important clues about the relative importance of various evolutionary processes, since some processes increase variation and others decrease it.

2). The manner in which new species arise may depend upon the amount of genetic variation harbored within populations.

3).Evolution by natural selection depends on the existence of genetic variation within a population.

4). Variation that is not subject to natural selection is useful in DNA fingerprinting, conservation biology, and empirical population genetics. Genetic variation can be quantified by estimating genotype and

allele frequencies and also by considering the proportion of polymorphic loci in a population (*P*)or by the observed heterozygosity. The genetic variation can be measured both at protein and DNA levels.

Measuring genetic variation via DNA sequence variation

Molecular markers provide useful tools for studying mutation rates, population sizes, natural selection, population structure, and other important ideas in evolution and conservation biology. The power of molecular markers to display genetic diversity between and within populations attract extensive interest due to usefulness of this information in breeding and conservation programs.

These techniques fall into two main kinds: Direct methods using DNA sequencing technology, and indirect methods using restriction enzyme analysis and other molecular techniques to infer variation in DNA sequence.

DNA sequencing has revealed high levels of variation in nucleotide sequences. Single nucleotide differences (SNPs) are the most common kind of variation at the DNA level. Newer techniques like DNA micro arrays or gene chips allow rapid automated detection of SNPs. The variation at DNA level can be quantified by calculating the nucleotide polymorphism (the proportion of nucleotide sites that are polymorphic in a sample) and nucleotide diversity in the population.

Indirect estimates of DNA variation are cheaper and faster than DNA sequencing and are useful for preliminary screening of a large number of individuals. Restriction fragment length polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Single Strand Conformation Polymorphisms (SSCPs) etc. come under this.

Certain kinds of satellite DNA in eukaryotic chromosomes are dispersed more or less randomly throughout the genome. Each cluster of repeats is considered a locus and the number of repeats at a particular locus is variable. Such loci are called the VNTR loci or variable number of tandem repeats. There are two kinds of VNTR loci depending on the length of repeat sequence *viz.* Minisatellite loci and microsatellite loci, both of which are useful genetic markers. Microsatellite loci are highly variable and can be analyzed locus by locus, hence more powerful for detection of genetic variation at population level. Variation in length of DNA sequences is also common and is frequently due to insertion or removal of transposable elements.

Population genetics has wide applications in fisheries.An important prerequisite for elaborating management strategies in fisheries is identifying the number of reproductively distinct populations, the presence of which can be confirmed by genetic assessments. Significant differences in allele frequencies will be detected when populations are isolated for a sufficient number of generations.

Population genetics has also role in the management of hatchery populations. Genetic changes to hatchery stocks can occur through selection, drift, or stock transfers. Inbreeding is a major problem in hatchery stocks. It is influenced by a number of factors like the number of breeding individuals in the population, sex ratio, variation in the reproductive success of individual spawners, and effective population size during previous generations. The effective size of hatchery populations should be as large as possible in order to minimize loss of genetic diversity. It is suggested that Ne of 500-1000 should be maintained to prevent inbreeding and genetic drift related problems and to maintain the genetic variance in fish populations that are used for stocking programmes. Yet, Ne has to be customized at the level of farmers as it may not always be possible to maintain the recommended numbers.

SUGGESTED READINGS

Allendorf, F. W., G. H. Luikart and S. N. Aitken. 2013. Conservation and the Genetics of Populations. Second edition. Wiley-Blackwell, U. K.

Beaumont, A., P. Boudry and K. Hoare. 2010. Biotechnology and genetics in fisheries and aquaculture. John Wiley and Sons. pp. 1-176.

Falconer, D. S. and T. F. C. Mackay. 1996. Introduction to Quantitative Genetics. 4th ed. Longman, London. pp. 1-375

Halliburton, R. 2004. Introduction to population genetics. Upper Saddle River: Pearson/Prentice Hall. pp. 1-587.

Hamilton, B. M. 2009. Population Genetics. Wiley-Blackwell, U.K.

Hauser, L., G. J. Adcock and G. Carvalho. Long-term genetic changes in exploited populations: Effects of fishing on New Zealand snapper. courses.washington.edu

Kapuscinski, A. R. and L. M. Miller. 2007. Genetic guidelines for fisheries management. Second Edition.University of Minnesota. pp. 1-113.

Morrison, B.P. 2012. An annotated bibliography on the impacts of fish hatchery supplementation and enhancement on wild populations. Ganaraska Region Conservation Authority, Port Hope, Ontario. pp. 54.

Tave, D. 1999. Inbreeding and brood stock management (No. 392). Food and Agriculture Organization.

Waples, R. S. 1991. Genetic interactions between hatchery and wild salmonids: lessons from the Pacific Northwest. Canadian Journal of Fisheries and Aquatic Sciences, 48: 124-133.

Advancements in molecular markers used for biological investigations

Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Understanding the genetic variation within and between species is important to make inferences regarding conservation needs in varied species. Genetic diversity and variation can be studied using molecular markers like allozymes, mitochondrial DNA markers, microsatellites, single nucleotide polymorphisms and many other advanced tools. Genetic variation leads to differentiation at the level of population, species or higher order taxonomic groups which can be accurately delineated using molecular markers. The use of molecular markers in fisheries and aquaculture include, species identification, genetic stock structure investigations, understanding the impact of hatchery released populations to wild habitats, comparing the genetic diversity of hatchery and wild populations, understanding demographic patterns of natural populations and adaptation and selection to different environmental conditions.

The molecular markers can be categorized as Type I and Type II markers. When molecular markers are associated with genes of known function they are called type I marker and those associated with genes of unknown function are known as type II markers. Allozyme markers can be categorized as type I marker as the proteins they encode are associated with some known functions. Microsatellites and other neutral markers are type II markers as they are in most cases, not associated with any known function.

ALLOZYMES

Allozyme method was one of the most popular method for analyzing genetic variation and stock structure in fishes. Allozymes are codominant markers which will get expressed in heterozygous individuals in a Mendelian fashion. Allozyme analysis will provide us information regarding single locus genetic variation among populations and hence ideal for genetic stock structure investigations. The method of allozyme analysis involves extracting allozymes from tissues using standard protocols. Subsequently allozyme variation can be detected using electrophoresis in an acrylamide or cellulose acetate gel. Homozygous individuals will exhibit a single band whereas heterozygous individuals will show two bands. Simplicity, low cost and requirement of little specialized equipments made allozymes the molecular markers of choice for genetic studies. Soluble proteins of any kind can be used for allozyme analysis and a large number of loci can be screened simultaneously. The main limitations of allozyme method are; requirement of

large tissues samples (samples like larvae could not be studied); invasive method of sampling; point mutations could not be detected as many point mutations will not result in amino acid change. In spite of all these limitations, allozyme analysis has been widespread in fisheries science for making inferences in fish systematic, population genetic structure, conservation genetic, mixed stock analysis and forensic investigations.

MITOCHONDRIAL DNA

Mitochondrial DNA is located in the mitochondria of the cells and hence is termed as non-nuclear DNA. It is inherited maternally with a haploid genome and entire genome undergoes transcription as one single unit. Mitochondrial DNA is not subjected to recombination and hence they are homologous markers. Mitochondrial DNA is selectively neutral and occurs in multiple copies in each cell. As they are physically separate from the rest of cell's DNA, it is easy to isolate them from any tissue samples. The effective population size of mitochondrial DNA is smaller than nuclear DNA due to their maternal inheritance which makes them sensitive to population bottlenecks and hybridizations. There are several direct and indirect methods to understand the variations in nucleotide sequence of mtDNA. Restriction Fragment Length Polymorphism analysis involves digesting the whole mtDNA with restriction endonucleases and visualizing the fragments on a gel. DNA sequencing of small fragments of mitochondrial genes and analyzing the sequences is another method. The simplicity and accuracy of these methods made mitochondrial DNA a marker of choice for many investigations.

Several sets of universal primers have been developed from conserved sequence regions of mitochondrial DNA which are widely used. Inter specific comparisons are made using slow evolving gene regions and intra specific comparisons using fast evolving gene regions. Mitochondrial control region is fast evolving and used for population comparisons. Cytochrome b and NaDH dehydrogenase genes (ND1, ND2) are also being used widely. Conserved nature of cytochrome c oxidase 1 gene has made it a universal barcode for species level differentiation. DNA barcodes are approximately 650bp and this region of mitochondrial DNA has been used for cataloguing the biodiversity of organisms. Mitochondrial genes have found wide applications in taxonomy, evolutionary biology and population genetics studies.

NUCLEAR DNA MARKERS – RANDOM MARKERS

Arbitrary nuclear markers target DNA fragments of unknown function. RAPD (Random Amplified Polymorphic DNA), ISSR (Inter- Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism) are widely practiced methods for amplifying unknown regions of DNA. An arbitrary primer is used to amplify unknown loci in RAPD. It is fast, cheap and exhibits high levels of polymorphism an prior knowledge of genetic constitution of an organism is not

required for using RAPD markers. The major drawback with RAPD markers is the lack of reproducibility and repeatability across labs and a large number of products are generated. It is considered as a dominant marker as homozygous and heterozygous loci cannot be differentiated. RAPD patterns are sensitive to minor changes in amplification conditions.

Inter- simple sequence repeats (ISSRs) are parts of the genome flanked by microsatellite sequences. When these regions are amplified using PCR primers several amplification products are obtained and this can be used as a dominant multilocus marker system for genetic diversity studies. Similar to RAPD markers, ISSR markers are easy to use, low-cost and less demanding methodologically which makes it an ideal marker system for beginners. It is also suitable for those organisms whose genetic information is lacking. Similar to RAPD markers, the dominant pattern of inheritance combined with its random nature make it less accurate.

Amplified Fragment Length Polymorphism (AFLP) markers combine the advantages of both RFLP and RAPD. Two restriction enzymes are used to digest total genomic DNA. Further, double-stranded nucleotide adapters are ligated to the ends of DNA fragments which will serve as binding sites for primers for PCR amplification. Primers complementary to the adaptor and restriction site sequence with additional nucleotide sequences at the 3'-end are used to amplify ligated fragments. The presence or absence of DNA fragments are detected on polyacrylamide gels to detect polymorphisms.

NUCLEAR DNA MARKERS – SPECIFIC MARKERS

Variable Number of Tandem Repeats are segments of DNA which are tandemly repeated tens, hundreds or thousands of times in nuclear genomes. Tandem repeats vary in number in many loci and between individuals. Two main classes of highly polymorphic and repetitive DNAs are found in individuals; minisatellite and microsatellite DNA. Genetic loci with repeats varying in length 9-65bp are called minisatellite DNA and those with repeats of 2-8bp in length microsatellite DNA. Microsatellites are found abundantly in genome than minisatellites. These loci are widely used in population genetics of vertebrates and invertebrates. Minisatellites are classified as multilocus and single locus and mainly used for parentage analysis. They are not very useful for population genetic studies due to the complexity of mutation processes undergone by them. Most of the research work on minisatellites are focused on single locus minisatellite probes and they have been successfully used to detect genetic variations between populations. Minisatellites have also been widely used for forensics, genetic identity testing, parentage analysis, confirming gynogenesis and androgenesis and several other processes.

MICROSATELLITES

Microsatellites are repeated DNA sequences and found several times across various positions of DNA of an organism. Since microsatellites are highly variable they can be used as markers. Microsatellites are found once in every 10kbp whereas minisatellite in every 1500kbp in fishes and hence microsatellites are very useful in genome mapping and genetic stock structure studies.

As microsatellites are highly variable, non-coding and considered as selectively neutral, the basic assumption on using microsatellite is that the sequence divergence is proportional to the length of time since separation. Microsatellites get inherited in a Mendelian fashion and they are codominant markers which evolve with 10-3-10-4 mutation/generation. Microsatellites have become very popular due to the high levels of polymorphism. Cross- amplification or in other words use of primers used in closely related species reduces the cost associated with detecting microsatellite sequences in a different species. Microsatellite loci is amplified using specific primers from genomic DNA in a PCR machine and visualized using poly acrylamide gel electrophoresis (PAGE). Automated genotyping using labeled primers are used to analyse the size polymorphisms which is carried out in genotyping machines. Due to automated genotyping large number of loci can be screened which increases precision, accuracy and speed. Major constraints while using microsatellite markers are presence of null alleles and stutter bands. Null alleles are formed due to mutations occurring at primer binding sites of microsatellite locus. Loci exhibiting null alleles should be removed from analysis as null alleles reduce accuracy especially when parentage or related analyses are being carried out. Stutter bands are bands which differ in 1-2bp and they occur due to slipped strands impairing during PCR or incomplete denaturation of amplification products. The problem can be solved by amplifying tri-nucleotide and tetra-nucleotide repeats as these loci do not show significant amounts of stuttering. Microsatellite markers are very important for fisheries research especially for phylogenetic investigations, phylogeography, genetic stock structure, conservation of biodiversity, impacts of stocking and hybridization. It can also be used for forensic identification of individuals, mapping of genome and understanding kinship and behavioural patterns.

SINGLE NUCLEOTIDE POLYMORPHISMS

Single nucleotide substitutions like transitions or transversions and insertions or deletions bring about single nucleotide polymorphisms in the genome. These point mutations produce different alleles with alternative bases at specific nucleotide positions. SNPs are the most abundant polymorphisms in the coding and non-coding parts of the genome which can be detected using PCR, microchip arrays or fluorescence technology. SNPs are markers of next generation and can be employed for varied applications like genomic

investigations, climate change impacts and detection of diseases.

DNA MICROARRAYS OR DNA CHIPS

DNA microarrays are small glass microscope slides, silicon chip or nylon membranes which contain many immobilized DNA fragments arranged in a standard pattern. They are used as a medium for matching reported probes for known sequences against the DNA isolated from the unknown target sample. It is possible to incorporate species-specific DNA sequences to a DNA microarray for identification purposes. DNA isolated from target samples should be labeled with specific fluorescent molecules and hybridized to the microarray DNA so that when the hybridization is positive a fluorescent signal is detected with appropriate fluorescent scanning or imaging equipment.

EXPRESSED SEQUENCE TAGS (ESTS)

ESTs are single pass sequences which could be generated from random sequencing of cDNA clones. ESTs are very useful for identification of genes and understand their expression by means of expression analysis. These markers can be used for studying the genes expressed in particular tissue types under specific physiological conditions or developmental stages with speed and accuracy. cDNA microarrays can be used to identify differentially expressed genes in a systematic way. ESTs are very valuable for linkage mapping.

Inferring phylogenies using Molecular systematic tools

Sandhya Sukumaran
Marine Biotechnology Division, Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The evolutionary biology of an organism is very important as it is the foundation upon which species exist. Systematics explores the evolutionary relationships between organisms using scientifically validated methods and classifies them into discrete groups. Classification is carried out by considering their phenetic or phylogenetic characters, naming them and placing them into previously described groups. Systematics is in other words studying the evolutionary patterns on a temporal scale. Tremendous advancements have been made in molecular biology and bioinformatics during the past decade which provided powerful tools for the study of molecular systematics. Molecular systematics use the informationcontained in molecular data for reconstruction of phylogenetic relationships. Phylogenies are increasingly being used by many to solve problems in evolutionary biology.

Analysis of DNA or protein sequence variation has become the standard practice for many molecular biology investigations as it has many advantages compared to traditional morphology based methods. The advantage of molecular systematics is that there are character types and states that are universal (many times character states will be similar due to homology or in other words inheritance from a common ancestor), increased availability of character states, substitution rates vary among regions of gene, molecular evolution is well studied, availability of advanced statistical packages and easiness of collection of data from various taxa. A large number of sequence data is available for many taxa which augmented possibilities for phylogenetic comparisons and analysis. But even these data have limitations as character states for DNA data is only four and amino acid data 20. So a high probability of homoplasy or similarity in character states is possible due to the substitution processes getting saturated.

HOW PHYLOGENETIC INFORMATION IS INFERRED?

Molecular systematics relies on inferring phylogenetic information using evolutionary models. Most of the individuals are related directly or indirectly by sharing common ancestors and the extent of genetic divergence provides clues to the type and degree of relatedness among and between individuals in populations. The first step in molecular phylogenetic inference is acquiring sequence information by sequencing a particular gene or it can be

retrieved from NCBI, GenBank. The sequences are to be then aligned and subsequently mutations like insertions or deletions could be identified. If needed gaps could be inserted to the sequences to increase similarity between sequences. Several algorithms are available to align multiple sequences and these algorithms try to reduce the total effect of all the possible changes in pair wise sequence comparisons. If some sequences are not properly aligned, they shall be removed from the dataset. A phylogenetic tree could be constructed from sequence alignment data and the tree rooted using outgroup sequences from distantly related species which will provide insights into the evolutionary relationships. The tree topology will be affected depending on the outgroup.

In addition to sequence data and alignment, a suitable model of sequence evolution must also be selected as there are several assumptions about substitution processes in molecular phylogenetics.

COMMONLY USED METHODS FOR PHYLOGENETIC INFERENCE

Phylogenetic inference has been widely carried out mainly by four methods; maximum parsimony, neighbor-joining, maximum likelihood and Bayesian inference.

Maximum parsimony is one of the oldest inference methods for phylogeny reconstruction which uses character states as compared to distance methods. It is method based on the fewest assumptions. Parsimony is a non-parametric method as it does not require any parameters and does not estimate branch lengths. The optimality criterion is used to decide the best alternative tree by selecting fewest character state changes minimizing homoplasy. The length of an unrooted tree is calculated directly using Fitch's algorithm which moves through the tree assigning one or more character states to each of the internal nodes. Searching the tree space (possible tree topologies for a given number of taxa) is done by heuristic or exact searches. Exact searches look for all possible trees (carry out exhaustive searches) or parts thus guaranteeing the optimal tree whereas heuristic searches do not search for all possible trees. The advantage of maximum parsimony analysis is that it takes very less time for large data sets containing many sequences and robust estimates are obtained when branches of trees are very short. But when there is substantial variation in evolutionary rates, maximum parsimony performs poorly.

Distance trees

Distance methods focus on pairwise dissimilarities in sequences between species. A distance matrix is defined and depending on the model of evolution and the hierarchical associations among species is defined. Closely related species have more similarity and higher similarity indices than distantly related species. But the difficulty with these methods is that one distance matrix may define more than one tree mathematically and we need some algorithms to find out the most

optimal tree for a given matrix. Either the most closely related species are aggregated or distantly related are split.

Neighbour joining is a distance method which is widely used. Neighbour joining method is based on the assumption that dissimilarity between any sequences is related directly to their phylogenetic relationship. Dissimilarity is arisen from the evolutionary distance between two species. DNA or amino acid sequences are first converted to distance matrix and phylogenetic tree explored. In optimality methods, the score of a tree is evaluated based on the squared deviation of the pair wise observed distances between each pair of taxa estimated from the data matrix and the distance separating those taxa on the tree. Distance methods work faster than all other methods andperform well when divergence between sequences is low. When sequences are converted to distances loss of information occurs and reliable estimates cannot be obtained when the sequences are highly divergent.

Maximum likelihood is based on the estimation of a likelihood function which describes how a given tree can be fitted to the sequence data. An explicit model is chosen for character state evolution and a phylogenetic tree proposed for with branch lengths. The likelihood of a phylogenetic tree is the probability of observing the data given the tree and the model of evolution. This also uses an optimality method. Finding the tree with highest likelihood will estimate the phylogenetic hypothesis that has the highest probability of producing current sequences under the probabilistic model of sequence evolution considered. This method is considered generally as a robust method even though evaluating likelihood surface may take a long time for large data sets. The best tree produces the observed sequences most likely to have evolved under the expected evolutionary model. Heuristic searches are used to explore the tree space and likelihood allows the inference of phylogenetic trees using complex models of sequence evolution. The disadvantage of maximum likelihood methods are that the results are highly dependent on the model of sequence evolution used and when sequences are large, the method is computationally highly demanding.

Bayesian inference is based on the Bayesian theorem and it is the most recent phylogenetic inference method. It is most closely related to the maximum likelihood method and the optimal hypothesis is the one which can maximize the posterior probability. According to Bayes' theorem, the posterior probability for a hypothesis is proportional to the likelihood multiplied by prior probability of the particular hypothesis. In Bayesian analysis, complex models of sequence evolution can be applied for the whole sequence data set and different partitions of it. A model along with a prior distribution can be specified and the product of these quantities can be integrated over all parameter values that are possible and based on these the posterior probability values of each could be determined. Analytical integration of likelihood functions for phylogenetic

models are too complex and hence Bayesian approaches dependonMarkov chain Monte Carlo (MCMC) procedures. In MCMC procedure, trees are sampled for the distribution of posterior probabilities. Bayesian MCMC looks for the "best set of trees" in the tree space unlike maximum likelihood which looks for the most likely tree. Bayesian analysis works on a strong statistical foundation as in maximum likelihood method and gives a robust approximation of the evolutionary tree. The disadvantage of Bayesian methods is that prior distributions for the parameters have to be specified and difficulties in determiningwhether the MCMC approximation has run for sufficient number of cycles.

A Monte Carlo simulation is used to generate the data in parametric bootstrapping method. A simulation is performed using replicate data sets of the same size as original based on the null hypothesis being tested. For each replicate data set, likelihoods according to both the null and alternative hypotheses are estimated and the LRT (Likelihood Ration Test) statistic is derived and significance test implemented. Parametric bootstrap is computationally very demanding when the data sets are large. Non-parametric tests appear to be conservative and parametric tests appear to be liberal in empirical comparisons. But generally, to assess branch support for phylogenetic trees non-parametric bootstrapping is used.

Conclusion

Molecular systematic have contributed tremendously to the world of evolutionary biology and taxonomy. Next Generation sequencing methods are also contributing substantially to this branch of science and the challenge will be to handle large volume of data and integrating information at different levels of genome.

SUGGESTED READING

Askari, G.H., Shabani, H. and Miandare, H.K. Application of molecular markers in fisheries and aquaculture. Scientific Journal of Animal Science, 2(4): 82-88.

Chauhan, T and Rajiv. K. 2010. Molecular markers and their application is fisheries and aquaculture. Advances in Bioscience and Biotechnology 1: 281-291.

Davis, G.P. and Hetzel, D.J.S. 2000. Integrating molecular genetics technology with traditional approaches for genetic improvement in aquaculture species. Aquaculture Research, 31: 3-10.

Edwards, S.V. 2008. Is a new and general theory of molecular systematics emerging? Evolution 63(1): 1-19.

Hallerman, E.M. 2006. Use of molecular tools for research and improvement of aquaculture stocks. The Israeli Journal of Aquaculture – Bamidgeh, 58(4): 286-296.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematic. Annual Reviews in Ecology and Systematics 18: 23-42.

Liu, Z.J. and Cordes, J.F. 2004. DNA marker technologies and their applications in aquaculture genetics. Aquaculture 1-37.

Mauro, D.S. and Agorreta, A. 2010. Molecular systematic: A synthesis of the common methods and the state of knowledge. Cellular and Molecular Biology Letters 311-341.

Okumus, I. and Ciftci, Y. 2003. Fish population genetics and molecular markers: II-Molecular markers and their applications in fisheries and aquaculture. Turkish Journal of Fisheries and Aquatic Sciences 3: 51-79.

Presti, R.L., Lisa,C. and Stasio, L.D. 2009. Molecular genetics in aquaculture. Italian Journal of Animal Science 8: 299-313.

Schwartz, J.H. and Maresca, B. 2006. Do molecular clocks run at all? A critique of molecular systematics. Biological Theory 1(4): 357-371.

Teletchea, F. 2009. Molecular identification methods of fish species: reassessment and possible applications. Reviews in Fish Biology and Fisheries 19: 265- 293

An overview of Next Generation Sequencing methods

Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Gathering DNA sequence information has become fundamental to answer questions in biology and with the advent of Next Generation Sequencing methods, this has become more and more important. DNA sequencing and sequence analysis helps in making inferences in population genetics, disease resistance investigations, molecular breeding, healthcare and comparative evolutionary studies. DNA sequencing technologies have moved from first generation to third generation during the last 30 years. First draft human genome was completed in 2003 using sanger sequencing method and from then onwards the era of genomics started and progressed. Modern sequencing machines made genome sequencing fast and cheap. Personalized medicine has become very popular now due to the availability of technologies to sequence genomes of individuals.

HISTORY OF SEQUENCING

First generation sequencing machines

Frederick Sanger introduced Sanger sequencing in 1977 which is based on chain termination method. Over years, this technology has undergone tremendous improvements and the first automatic sequencing machine (AB370) was introduced by Applied Biosystems Company in 1987 based on capillary electrophoresis making sequencing faster and accurate. Based on this machine, 96 bases could be detected at a time with read length of 600 bases. After many improvements and improvisations, up to 900-1000 bases could be detected at a time.

DNA preparation for sequencing

DNA to be sequenced is amplified either by cloning or PCR amplification. For shot gun de novo sequencing, DNA which is fragmented randomly is cloned into a high copynumber plasmid and consequently used to transform *Escherichia coli*; In the second approach, PCR amplification of the target segment of DNA is carried out using primers that flank the target. The amplified template produced through either of these methods is used for sequencing.

Sequencing reactions

Sequencing reactions are carried out in a “cycle sequencing mode” and the cycles consist of template denaturation, annealing of primers and primer extension and the primers are complementary to the sequences that flank the region of interest. Primer extension is terminated at every step stochastically by incorporating

fluorescently labeled dideoxy nucleotides (ddNTPs). The final mixture contains extension products that are end-labelled and the label on the terminating ddNTP corresponds to the identity of the nucleotide at its terminal position. The sequence is then determined using capillary based polymer gel by high-resolution electrophoresis separation of the single stranded fragments which are end-labeled. The fluorescent fragments with discrete lengths get laser excited as they exit the capillary and with the help of a four colour detection of emission spectra, a read out is obtained in the Sanger sequencing trace. A software translate these traces into DNA sequence along with error probabilities for each base call. Parallelization is possible at a limited level by carrying out simultaneous electrophoresis in 96 or 384 independent capillaries.

Second Generation/Next Generation Sequencing Technologies

Second generation sequencing takes advantage of many strategies in sequencing like
1) micro-electrophoretic methods 2) sequencing by hybridization 3) real time monitoring of single molecules and 4) cyclic array sequencing. Cyclic array sequencing is employed in 454 genome sequencers by Roche Applied Science which is also called pyrosequencing. Reversible terminator sequencing is employed by Illumina. Sequencing by ligation technique is performed in ABI/SoLiD machines and single molecule sequencing in Helicos.

Sequencing in Roche454 platform/pyrosequencing

The first next generation sequencing platform available as a commercial product was the 454 system. An method can be employed for construction of DNA libraries giving rise to a mixture of short, adaptor flanked fragments. Emulsion PCR is used for clonal amplification of target sequence and the amplicons are captured to the surface of 28µm beads. The emulsion is then broken to fragments and the beads susbsequently treated with denaturants to remove untethered strands which are then subjected to a hybridization based enrichment for amplicon bearing beads. Hybridization reactions are carried out with a sequencing primer using a universal adapter at the position and orientation which is immediately adjacent to the start of unknown sequence. Pyrosequencing method is used to carry our sequencing reactions. The beads which are amplicon-bearing are pre-incubated with the enzyme *Bacillus stearothermophilus* (Bst) polymerase along with single-stranded binding protein and subsequently deposited to a microfabricated array of picolitre scale wells (the dimensions will suit for only one bead per well) so as to make this biochemistry compatible with array-based sequencing. In addition smaller beads bearing immobilized enzymes like ATP sulfurylase and luciferase are also added as they are required for pyrosequencing. One side of the array will function as a flow cell for introducing and removing sequencing

reagents and other side bonded to a fiber-optic bundle for CCD (charge coupled device) based detection of signals while sequencing. While sequencing, a single species of unlabeled nucleotide is introduced at each of several hundred cycles. Pyrophosphate is released at each incorporation event in templates. The enzymes ATP sulfurylase and luciferase help in generating a burst of light at each incorporation event in templates during the course of sequencing which is further detected by the CCD corresponding to the array coordinates of specific wells. During multiple cycles, (eg: A-G-C-T…..) the

incorporation events reveals the sequence of templates that is represented by individual beads. 454 sequencing produces good read lengths. The 454 FLX sequencer can produce approximately 4,00,000 reads per instrument with lengths of 200-300bp. Rate of error will be increased by the presence of homopolymers which are consecutive

presence of same bases like AAA or GGG. When there is absence of a terminating moiety which can prevent multiple consecutive incorporations at a given cycle inference regarding length of homopolymers has to be made from intensity of signals. This will increase the error rate and the dominant error type in 454 platforms is insertions and deletions.

Illumina Genome Analyzer

Four companies were merged together mainly; Solexa (Essex, UK), Lynx Therapeutics (Hayward, CA, USA), Manteia Predictive Medicine (Coinsins, Switzerland) and Illumina and the Illumina genome analyzer has become very popular now. Libraries can be produced by any method giving rise to a mixture of adapter-flanked fragments which are several hundred base pairs in length. Amplified sequencing features are produced using a bridge PCR. In this method, forward and reverse PCR primers are secured to a solid substrate by a flexible linker and the amplicons arising from template molecules during amplification will be immobilized and clustered to a single physical location on the array. Bridge PCR is based on alternating cycles of extension with Bst polymerase followed by denaturation with formamide. Around 1000 clonal amplicons are found in each cluster. Thus several million clusters are amplified to specific locations within each of eight independent lanes on a single flow-cell and eight independent libraries can be sequenced at a time in parallel during each run of the instrument. After generating clusters, amplicons are single stranded or linearized followed by hybridization by a sequencing primer to the universal sequence which flanks the region of interest. Sequence interrogation at every step is composed of single base extension with a modified DNA polymerase and mixture of four nucleotides. The modification of nucleotides are done in two ways; using reversible terminators in which a chemically cleavable moiety at the 3’ hydroxyl position permits only a single base incorporation at each cycle and one in four fluorescent labels that are chemically cleavable corresponds to the

identity of each nucleotide. Currently read-lengths of upto 36bp are possible and higher error rates are found at longer reads. Substitutions are the common error type in this system than insertions and deletions.

AB SOLiD

Sequencing of libraries can be done by any method giving rise to a mix of short, adaptor flanked fragments. Emulsion PCR is carried out for clonal sequencing and amplicons are captured to the surface of 1µm paramagnetic beads. Subsequently, the emulsion is broken and the beads with amplification products are selectively recovered and immobilized to a solid planar substrate generating a dense and disordered array. DNA ligase is used to carry out sequencing by synthesis and not polymerase. A universal primer which is complementary to adapter sequence will be hybridized to the array consisting of amplicon bearing beads and each cycle of sequencing involves the ligation of a degenerate population of fluorescently labeled octamers. The mixture of octamers is arranged in such a way that identity of specific positions within the octamer (base 5) correlate with the identity of the fluorescent label and during the course of octamer ligation, every fifth base is sequenced. Different base positions are identified at subsequent steps and sequencing completed.

HeliScope

HeliScope also works on the principle of cyclic interrogation of a dense array of sequencing features. Clonal amplification is not required in this case. Sequencing by synthesis is carried out using a highly sensitive fluorescence detection system which directly interrogates single DNA molecules. Template libraries are prepared by random fragmentation along with poly A- tailing (without PCR amplification) and they are captured to surface bound poly-T oligomers by hybridization and a disordered array of primed single molecular sequencing templates are produced. DNA polymerase and a single species of fluorescently labeled nucleotides are added at each cycle resulting in template dependent extension of surface-captured primer-template duplexes. As acquisition of images tiling the full array is finished, chemical cleavage followed by release of fluorescent label permits subsequent cycle of extension and imaging. Thus several hundred cycles of single-base extension (A, G, C, T, A, G, C, T) produces average read lengths which are 25bp or greater. Deletion is the dominant error type in this method.

THIRD GENERATION SEQUENCING TECHNOLOGIES

Third generation sequencing technologies have brought about substantial improvement on methods characterized by new chemistry, reduced operation time, desktop design and reduced cost of operation. The major sequencers of third generation are; Pacific Biosciences real time single molecule sequencing (PacBioRS), Compete Genomics combined pre anchor hybridixation and ligation

(cPAL) and Ion Torrent of Life Technologies, Inc., MinION sequencer from Oxoford Nanopore technology. Third generation sequencing is otherwise known as long-read sequencing and the nucleotides are read at single molecule level unlike second generation methods that require breaking of long fragments of DNA into small segments and inferring nucleotide sequences by amplification and synthesis.

PacBioRS is a real time single molecule-single polymerase sequencing platform which can produce reads upto 1000bp. The chips constitute zero-mode wave guided (ZMW) nano structures with holes of size 100nm. Inside this hole, sequencing by synthesis is performed by DNA polymerase with the help of phospholinked nucleotides labeled with fluorophores introduced sequentially. The kinetics of nucleotide incorporation could be monitored using this instrument helping to gather epigenetic information in the future.

A combined approach of probe-anchor hybridization and ligation sequencing (cPAL) is employed by Complete Genomics and the highest throughput among third generation sequencers is claimed by them. The method is based on rolling circle amplification of small DNA sequences into the form nanoballs. Further, the sequence of nucleotides is determined by ligation method. Several DNA nanoballs could be sequenced per run with low costs using this approach.

DNA sequencing by Ion Torrent technology (Life Technologies Inc.) is considered as one of the most versatile and cheap method and at present it is being supplied as a personal genomic machine (PGM). It is like a benchtop instrument and it used widelyby those involved in research and medicine. The technology is based on proton release during incorporation of each nucleotide by DNA polymerase.

Fourth Generation Sequencing Technologies

Nanopore based sequencers

Nanopore based sequencers can be described as the fourth generation sequencing technology as they are able to sequence entire genome of any organism rapidly and with a very low cost. The origin of nanopore based technologies is from Coulter counter and ion channels. When an external voltage is applied, particles with sizes slightly smaller than the pore size are passed through the pore and these pores which are nanometer-sized are either embedded in a biological membrane or formed in solid state film separating the reservoirs containing conductive electrolytes into cis and trans compartments. When the voltage is biased, electrolyte ions in solution moves through the pore electrophoretically which generates an ionic current signal. When an analyte like negatively charged DNA molecule added into the cis chamber blocks the pore, current flowing through the pore gets blocked which interrupts the current signal. Consequently, the physical and chemical properties of the target

molecules are calculated by analysing statistically the amplitude and duration of current blockades occurred transiently from translocation events. The popular sequencing machines available at present utilising the nanopore technology are portable MinION machine, the benchtop GridION and high-throughput, high sample number PromethION offered by Oxford Nanopore systems.

FUTURE OF NGS METHODS

NGS methods find numerous applications in human and animal medicine along with ecological and evolutionary studies as they are fast, efficient and cheap. The major constraints with NGS data are the numerous short reads generated which needs to be assembled and annotated with the help of reference sequences. In contrast, longer reads generated using advanced machines may have more error reads towards the end. Main problem is with the repetitive sequences as they are difficult to read ending up in more errors. Even with all these limitations, the amount of information generated using NGS technologies is so huge that these method finds applications in human medicine mainly in diagnostics, prognostics and therapeutics. In addition, NGS techniques are also valuable in whole genome sequencing, targeted sequencing of exomes or selected genes responding to specific disorders or diseases, epigenetic mapping, transcriptome sequencing and metagenome sequencing.

SUGGESTED READING

Amish, S.J., Hoehenlohe, P.A., Painter, S., Leary, R.F., Muhlfeld, C., Allendorf, F.W. and Luikart, G. 2012. RAD sequencing yields a high success rate for westslope cutthroat and rainbow trout species-diagnostic SNP arrays. Molecular Ecology Resources 12: 653-660.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A. and Cresko, W.A. 2013. Stacks: an

analysis tool set for population genomics. Molecular Ecology 22: 3124-3140.

Davey, J.W. and Blaxter, M.L. 2011. RADSeq: next generation population genetics.

Briefings in Functional Genomics 9(5): 416-423.

Davey, J.W., Cezard, T., Fuentes-Utrilla, P., Eland, C., Gharbi, K. and Blaxter, M.L. 2013. Special features of RAD sequencing data: implications for genotyping. Molecular Ecology 22: 3151-3164.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L. and Law, M. 2012. Comparison of next-generation sequencing systems. Journal of Biomedicine and Biotechnology 2:11.

Metzker, M.L. 2010. Sequencing technologies-the next generation. Nature Reviews Genetics 31-46

Pocwierz-Kotus, A., Bernas, R., Kent, M.P., Lien, S., Leliuna, E., Debowski, P. and Wenne, R. 2015. Restitution and genetic differentiation of salmon populations in the sourthern Baltic genotyped with the Atlantic salmon 7K SNP array. Genetics Selection Evolution 47: 1-9.

Quail, M.A., Smith, M., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., Bertoni, A., Swerdlow, H.P. and Gu, Y. 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 13: 1-13.

Shendure, J. and Ji, H. 2008. Next generation DNA sequencing. Nature Biotechnology 26: 1135-1145.

Application of Population genomic tools in fisheries and aquaculture

Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Genomic investigations are gaining momentum in fisheries and aquaculture due to the enormous amount of information that could be generated by genomic tools. Fishes are considered as the most diversified and successful vertebrates in the planet with around 28,600 extant species in contrast to 4,629 species in mammals and 9,946 species in birds. Fishes occupy habitats ranging from below freezing to more than 40OC and from freshwater to hypersaline waters exhibiting varied life history, reproductive patterns and prey-predator relationships. Due to their enormous diversity and divergence, they are considered as excellent models for genomic investigations on selection, adaptation and evolution. Several model species with genomic information are available at present and understanding the genomic interactions among these fishes will provide important insights into ecological speciation and adaptation.

POPULATION GENETICS

Population genetic tools are well established as it uses neutral markers like microsatellites and mitochondrial DNA genes to infer characteristics of a population. Inferences regarding geneticdrift, gene flow and inbreeding could be made using the tools of population genetics with reasonable precision which has contributed significantly to the knowledge regarding evolutionary processes important in influencing the formation of population structure in diverse oceanic habitats. Population genetics inferences were based on a few well characterized neutral genes and the main limitation is that it does not provide information regarding adaptive mechanisms operating at genome level. Next generation sequencing techniques have revolutionized the field of genetics and it is now moving on to more inclusive genomics or population genomics.

POPULATION GENOMICS

Information from many genome wide markers is used in population genomics in contrast to population genetics where only a few markers are used. Information regarding both genic and non-genic regions could be generated using population genomic tools and hence effects due to selection, mutation and recombination on populations could be distinguished from effects due to genetic drift, inbreeding and gene flow. When only neutral markers used,

information about adaptive evolution is missing as adaptive divergence plays a very important part in local adaptation. Local adaptation provides optimum fitness for adapting to a particular environment or habitat which is driver for subpopulation structuring in fishes. Knowledge about locally adapted populations is also important to suggest conservation measures aimed at conserving genetic diversity at ecological time scales. Climate related habitat shifts and movements of marine fishes can be predicted if we have information about environment induced microevolution. Genome level prediction of footprints of selection associated with a particular trait is not very easy as several genes are involved in determining the characteristics of an adaptive trait. Population genomics becomes more relevant in this context as genomic variations in many functional genes linked to adaptation and selection within and between populations in space and time could be delineated using genomic tools. Several methods like Expressed Sequence Tags Sequencing (EST), transcriptome sequencing and whole genome sequencing will help in inferring important information regarding functional gene modifications linked to environmental alterations.

Approaches in population genomics

Studying the variations in expression of functional genes which has important roles in adaptation is one of the best approaches to understand variations in real time, which is otherwise known as candidate gene approach. Functional genes having a known function and influence on an adaptive trait can be selected for these studies. Genes which are linked to variations in structure of fishes or related to physiologicalprocesses and consequent phenotypic variations can be selected. Sequence information or allele frequency based tests can be used to derive information regarding functional gene variations. Action of evolutionary forces can be understood based on sequence data or allele frequency based tests. Candidate gene approach is relatively faster as comparative genomic approaches using candidate genes enable efficient characterization and comparison of different genes and controlled experimental set up is required only if a direct linking with phenotypic variation is desired. Efficient detection of variations across populations could be carried out which will help in understanding the influence of selective pressures in relation to environmental fluctuations. The major challenge in candidate gene approach is to identify the correct candidate gene related to the trait under study and the time consuming sequencing to understand variations in functional gene expressions.

In genome scan approach, regions or loci exhibiting high amount of structuring due to the effect of genetic hitchhiking is studied. Balancing selection or direction selection will bring about variable patterns on such loci and

hence studying those loci will provide insights into selective forces acting on the genome. Large number of genetic markers widespread in the genome like microsatellites, SNPs, AFLPs or functional markers like genes linked to ESTs could be employed for this method. Transcriptome sequencing could be used to detect genes linked to ESTs. Thus, genome scan approaches help in detecting loci under selection.

QTL mapping relies on information from linkage maps which are constructed using a dense set of markers providing clues with respect to the genetic basis which determine phenotypic traits and this is achieved through family crosses under controlled conditions in the laboratory. QTL information could be compared among closely related species minimizing the cost associated with it. Since QTL investigations require controlled experimental conditions, species amenable to aquaculture will be the best candidates for QTL related experiments. Association mapping is based on the information regarding linkage disequilibrium between large numbers of genetic markers which is linked to phenotypic variations. Admixture mapping helps indetecting natural events of admixture like intra-specific hybrid zones and we make use of the information regarding linkage disequilibrium to map admixture zones in the wild.

Seascape or landscape genomics tries to correlate information from genetic markers with geographic information for deriving insights regarding geographic patterns of genetic differentiation. Both neutral and non-neutral or functional gene markers can be employed in sea scape genomics so that information regarding selection could be deduced based on the divergence level of functional genes at each geographic location. Genetic divergence in non-neutral markers if any is indicative of locally adapted populations.

Data regarding differential gene expression patterns are very important to understand the role of key functional genes in modulating vital physiological processes. Transcriptomic approaches like mass scale RNA sequencing are very useful to understand variations in gene expression patterns in real time and these methods can be applied to all species due to the ease of transcription profiling and annotation. Micro-arrays can be used to measure the differential gene expression of hundreds or thousands of genes simultaneously whereas real time PCR measure gene expression of one or a few candidate genes.

Real time PCR is based on the principle of normal PCR and the product measured in real time by tagging the reaction mixture with fluorescent dyes. Flourescence can be measured after each PCR cycle which corresponds to the expression levels of a gene. The main drawback with real time PCR is that only very few genes within a genome could be studied using this method. But, microarrays enable

investigation of gene expression of thousands of genes simultaneously. Microarrays consist of thousands of single stranded DNA spots attached to surfaces like glass slides with each spot corresponding to a gene. Gene expression can be measured by washing fluorescently tagged cDNA over the slides so that complementary strands hybridize on to the array and fluoresce. The intensity of each spot will be proportional to the expression of thatparticular gene. All these investigations require controlled experimental conditions to relate gene expression data with adaptive variation.

Present status of fish genomics research

The enormous diversity and heterogeneity of marine fishes make them excellent models for fish genomics research. Marine fishes also exhibit high levels of gene flow mainly due to the pelagic larval phase and consequent dispersal of many pelagic and demersal resources. Population genetic structure studies on many fish groups like clupeids(eg: herring, anchovy and sardine), scombrids (eg: mackerel, tuna and bonito), pleuronectids (eg: plaices, soles and flounders) and gadids (eg: cod, hake and haddock) have been carried out for defining management units. The outcome of these genetic investigations revealed a lack of structuring with high levels of intra-population diversity values. In addition, the effective population size of many marine fishes is very high which also contribute to substantial gene flow and allele homogenization. In spite of all these factors, presence of locally adapted populations with substantial divergence in morphological and physiological has been indicated in marine fishes.

As of now, genomic resources are available for model species like;
Zebra fish, *Danio rerio*
Medaka, *Oryzias latipes*
Japanese puffer fish, *Takifugu rubripes*
Three-spined stickleback, *Gasterosteus aculeatus*
Spotted green puffer fish, *Tetraodon nigroviridis*

In addition, large scale genome sequencing of flat fish (PLEUROGENE) and Atlantic cod is also being carried out. Microarrays, BAC libraries and linkage maps are being constructed worldwide for species important in aquaculture.

EST information is available for the following species;
Atlantic cod (*Gadus morhua*)
Killifish (*Fundulus heteroclitus*)
Three spined stickle back (*Gasterosteus aculeatus*)
European sea bass (*Dicentrarchus labrax*)
Gilt head seabream (*Sparus aurata*)
Piked dog fish (*Squalus acanthias*)
Little skate (*Leucoraja erinacea*)
Japanese pufferfish (*Takifugu rubripes*)
Copper rock fish (*Sebastes caurinus*)
Blue fin tuna (*Thunnus thynnus*)
Japanese flounder (*Paralichthys olivaceus*)

Barramundi (*Latescalcarifer*)
Atlantic halibut (*Hippoglossus hippoglossus*)
Turbot (*Scophthalmus maximus*)
Grass rock fish (*Sebastes rastrelliger*)
Sheepshead minnow (*Cyprinodon variegates*)
European flounder (*Platycthys flesus*)
Long-jawed mudsucker (*Gillichthysmirabilis*)
Senegalese sole (*Solea senegalensis*)
Pacific electric ray (*Torpedo californica*)
Striped seabream (*Lithognathus mormyrus*)
Tongue sole (*Cynoglossus semilaevis*)
Large yellow croaker (*Pseudosciaenacrocea*)

There are many more EST sequences of teleost fishes in the dbEST database of NCBI.

Candidate functional genes have been studied in wild populations of many fishes. Pantophysin (Pan I) in Atlantic cod, Ectodysplasin (EDA) gene in stickle back and Lactate dehydrogenase B (Ldh-B) in killifish has been well characterized and studied. Haemoglobin polymorphism investigations in Atlantic cod revealed clear genetic differentiation across populations. Several insertion/deletion polymorphisms have been detected in Heat shock cognate gene Hsc70 in European flounder (*Platichthysflesus*). Genome scans in three-spined sticklebacks provided insights into theiradaptation to fresh and marine environments. Divergence in different ecotypes hasbeen studied using EST-based microsatellites and EDA gene linked indels. Outlier loci subjected to selection has been identified in Atlantic cod using mass scale genome scans. Adaptive population divergence corresponding to different geographic locations has been detected in many marine fishes.

Whole genome sequencing of 10000 vertebrates was aimed by the project Genome 10K (Genome 10K community of scientists, 2009) and genome characterization of many of the fishes are progressing. Whole genome of cartilaginous elephant fish, *Carcharhinusmilii* which is a member of the Holocephali has been assembled. Otherendangered and threatened aquatic organisms being sequenced by Genome 10K and Bejing Genomics Institute (BGI) are *Acipenser sinensis* and *Hippocampus comes*. Recently whole genome of *Hippocampus comes* has been published.

Genomic signatures associated with migratory and resident ecotypes of endangered sock eye salmon *Oncorhynchus nerka* were studied using 2600 SNPs derived from Restriction site associated DNA sequencing which provided vital clues to adaptive genetic variation between ecotypes. Putative neutral and divergent adaptive markers were used to determine population differentiation in an anadromous Pacific smelt using RAD sequencing. The study provided signatures of divergent selective pressures in differing freshwater and marine environments

between regional eulachon populations. This information was helpful in defining management units. Cryptic population differentiation was studied in the Baltic Sea herring *Clupea harengus* using a total of 4756 divergent SNPs derived through RAD sequencing approach and they could detect high degree of differentiation. RAD sequencing has also been employed for understanding demographic history, quantifying population connectivity and detection of loci subject to local adaptation.

Future of population genomics

Fishes face many physical, chemical and biological challenges in the dynamic environment in which they live. Surviving in these conditions requires alterations in phenotypic characteristics and the major driver for these variations is natural selection occurring at varying degrees. Genome investigations provide insights regarding micro-evolutionary patterns and the advent of next generation sequencing technologies have revolutionized these approaches. We are witnessing an era of genomics and the floodgates for genomic technologies being opened now, a revolution in fish genomic research is imminent with more and more fish genomes being sequenced.

SUGGESTED READING

Allendorf, F.W., Hohenlohe, P.A. and Luikart, G. 2010. Genomics and the future of conservation genetics. Nature Reviews Genetics 11: 697-709.

Cossins, A.R. and Crawford, D.L. 2005. Fish as models for environmental genomics.

Nature Reviews Genetics 6:324-340.

Crollius, H.R. and Weissenbach, J. 2005. Fish genomics and biology. Genome Research 15: 1675-1682.

Ding, F., Chu, W., Cui, P., Tao, M., Zhou, R., Zhao, F., Hu, S. and Zhang, J. 2011. EST-Based identification of genes expressed in skeletal muscle of the Mandarin Fish (*Sinipercachuatsi*). Genomics, Proteomics and Bioinformatics 9(1-2): 30-36.

Guo, B., DeFaveri, J., Sotelo, G., Nair, A. and Merila, J. 2015. Population genomic evidence for adaptive differentiation in Baltic Sea three-spined sticklebacks. BMC Biology 13: 19

Hansen, M.M.,Olivieri, I., Waller, D.M., Nielsen, E.E. and The GeM working group. 2012. Monitoring adaptive genetic responses to environmental change. Molecular Ecology 21: 1311-1329.

Helyar, S.J., Hemmer-Hansen, J., Bekkevold, D., Taylor, M.I., Ogden, R., Limborg, M.T., Cariani, A., Maes, G.E., Diopere, E., Carvalho, G.R. and Nielsen, E.E. 2011. Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. Molecular Ecology Resources 11(Suppl.1), 123-136.

Hemmer-Hansen, J., Therkildsen, N.O. and Pujolar, J.M. 2014. Population genomics of marine fishes: Next – Generation prospects and challenges. Biological Bulletin 227: 117-132.

Hohenlohe, P.A., Bassham, S., Etter, P.D., Stiffler, N., Johnson, E.A. and Cresko, W.A. 2010. Population genomics of parallel adaptation in Threespine stickleback using sequenced RAD tags. PLoS Genetics 6(2): 1-23.

Nielsen, E.E., Hemmer-Hansen, J., Larsen, P.F. and Bekkevold,D. 2009. Population genomics of marine fishes: identifying adaptive variation in space and time. Molecular Ecology 18: 3128-3150.

Quere, N., Guinand, B., Kuhl, H., Reinhardt, R., Bonhomme, F. and Desmarais, E. 2010. Genomic sequences and genetic differentiation at associated tandem repeat markers in growth hormone, somatolactin and insulin-like growth factor-1 genes of the sea bass, *Dicentrarchus labrax*. Aquatic Living Resources 23: 285-296.

Zhu, M. and Zhao, S. 2007. Candidate gene identification approach: progress andchallenges. International Journal of Biological Sciences 3(7): 420-427.

Genomics for conservation of fin fishes and shell fishes

Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

India is a major producer of marine and fresh water fishes and largest supplier of fish in the world. The country has 7517 kilometers of marine coastline, 3,827 fishing villages, and 1,914 traditional fish landing centers. Among 65 commercially important species harvested in India, pelagic and mid-water species contributed about 50% of the total marine fish. Fishing and aquaculture in India employs about 14.5 million people and it is an important sector for food security.

Indian fisheries and oceans face a number of challenges including the rapid reduction of major wild fish stocks, increasing market demands, and environmental challenges, such as pollution and climate change. For feeding a growing world population with fish, it is necessary to develop new approaches to managing wild fisheries and practising aquaculture.

Genomics can provide markers and other approaches to improve the management of wild fisheries and allow for the protection and enhancement of biodiversity and aquatic habitats. For the aquaculture industry, marker assisted selection can be used to establish breeding programs to produce fish with desirable traits, including faster growth rates, disease resistance, thermal tolerance etc. Genomics can also be used to detect, monitor and minimize the impact of pathogens and thereby improve the health of aquatic animals.

It is already clear that large-scale changes to natural communities are occurring and will accelerate over the coming decades by environmental stress resulting from human activities. The world is facing an increase in temperature, coupled with ocean acidification, increases in the length and intensity of drought and flood conditions, as well as changes in the salinity of coastal areas. The distributions of many species are expected to shift during this period and it predicts that many areas currently occupied by species will no longer be suitable for them. At the same time, some species are expected to benefit from the effects of climate change; groups of invasive species and even some native species are expected to benefit in this way.

Three interacting demographic factors are widely identified to have major effects on the successful adaptation to rapid environmental change - generation time, population size and population structure. Therefore natural populations are threatened by three forces: (1) a reduction in genetic variation as a consequence of decreases in population size affecting in situ evolution, (2) a reduction in

gene flow preventing an influx of genetic variants from other populations and (3) a reduction in environmental heterogeneity that can lead to a decrease in adaptive capacity of the species as a whole.

POPULATION GENOMICS APPROACHES

The resent revolution in genomics and other omics technologies is providing better methods to insights into evolutionary processes to above mentioned environmental stress and offers an opportunity to improve conservation planning and management decisions. The next-generation sequencing (NGS) has changed genomic and transcriptomic approaches to fish biology. These new sequencing tools are also valuable for the discovery, validation and assessment of genetic markers in populations. Population level genotyping and transcription profiles study has provided opportunities to identify the widespread genomic variation within species. With high quality genome (multi-individual Whole Genome Sequencing - WGS) and transcriptome assemblies, the accuracy and power of characterization of genomic diversity will be improved. This will also contribute to understanding association of genotypes with desirable traits and environmental resilience. But still there is a significant financial challenge when multiple populations are under investigation. There are several economical alternatives to multi-individual WGS.

There are some NGS methods based on Genotyping by Sequencing(GBS) for genome-wide genetic marker development and genotyping that use restriction enzyme digestion of target genomes to reduce the complexity of the target. These include reduced-representation sequencing (RRS) using reduced-representation libraries (RRLs) or complexity reduction of polymorphic sequences (CRoPS), restriction-site-associated DNA sequencing (RAD-seq) and low coverage genotyping. These are applicable to both model organisms with reference genome sequences and non-model species with no existing genomic data. Genotyping by sequencing (GBS) has revolutionized fish genotyping by providing powerful tools for rapid and high-throughput identification of genetic variation (candidate loci/QTL) underlying fish with desirable traits, including faster growth rates, disease resistance, thermal tolerance etc. For the aquaculture industry, these genetic variations can be used as candidate loci/QTL for marker assisted selection to establish breeding programs to produce fish with desirable traits.

Table 1 Applications of genomics data and method for decision making in conservation genetics
(Source- Hoffmann et al. 2015)

Decisions	Biological issue	How genomics can help inform decisions	Data type	Analysis method
Species tolerate change in situ?	Determining if a species is currently experiencing stress which suggests it is approaching the limit of physiological tolerance. Testing whether a species has sufficient phenotypic plasticity to tolerate projected change. Testing whether a species has sufficient phenotypic plasticity to tolerate projected change	Screen biomarkers indicative of stress. Understand the limits to plasticity under environmental change. Greater numbers of loci provide the opportunity to reconstruct demographic history deeper in time.	[TS] with [IG] [TS] with [IG] [WGS], [RRS], [DE] with [IG] or [WGS], [DE] with [PPS]	Gene expression analyses to identify abundance of key gene transcripts. Bayesian skyline plots/coalescent simulations or likelihood-baseddiffusion modeling from SNP data
Do populations have enough genetic diversity for an evolutionary response?	Determining whether the species or population is currently experiencing inbreeding, which can lead to loss of genetic diversity essential for evolution. Assess whether there is enough standing genetic diversity to provide opportunities to adapt. Determining whether selection has acted on genetic variation in the species	Genome-wide sequencing allows accurate estimation of heterozygosity in individuals and populations. Accurately estimate the levels of genetic diversity in populations. Test whether major events have resulted in selection on genetic diversity.	[WGS], [RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS]	Estimate F-statistics and heterozygosity from SNP data. Estimate heterozygosity, DNA sequence diversity estimates (pi, theta) from SNP or sequence Data. Allele frequency spectrum tests (e.g. Tajima's D), linkage disequilibrium, non-synonymous to synonymous polymorphism ratios (e.g. Kn/Ks) from sequence data.
Is genetic diversity strongly distributed across populations?	Identification of centre of genetic diversity, or genetically distinct regions, for prioritized conservation	Examine patterns of population genetic structure to identify outlier populations.	[WGS], [RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS]	Estimation of population differentiation based on SNP data using classical F-statistics, PCA or MCMC and Bayesian derived estimates of admixture (e.g. STRUCTURE)
Are some populations adapted to local climate?	Identifying whether populations show adaptation to local climate (or other environmental	Identify loci that have been under selection in populations conditioned on local environment. Identify rates of genetic	[WGS], [RRS], [DE], [TS] with [IG] or [WGS], [DE]	Population level or landscape genomics methods based on SNP data: outlier tests, relative rate tests, allelic association with environment

	variables) How quickly can genetic adaptation occur?	adaptation to environment by screening adaptive variationin natural populations experiencing environmental change, or through simulated or experimental evolution.	with [PPS]	and allelic association with adaptive traits. Computational modelling of genomic diversity evolution under environmental change
Is gene flow high enough? (or too high?)	Determining the extent of gene flow between existing populations to inform on dispersal capability and also potential for adaptive alleles to spread or be swamped	Provide estimates of ongoing gene-flow and admixture among populations.	[WGS], [RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS]	Coalescent genealogy sampling to generate Bayesian and maximum likelihood estimates of migration and gene flow (e.g. Lamarc, Migrate), or MCMC and Bayesian-derived estimates of admixture (e.g. STRUCTURE) based on SNP data. Genomics also has the power to identify recent migrants and so test the efficacy of movement pathways
Is a positive evolutionary response possible through natural hybridization with sympatric species?	Determining whether hybridization occurs in nature. Assess how quickly beneficial hybridization is adaptive alleles can move into a population or species	Estimate rates and genomic extent of hybridization/gene flow between species in situ. Track introgression of genomic regions under selection following documented hybridization events. Identify potential for hybrid incompatibilities or swamping.	[WGS], RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS] [WGS], [RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS]	Identify hybrid ancestry via comparison to known non-hybrids. Estimate migration and gene flow (e.g. Lamarc, Migrate) and admixture (e.g. STRUCTURE) between species. Admixture quantification also confirms F1 hybrid fecundity Track distribution of species specific alleles in population with regard to null selection models. Transmission distortion in artificial F2 hybrids can indicate genetic incompatibilities
Can species migrate quickly enough?	Assess potential for migration in to climatic refuge given ecological constraints and known rates of gene flow	Provide accurate estimates of gene flow.	[WGS], RRS], [DE], [TS] with [IG] or [WGS],[DE] with [PPS]	Genomic estimates of gene flow can be coupled with data on rates of dispersal or movement and habitat analysis (path analyses, resistance models) to predict viability of dispersal pathways

[WGS] whole-genome sequencing.
[RRS] reduced representation sequencing (e.g. RADseq, GBS, DArTseq).
[DE] DNA enrichment (e.g. exon capture, SureSelect, anchored hybrid enrichment).
[TS] genotypes called from transcriptome sequencing or gene expression data.
[IG] sequencing and analyses performed on individual genotypes.
[PPS] sequencing and analyses performed on pooled population samples.

Importance of restriction site associated DNA (RAD) sequencing in population genomics

Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

Restriction site associated DNA sequencing is a method where restriction enzymes are used to cut genomes at specific sites and this provides a genome wide representation of every site cut by a particular restriction enzyme. This is also termed as a genome "complexity reduction protocol". This method has become very popular in recent years for genome wide discovery of polymorphic markers from several individuals.

RAD sequencing tries to combine two techniques in molecular biology with Illumina sequencing. DNA is cut into fragments using restriction enzymes and sequence reads are related to specific individuals using molecular identifiers (MID). Fragmentation of DNA from one individual is carried out with specific restriction enzyme which produces a set of sticky ended fragments. Further, these sticky ended fragments are ligated to a P1 adapter which contains a matching sticky end along with a molecular identifier. The tagged restriction fragments from different individuals are pooled and randomly sheared to produce fragments having average length of a few hundred base pairs. The sheared fragments are then ligated to a second P2 adapter and amplified by P1 and P2 primers using PCR. The P2 adapter has a divergent "Y" structure which cannot bind to P2 primer unless it is completely amplified by the P1 adapter. This ensures that all the amplified fragments consist of the P1 adapter and MID, partial restriction site, the flanking sequence characterized by a few hundred bases and a P2 adapter. The amplified fragments which are then ready for sequencing shall be selected for size (approximately 200-500 base fragments) and the RADseq library is sequenced on an Illumina platform. Consequently, sequence is generated from the molecular identifier in the P1 adapter and across the restriction enzyme site generating a data set of RAD tags derived from a reduced part of the original genome.

There are many advantages for RAD sequencing as compared to whole genome sequencing since it allows regions of interest to be sequenced. RAD sequencing is a reduced representation method which samples only a shared set of sites across the genome in many individuals. This allows population level sequencing at a fraction of the cost required for whole genome sequencing. In addition, fine scale linkage mapping, phylogenetics, phylogeography, genome scaffolding and population genetics investigations could be carried out using NGS RAD sequencing. Recently, it has been applied in species like salmon, cutthroat and rainbow trout in order to deduce inferences regarding population genetic structure.

In developed countries genomic approaches become routine components of their conservation programmes, but Indian fisheries sector has not utilising the benefit of genomics tools efficiently. Genomic approach has great potential to support conservation programs for our non-model species. New levels of understanding of genetic diversity and climate change adaptation will help us to protect our biodiversity and keep ecosystem healthy under a rapidly changing climate.

SUGGESTED READINGS

Hoffmann, A., Griffin, P., Dillon, S., Catullo, R., Rane, R., Byrne, M., Jordan, R., Oakeshott, J., Weeks, A., Joseph, L. and Lockhart, P., 2015. A framework for incorporating evolutionary genomics into biodiversity conservation and management. Climate Change Responses, 2(1), p.1.

Amish, S.J., Hoehenlohe, P.A., Painter, S., Leary, R.F., Muhlfeld, C., Allendorf, F.W. and Luikart, G. 2012. RAD sequencing yields a high success rate for westslope cutthroat and rainbow trout species-diagnostic SNP arrays. Molecular Ecology Resources 12: 653-660.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A. and Cresko, W.A. 2013. Stacks: ananalysis tool set for population genomics. Molecular Ecology 22: 3124-3140.

Davey, J.W. and Blaxter, M.L. 2011. RADSeq: next generation population genetics.Briefings in Functional Genomics 9(5): 416-423.

Davey, J.W., Cezard, T., Fuentes-Utrilla, P., Eland, C., Gharbi, K. and Blaxter, M.L. 2013. Special features of RAD sequencing data: implications for genotyping. Molecular Ecology 22: 3151-3164.

Pocwierz-Kotus, A., Bernas, R., Kent, M.P., Lien, S., Leliuna, E., Debowski, P. and Wenne, R. 2015. Restitution and genetic differentiation of salmon populations in the sourthernBaltic genotyped with the Atlantic salmon 7K SNP array. Genetics Selection Evolution 47: 1-9.

Amish, S.J., Hoehenlohe, P.A., Painter, S., Leary, R.F., Muhlfeld, C., Allendorf, F.W. and Luikart, G. 2012. RAD sequencing yields a high success rate for westslope cutthroat and rainbow trout species-diagnostic SNP arrays. Molecular Ecology Resources 12: 653-660.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A. and Cresko, W.A. 2013. Stacks: ananalysis tool set for population genomics. Molecular Ecology 22: 3124-3140.

Davey, J.W. and Blaxter, M.L. 2011. RADSeq: next generation population genetics.Briefings in Functional Genomics 9(5): 416-423.

Davey, J.W., Cezard, T., Fuentes-Utrilla, P., Eland, C., Gharbi, K. and Blaxter, M.L. 2013. Special features of RAD sequencing data: implications for genotyping. Molecular Ecology 22: 3151-3164.

Pocwierz-Kotus, A., Bernas, R., Kent, M.P., Lien, S., Leliuna, E., Debowski, P. and Wenne, R. 2015. Restitution and genetic differentiation of salmon populations in the sourthernBaltic genotyped with the Atlantic salmon 7K SNP array. Genetics Selection Evolution 47: 1-9.

An overview of software packages used in population genetics

Sandhya Sukumaran, Jeena N.S., Reynold Peter, Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

There are many freely available software packages. These packages can be used for aligning sequences, analysis of genetic differentiation, construction of phylogenetic trees using different methods, constructing network diagrams, analysis of historical demography, analyzing genetic population structure and a number other analyses. These packages are available as freely downloadable files. The major software packages used for population genetic analyses are listed below.

BioEdit

Bio Edit is a user-friendly biological sequence alignment editor and analysis program and is an important bioinformatics tool for molecular biologists. It was developed initially as a graphical biological sequence alignment editor written for Windows only. The purpose of this program is to provide a useful molecular biology tool which can be started up and used easily. However, in the last few years it has been improved dramatically to integrate many other features and functions such as several modes of hand alignment, plasmid drawing and annotation, restriction mapping and much more.

BioEdit can accept a wide variety of formats that is commonly used with other bioinformatics application. This allows the swapping of Data files between BioEdit and other programs. It contains many features for sequence alignments like split window view, user defined color, information based shading and auto integration with other programs such as Clustal W and Blast. Most of sequence alignment programs do not have many other functions or useful tools for molecular biologist comparing to BioEdit. BioEdit (Current version 7.2.5 Last updated 12/11/2013) can be downloaded from the following Server: http://www.mbio.ncsu.edu/BioEdit/bioedit.html

MEGA (Molecular Evolutionary Genetics Analysis)

The Molecular Evolutionary Genetics Analysis software is a multi platform desktop application designed for comparative analysis of homologous gene sequences either from multi-gene families or from different species with a special emphasis on inferring evolutionary relationships and patterns of DNA and

protein evolution. In addition to the tools for statistical analysis of data, MEGA provides many convenient facilities for the assembly of sequence data sets from files or web-based repositories, and it includes tools for visual presentation of the results obtained in the form of interactive phylogenetic trees and evolutionary distance matrices.

The Molecular Evolutionary Genetics Analysis (MEGA) software aims to serve both of these purposes in inferring evolutionary relationships of homologous sequences, exploring basic statistical properties of genes and estimating neutral and selective evolutionary divergence among sequences.

MEGA is an integrated workbench for biologists for mining data from the web, conducting automatic and manual sequence alignment, inferring phylogenetic trees, estimating rates of molecular evolution, testing evolutionary hypotheses and generating publication quality displays and descriptions. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species.

MEGA is distributed in two editions: a graphical user interface (GUI) edition with visual tools for exploration of data and analysis results and a command line edition (MEGA-CC), which is optimized for iterative and integrated pipeline analyses. Both GUI and command-line versions of MEGA6 (current version) can be downloaded from www.megasoftware.net free of charge.

Genepop

Genepop is a population genetics software package. The basic tools of this program are designed for multi-allelic markers like microsatellite, short tandem repeat-STR etc. It is used for descriptive statistics, estimates of genetic differentiation (traditional & microsat specific estimates of genetic differentiation), Hardy-Weinberg equilibrium (pairwise comparisons of single locus and global tests), geographic vs. genetic distance.

Three major tasks can be performed in Genepop. 1) It computes exact tests: for Hardy-Weinberg equilibrium, for population differentiation and for genotypic disequilibrium among pairs of loci. 2) computes estimates of classical population parameters, such as Fst and other correlations, allele frequencies, etc. and 3) converts the input Genepop file to formats used by other programs, like Biosys, Fstat and Linkdos.

The latest version of Genepop (4.2) is now available from http://kimura.univmontp2.fr/~rousset/Genepop.htm.
Genepop 4.2 runs under Windows, and can also be compiled to run under Unix or Linux. The Genepop programs is a simple command-line executable files, where the settings of the computations can often be specified on a command line. Even though being less user friendly than graphical packages, command-line programs have

the advantage that they can be easily launched in parallel on computer clusters and can be used to automatically analyze a large number of input files, like those resulting from large genomic studies or simulations.

A web-based front-end is available for teaching purposes and for those who, for some reason, cannot run the Genepop on their local PC or Mac.

GenAlEx

GenAlEx 6.5 is used for a range of population genetic analysis with a wide range of molecular markers within the Microsoft Excel environment on both PC and Macintosh computers. It has a user friendly interface with rich graphical outputs for data exploration and publication, tools for data manipulation and export options to many other software packages. GenAlEx offers analysis of codominant, haploid and binary

genetic loci and DNA sequences. Both frequency-based (F-statistics, heterozygosity, HWE, population assignment, relatedness) and distance-based (AMOVA, PCoA, Mantel tests, multivariate spatial autocorrelation) analyses are provided. In GenAlEx 6.5 there are new features including calculation of new estimators of population structure: G'ST, G''ST, Jost's Dest, and F'ST via AMOVA, Shannon Information analysis, linkage disequilibrium analysis for biallelic data, and heterogeneity tests for spatial autocorrelation analysis. Direct data export is provided to more than 30 other software packages, and indirectly via common formats to many more packages.

DnaSP

DNA sequence polymorphism (DnaSP) is an interactive computer program for the analysis of DNA polymorphism from nucleotide sequence data. It calculates several measures of DNA sequence variation within and between populations; linkage disequilibrium, recombination, gene flow and gene conversion parameters. In addition, DnaSP performs some neutrality tests. DnaSP is written in Visual Basic v. 6.0 (Microsoft) and DnaSP version 5.10.1 is freely downloadable from http://www.ub.edu/dnasp/

DnaSP can automatically read the following types of data file formats: FASTA, MEGA, NBRF/PIR, NEXUS, PHYLIP and HapMap3 Phased Haplotypes. In all cases one or more homologous nucleotide sequences should be included in just one file (ASCII file). The sequences must be aligned (i. e. the sequences must have the same length).

Various analysis options are included in the software such as DNA Polymorphism analysis, GC content at non-coding and coding positions, Haplotype/Nucleotide Diversity and Divergence. There are options to find out the number of Segregating Sites (S), total number of mutations (Eta), the number of haplotypes, Haplotype (gene) diversity and its sampling variance, Nucleotide diversity, Pi (π), The average number of nucleotide

differences (k), Nucleotide divergence with Jukes and Cantor, Theta (per gene or per site) from Eta (η) or from S and ZnS statistic, Neutrality tests such as Tajima's *D* with its statistical significance, Fu and Li's *D*, Fu's *Fs* etc. This software also

estimates the raggedness statistic (r) which quantifies the smoothness of the observed pairwise differences distribution. More powerful statistics for detecting population expansion such as the Fu's *Fs* Test and the Ramos-Onsins and Rozas's R2 can also be carried out using the software.

Arlequin

The free population genetic software Arlequin provides the user with quite a large set of basic methods and statistical tests, in order to extract information on genetic and demographic features of a collection of population samples. The latest version Arlequin ver 3.5.2.1 can be downloaded in Windows XP/Vista/7 based systems with a minimum of 256 MB RAM from
http://cmpg.unibe.ch/software/arlequin35/Arl35Downloads.html

Arlequin can handle several types of data either in haplotypic or genotypic form. The basic data types are DNA sequences, RFLP data, Microsatellite data, Standard data and Allele frequency data. The analyses Arlequin can perform on the data fall into two main categories: intrapopulation and inter-population methods. In the first category statistical information is extracted independently from each population, whereas in the second category, samples are compared to each other. Intra-population methods include standard diversity indices like the number of polymorphic sites, gene diversity, molecular diversity indices like nucleotide diversity, different estimators of the population parameter θ, mismatch distribution of the number of pairwise differences between haplotypes, Haplotype frequency estimation present in the population by maximum likelihood methods, linkage disequilibrium which is a test of non-random association of alleles at different loci., Hardy-Weinberg equilibrium which is the test of non-random association of alleles within diploid individuals, Tajima's neutrality test, Fu's *Fs* neutrality test, Ewens-Watterson neutrality test, Chakraborty's amalgamation test and Minimum Spanning Network (MSN) analyses. Inter-population methods include searching for shared haplotypes between populations, different hierarchical Analyses of Molecular Variance (AMOVA) to evaluate the amount of population genetic structure, *FST* based pairwise genetic distances for short divergence time, exact test of population differentiation, assignment of individual genotypes to particular populations according to estimated allele frequencies, detection of loci under selection from *F*-statistics and mantel test to test for the presence of isolation-by-distance.

Network

Network is a free phylogenetic network software available from fluxusengineering.com. It is used to

reconstruct phylogenetic networks and trees, infer ancestral types and potential variants and evolutionary branching patterns. The Network programs have user-friendly graphical interfaces, allowing users to easily choose the types of analysis and computation parameters to be performed. The basic algorithms of this program are designed for non-recombining bio-molecules, thus it can be effectively used for mtDNA, Y-STR, amino acid, RNA, viral DNA, bacterial DNA, some non-recombining autosomal DNA, and also for non-biomolecule data such as linguistic data.

The Network software was developed to reconstruct all possible least complex phylogenetic trees (all maximum parsimony or MP trees) from a given data set. The data can be DNA sequences or nucleotide sequences, amino acid sequences or protein sequences, STR (short tandem repeat) or microsatellite data, language/linguistic or manuscript data.

Two independent network-building options are included for arriving at optimal networks; the Reduced Median networks or *RM* network algorithm for binary data and the Median Joining networks or *MJ* network algorithm for all types of data. *MJ* networks are commonly used to visualize relationships of closely related mitochondrial or nuclear haplotypes, for which traditional phylogenetic methods yield multiple possible trees. For interpretation of complex and large data a neat skeleton phylogeny can be constructed using Star Contraction pre-processing. Age estimation for ancestral nodes or branching points is also possible in Network software.

Current Version is Network 4.6.1.3., released on 24 December 2014, and it is available from www.fluxusengineering.com. Network runs on Windows 7, Vista, XP, 2000, many Linux versions with WINE Windows emulation, and Macs with Parallels Desktop Windows, or VirtualBox with Windows. Network can run from memory stick no Windows registry entries and no Windows Administrator required for installation. Network software package includes a data editor and a graphics program. FASTA files can be imported and prepared for Network using Fluxus' DNA Alignment software. Higher-quality graphics of Network's results files can be prepared using Fluxus' Network Publisher software.

Mr Bayes

MrBayes (Ronquist and Huelsenbeck 2003) **is** a program for doing Bayesian phylogenetic analysis. The posterior probability distribution is sampled using Markov Chain Monte Carlo (MCMC) techniques. Metropolis-coupling is used to accelerate convergence in MrBayes. In parallel with regular “cold” chain, three heated chains are also run in parallel. The heated chains will sample from distributions obtained by raising the posterior probability with some factor smaller than 1, which result in flattening ("melting") of the peaks in the landscape defined by the posterior distribution. At specified intervals, the parameter values (the locations in the landscape) are

swapped between cold and heated chains, making it possible for the cold chain to escape local peaks. This comes at the cost of increased computational complexity.

The last official release of the program is version 3.1.2. The most recent development version of the program (version 3.2) includes a number of significant new features, such as check-pointing, strict and relaxed clock models, and functionality for dating. Version 3.2 can be compiled from source code freely available on SourceForge (see instructions below).

MrBayes version 3.1.2 is available for Windows, OSX, and Linux. All versions use a command-line interface and the program looks virtually the same on all platforms.

MrBayes 3 is a program for Bayesian inference and model choice across a large space of phylogenetic and evolutionary models. The program has a command-line interface and should run on a variety of computer platforms, including large computer clusters and multicore machines. Depending on the settings, MrBayes analyses may demand a lot on the machines, both in terms of memory and processor speed. Many users therefore run more challenging analyses on dedicated computing machines or clusters. Several computing centers around the globe provide web access to such services. MrBayes 3 is distributed without charge by download from the MrBayes web site, http://mrbayes.net.

Structure

Structure is freely downloadable software which uses a model based clustering method to infer genetic population structure. It uses multi-locus genotype data for investigations on genetic population structure with a Bayesian approach (MCMC: Markov Chain Monte Carlo). It can be used for knowing the presence of distinct populations, assigning individuals to populations, studying hybrid zones, identifying migrants and admixed individuals, and estimating population allele frequencies. Results based on most of the commonly used genetic markers like SNPS, microsatellites, RFLPs and AFLPs could be analyzed using this software. This software could also be used to compute the proportion of the genome of an individual originating from each inferred population (quantitative clustering method).

It can be downloaded from:
(http://pritch.bsd.uchicago.edu/software/structure2_1.html).

Functional Genomics

M. P. Paulton
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The sequence information comprising of billions of bases do not communicate the role of the genes and how they are influencing the formation of organism, aging, biological pathways and what changes in them lead to diseases etc.Functional genomics comes into action to answer the above queries and it provides information about the role of different genes in comprising the function of cells and organism. The science of functional genomics helps the researchers to find out the gene expression and its regulationand its impact on complex production traits at the genome level.The goal of functional genomics could also be described as to find out the biological factors behind a phenotype and its expression in different situations or environment. With the advancement of sequencing techniques, researchers can directly go into the sequence variant lines and sub types and thereby to identify the polymorphic forms such single nucleotide polymorphisms (SNPs) and their impact in regulating the expression of certain genes. Functional genomics provides a reliable platform to understand gene environment interaction at the genome level and thereby to design strategies in breeding and production. Further, the elements such as non-coding RNAs (ncRNAs) and introns conventionally believed as non-functional are getting into limelight with the advancement in sequencing technologies. Many such hitherto unrevealed entities are coming up and even now the real challenge in functional genomics is not in obtaining new data but in analysing it. A series of techniques are available and also emerging in functional genomics with specific focus on their application to enhance production in agricultural, livestock and aquaculture systems.

Functional genomic approaches

The targets in functional genomics is achieved through the integration of different branches such as transcriptomics,proteomics, metabolomics, nutrigenomicsand epigenetics.Transcriptomics deals with the quantification gene expression at the transcript level. Proteomics focus on the expression of specific proteins and their structures. Metabolomics explains various metabolites produced in cell during various phases in cell cycle.Interactomics is another branch with applications in agriculture as they focus on the host pathogen interaction. Another area namely epigenetics studies the non-inheritable changes taking place at the genome and its impact in

phenotype.Nutigenomics deals with the how diets are affecting geneexpression. All these branches are working together to meet the challenges in food security and to ensure sustainable production. Further it is anticipated in long term that the results of functional genomics are properly integrated with quantitative genetics and ultimately expected to contribute improved agricultural productivity.

Single and multiple gene expression profiling methods

Analysis of the single gene expression is normally carried out by a variety of techniques which include Northern Blotting,RNAse protection assay(RPA),Reverse transcription PCR and quantitative real time PCR. Expression profiling of more number of known mRNA is possible by procedures like Differential Display (DD), Representational difference analysis (RDA) and suppression subtractivehybridization(SSH). As the technological advancement increases, following techniques based on sequencing the transcriptome is getting popular.

Transcriptomic techniques

Transcriptome constitute the whole population of RNA such as mRNA, rRNA, micro RNA and other regulatory RNAsand the study of this contribute the field of tanscriptomics.The popular techniques in transcriptome studies are Expressed sequence Tag(EST), microarray, serial analysis gene expression (SAGE).Many candidate genes of commercial importance which could be used in breeding programmes are being isolated. Further the evolution of inflammatory response markers through transcriptomic studies helped to detect the nutritional additives negatively regulating the growth of farmed animals like Atlantic salmon.

EST and Microarray analysis

Genomics involve the generation of DNA sequences and DNA markers, genetic linkage, physical maps and QTL mapping, gene discovery and the genetic control of complex traits and metabolic pathways .There exist three levels of genomic branches namely structural genomics, functional genomics and comparative genomics. Essential steps or targets of functional genomics involves the gene expressionprofiling though monitoring RNA and protein expressions following proteomic and transcriptomic tools and also find out mutant forms of gene. It co relates the gene function and gene trait relationships through gene expression analysis with the advanced procedures like DNA microarray.Expressed Sequence Tags (ESTs) are generated by creating libraries of mRNAs represented as different clones. The sequences from ESTs are properly clustered to create EST assemblies which form the basis to develop microarray probes and also to generate data for gene identification. National center for Bio-technology information (NCBI) keeps ESTs numbering into millions and serve as a major database support.

Serial analysis of gene expression (SAGE)

SAGE works on the principle that each RNA molecule could be identified through a unique short sequence tag of 10-14 Bp in

length. In this technique C-DNA is synthesized from mRNA through reverse transcription and the unique identifying sequence is extracted, ligated into a concatemer and the quantification of the same done through sequencing. This sequencing based technique is useful in developing bio markers and also enable the identification of differentially expressed genes not revealed through Micro array.

Massively Parallel signature sequencing (MPSS)

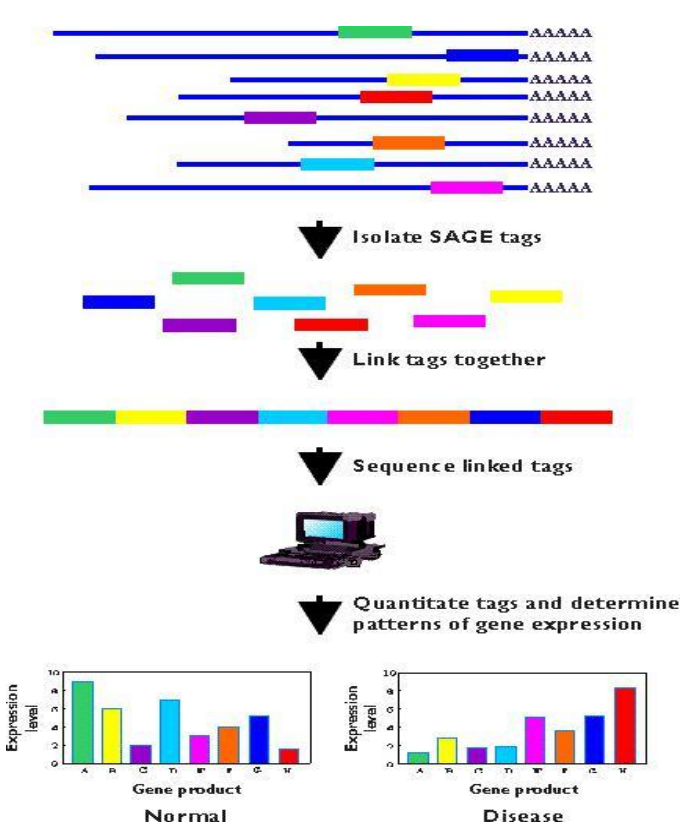
In this process, the C-DNA produced from experimental RNAs are tagged and attached to microbeads for flow cell sorting to quantify RNAs. This is useful in studying gene expression of species with little knowledge on sequences like pacific oyster.

Transcriptome sequencing (RNA-Seq)

This is the novel and emerging technology based on next-generation sequencing enable the researcher to simultaneously identify the novel regions of the sequences generated as wellas the quantification of the transcript and therebymeasures relative abundance. These advantages make it more suitable than microarray and the use of this technique is getting popular.

Comparative genomics

Vulnerable steps in the evolution of life begun more than 3.5 million years ago is recorded in the genomes of present day organisms and comparative genomics



deals with determining commonality among coding and non-coding genes between different species in relation to observed traits. Series of additions, deletions, duplications had been occurred during the evolution at gene level and among coding sequences itself, few sequences are ultimately expressed as proteins, while the left over is engaged in regulating gene expression. Carp (*cyprinuscarpio*) beta Actin gene shows 88-91% similarity at DNA level with other vertebrates including human beings and interestingly 100% conservation is observed among the sequences of transcriptional regulatory mottifs. This high lights the importance of regulatory sequences than the actual coding sequences ,point mutation of latter may not always critical in making a change in protein structure. Hence regulatory sequence divergences play an important

role in “switch on” and “switch off” mechanisms of genes which could be suitable to select natural mutants linked to positive traits to be used in marker assisted selection through genomic approach.

Experimental Determination of Gene Function

In order to define the function of a gene product and the true roles being played by the gene, functional assays which provide the exact physiological role of genes in managing the health and development of an organism are important. Essentially two approaches are common namely forward genetics and reverse genetics. Forward genetics approach deals with randomly mutating a DNA, observing resultant phenotype due to inactivation of gene followed by identification of mutated gene. Reverse genetics essentially deals with knocking out the gene of interest and follow the phenotype. Single mutation may not result in significant expression that can be noticed phenotypically. Three generation screening of post mating between populations of mutant and wild types in order to find out the mutant gene signals is functional breeding approach to raise mixed populations of mutant varieties. In this approach, mutant populations are mated with wild type and F1 and F2 are generated. Mutant expression signals could be detected from F1 generation onwards. Other methods such as Insertional mutagenesis, RNAi-mediated inhibition of gene expression or post transcriptional gene silencing etc are being employed to understand the gene function to work out strategies.

Functional genes related growth studied in fishes

Specific genes associated with growth related traits in fishes involve in regulating the growth of fishes in wild as well as in culture. Their expression pattern is also depending on the environmental parameters. Hencespecific strategies are essential to study the genes controlling the harvest traits living in diverse habitats.In many cases, the polymorphism of these genes negatively or positively correlates the growth of the fishes. In addition to the growth hormone gene (GH) genes like myostatin(MSTN), a member of the transforming growth factor-β superfamily and the insulin-like growth factor 1(IGF1) are two important genes which influence the growth.

IGF

IGF1 and 2 along with few binding proteins and receptors work together under the stimulation by growth hormone to development and normal growth. Polymorphism within this highly conserved IGF gene. Genetic variations were detected in the IGF1 gene in Artic charr and sinipercid species where they are found to be associated with growth traits. Single nucleotide polymorphisms (SNPs) regulating growth trait positively were also detected in farmed atlantic salmon, making them suitable for developing markers for marker assisted selection in aquaculture industry.

Myostatin (MSTN)

Myostatin is a member of TGF-β superfamily that inhibits or negatively regulates muscle growth in animals and it is conserved in vertebrates. Interestingly a mutant or polymorphic form of the gene

has gained attention as the phenotypic growth is enhanced to a great percentage in muscle growth. Hence the much focus is given to this gene in farmed animals. Recent years have witnessed research findings from fishes almost in the same line as in livestock animals. This has made the research groups to initiate work in detecting myostatin polymorphisms from wild to be used in marker assisted selection of the brood stock. Many results co relate the presence of SNPs within the exon of the gene and incidence of higher growth in many fishes including sea bass, Atlantic salmon., common carp etc.

Genes from finfishes with therapeutic use

Anti-microbial peptide with antimicrobial, antiviral and anti-tumour activities is distributed in mammals, birds, amphibians and fishes. Unlike in mammals, several variants /isoforms of this gene are found in fishes with expression in different tissues indicating an important role in fish immune defence system to confront complex aquatic environment. Hepcidin is a cysteine rich anti-microbial peptide with antimicrobial, antiviral and anti-tumour activities. A new four cysteine hepcidin isoform was recently isolated from the grouper species *Epinephelusdiacanthus,* The 270 bp ORF coding for a micro peptide with 89 amino sequences have shown 10 base substitutions with related fish species. Synthetic mature peptides are also found to show the therapeutically active properties and thereby opening a new area of developing drugs from oceans. Hence identifying more useful genes and expressing them in proper host systems will ensure natural bulk production and thereby availability at a lesser cost.

Examples genetic divergence and positive trait benefits

Growth Hormone gene length polymorphisms (17mer or15 mer) in Intron 1 &2 directly linked with growth traits (Sea bream).

Presence of microsatellite in promoter region of Growth Hormone GH directly linked with growth traits (Sea bream).

A novel polymorphism in GH intron 2 detected through RFLP is found to be linked to growth rate

Homozygous (Hb1*2/2) shows faster growth rate and earlier age of first spawning (non-synonymous mutations Hb-β gene).

Mitochondrial genes are playing important roles in adaptation which is readily reflected as sequence divergences and the same is used to differentiate species

SUGGESTED READINGS

TeresiaBuza and Fiona M. McCarthy. Functional genomics: applications to production agriculture. CAB Reviews 2013 8, No. 054.

Carolina Peñaloza, Alastair Hamilton, Derrick R Guy, Stephen C Bishop and Ross D Houston. A SNP in the 5' flanking region of the myostatin-1b gene is associated with harvest traits in Atlantic salmon (Salmosalar). BMC Genetics 2013, 14:112.

Zhanjiang (John) Liu. Aquaculture Genome Technologies. Auburn University, Blackwell Publishing, 2007.

Nutritional challenges in aquaculture and mariculture

P Vijayagopal
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The major challenges in aquaculture are ensuring availability of quality seed and feed consistently. All over the world recognizing the unprecedented growth potential of this food producing sector, efforts a directed towards research and development which forms the basis for addressing the problems in making available quality seed and feed in right quantities at the right time, cost-effectively. Mariculture in particular, imposes the constraints as well as opportunitiesby limiting the choice of food production from seawater.

Let us look at the challenges in mariculture nutrition under the broad headings, Larval and nursery phase, grow-out culture and broodstock nutrition.

START NUTRITION

This is the phase when the fish hatches out in nature or in a hatchery. The conditions are entirely different in both the aforementioned situations. In nature, they are subjected to the vagaries of nature. Most of the marine forms have prolific fecundity and low survival. They have access to all kinds of foods from bacteria to zooplankton as in a supermarket type of environment from where they can pick and choose ensuring nutritional completeness. In a hatchery, we limit space and the choice of food organisms and provide what we think is appropriate and cost effective. This is what challenges us. Thus, we limited ourselves to two live feed types, phytoplankton and zooplankton and found that we cannot manipulate the nutritional content of phytoplankton. Zooplankton used, like rotifers and artemianauplii were found to be deficient in certain nutrients andwe invented enrichment techniques albeit problems.

Where are we now amidst such a situation? Larval cycle of shrimp can be completed on a commercial scale without live feeds. Live feeds still remains the first choice, co-feeding formulated feeds with live feeds is next and completing the entire cycle with formulated feeds remains the last choice. For tiding over crisis situations caused due to crash of live feed cultures, safe alternatives and techniques are available for shrimp.

What about fish larviculture? There are lot of variations in feeding practices because of the nutritional disposition of each of them. The regimen remains to be hatching in green water, followed by feeding of rotifers of copepdites, then artemianauplii

and then to weaning diets. The status quo being so, the survival rates in fish hatcheries, especially marine fish, remains low. To state a comparison with shrimp – if the shrimp larval survival up to post-larvae is above 85%, in marine fish it remains below 50% under exceptional conditions. In general it is less than 25%. Let us look at the challenges here. Marine fish larvae are generally `altricial' – meaning undeveloped or underdeveloped. They lack sufficient yolk when hatched out. And with mouth opening within 3-5 days of hatching, they have to start feeding to survive. Alagal replacements do not work as well as in shrimp. Sizes of feeds range from <10 microns (microalgae) to 1.5 mm in the nursery phase, implying that the feeds meeting the nutritional requirements of the larvae of intermittent sizes is crucial. This choice of intermittent sizes can be met only by copepodites which not only satisfies the size but also the nutrients, especially the requirement of phospholipids. The importance of micro-feeds which will be discussed in the nursery phase is imminent here.

Nursery phase

In this phase, especially in marine food fish culture, the fries are grown to a stockable size either in ponds or cages. This is stage of fast growth and as growth progresses the mouth size of the fish also changes according to which the feed size has to be regulated. The entire range of feeds including larval feeds of the size ranging from 10 microns to 1.5 mm in particle size of diameter (if it is spherical) are known as microfeeds. Size reduction of feed ingredients to particle sizes less than the final feed is crucial in thisand technologies involve micro pulverizing equipment called micronizers. There are also specialized mills like ball mills which are utilized to achieve these particle sizes. Using such size reduction technical the feed ingredients are blended and extruded. The extrusion technology used to produce microfeeds is twin-screw extrusion. The drying technology for these feeds is using fluidized bed dryers. The micro feeds are in two forms either a crumble or a sphere. Crumbling is easier because it involves only crushing and sieving. Whereas, making it into sphere involves technologies like particle assisted rotational agglomeration (PARA), sphereonization or marumerization, all borrowed from the pharmaceutical industry. Floating, sinking and slow sinking are other physical properties which can be imparted during extrusion. It may be also noted that feeds finished as spheres is reported to be better nutritionally than crumbles because cracking during crumbling may lead to formation of crumbles with incomplete nutritional composition. At present feed technology is fool proof in production of nursery feed. However, microfeed production is yet begin in India. The entire requirement of quality microfeeds which include ornamental fish feeds also is imported. There are only two or three private entrepreneurs making ornamental fish feeds in India. We still draw a blank in the indigenous micro feed production for food fish. Shrimp feed crumbles for initial phases of their culture and pellets for

grow out phase are produced in India and we are self-sufficient.

Grow out

In the grow out phase, for finfish both marine and freshwater floating feeds are required. The current scenario in fresh water aquaculture is the availability of indigenously produced fresh water floating fish feeds is ensured. Production of the same is from about 15 single screw extruded feed plants in the private sector mainly located in and around Bhimavaram in Andhra Pradesh. Feeds for Indian major carps, cat fishes and tilapia are available. In spite of that the feeding of oil cake + bran by hanging it through perforated gunny bags in the water column is a popular indigenous feeding technology. With the advent of extruded feeds and its availability throughout the country, the economic gain through better feed management is well accepted at thefarmers level. Understanding the potential, there is already an overcapacity to the tune of 50% available for the production of extruded fish feeds in the country.

For marine fish, the accepted norm is floating fish feeds with high nutrient density i.e.,40% protein and $\geq$ 10% fat. Even though in our research, we found that this can be reduced without affecting the animals' growth; production of such feeds on a commercial scale is yet to take off. That is because the culture of marine fish either in open sea cages or coastal ponds is yet to grow to a really commercial scale. In that case, floating feeds with fat level above 6% calls for another technology add-on to the single screw extrusion know as post-pellet liquid application (PPLA) or*in-vacuo* fat coating or vacuum coating of fat. In order to produce floating feeds with more than 10% fat the added fat has to be infused in to the pellet after pellet formation using the vacuum coating technology. Other than the use of such kind of feeds, feeding of low value fish is a normal practice in most of the Asian countries which has been proven to be equally cost effective in some geographic locations in some scenarios. Wet feeds, however increases disease incidence and parasitic infestations.

Brood stock nutrition

This is an area which is researched the least because; developing a sizeable number of brood fishes, especially the huge marine finfish, involves efforts over a period of years.Protandry and protogynyexacerbate the problem. No one will spare such a stock for experimentation rather than using it for seed production. Even though scientific management of brood stock nutrition is possible, it gets relegated in such a scenario. Thus, the status now is – no single ingredient is successful as brood stock feed. It is a mix and match of several ingredients with inclusion of functional feeds and nutraceuticals. Many artisanal practices are in vogue. Raw sea food, commercial feeds and specialized feed additives in isolation and combination are applied with a multiplicity of irreproducible results. Nutrients proven to influence brood stock

development and accelerated and repeated spawning are,

– 1-2% of n-3 highly unsaturated fatty acids from marine oils,
– vitamin E (250 ppm),
– carotenoids like astaxanthin (100 pm),
– vitamin C (200 ppm) and
– amino acids of marine origin (80% of the diet)

For shrimp brood stock development and volitional spawning also there are several cues in nature such as rag worms and oligochaetes. Some research has pointed towards ARA, EPA and DHA compete with each other for enzymes that regulate synthesis of eicosanoids. Therefore, ratios of EPA/ DHA and ARA/ EPA are very important in production of PGs II and III involved in regulation of various physiological functions including reproduction in fish.

Deficiencies of ARA, EPA, DHA or imbalance in ratios can lead to impairment of reproduction. ARA supplementation has profound effect on reproduction. Above and below optimal levels are found to be detrimental. Optimal ARA increases fecundity, egg viability, hatching rate and larval survival. In fish larvae, optimal levels are defined as 1:1 for seabass and 10:1 for halibut and turbot. Such a ratio is not worked out in broodstock diets.

ARA-Arachidonic acid, EPA-Eicosapentaenoic acid, DHA-Docosahexaenoic acid, PG-Prostaglandins

Nutrigenomics in Fish Nutrition Research

Sanal Ebeneezar, D Linga Prabu, Chandrasekar S, Adnan Hussain Gora, Sayooj P
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Nutrigenomics is a branch of research which attempts to study the genome-wide influences of nutrition. From a molecular perspective, nutrients are considered as 'signalling molecules' that are detected by the cellular sensor systems and influence gene and protein expression and subsequently, metabolite flux and production. Hence, at a molecular level, several changes occur in the cells during normal feeding, starvation or over feeding. On a genomic level, the patterns of gene expression, protein expression and metabolite production in response to particular nutrients or nutritional regimes can be viewed as 'dietary signatures'. Nutrigenomics seeks to examine these dietary signatures in specific cells, tissues and organisms, and understand nutritional influence on homeostasis. Thus, this science seeks to understand the influence of dietary components on the genome, transcriptome, proteome, and metabolome.

Furthermore, nutrigenomics aims to identify the genes that influence the risk of diet-related diseases on a genome-wide scale, and to understand the mechanisms that underlie these genetic predispositions. Nutrigenomics also involves the studies on nutritional factors that act in protecting the genome. Nutrigenomics also focuses on the prevention of chronic diseases that are partly mediated by chronic exposure to certain food components. (Fig. 1)

Transcription factors are the main agents through which nutrients influence gene expression. The nuclear hormone receptor superfamily of transcription factors is the most important group of nutrient sensors which bind nutrients and their metabolites. These include retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR), vitamin D receptor (VDR), farnesoid X receptor (FXR), (also known as bile salt receptor), constitutively active receptor (CAR), pregnane X receptor (PXR) etc. (Table 1).

Nuclear receptors, such as the peroxisome proliferator activator receptor-α (PPARα) form heterodimers with the retinoid X receptor and bind to specific response elements in the promoter region of genes. In metabolically active organs (liver, intestine, adipose tissue), they act as nutrient sensors influencing the transcription of specific genes in response to nutrient changes. For instance, the PPAR group of nuclear receptors acts as nutrient sensors for fatty acids and influences gene expression. There are more than 3000 to 4000 target genes of PPARα that are involved in numerous

metabolic processes in the liver- fatty acid oxidation, ketogenesis, gluconeogenesis, amino acid metabolism, cellular proliferation and the acute-phase response.

Nutrigenomics research tools

The expression of genes influenced by nutrients are identified using genomics tools such as transcriptomics, proteomics and metabolomics, which subsequently allows the regulatory pathways through which diet influences homeostasis.(Fig. 2)

(i) Transcriptomics

Transcriptomics involves the study of specific or a complete set of activated mRNA transcripts. The mRNAs are produced by a given moment and in a given tissue of a selected organism; therefore, gene expression varies according to the different circumstances and periods of time. Transcription factors when activated, migrate to the nucleus and bind to a specific sequence of DNA in the promoter region of genes, thus inhibiting or facilitating transcription. These transcription factors can be stimulated by physiological signals, such as those triggered by nutrients/bioactive food compounds or the metabolites resulting from them; or by hormones, pharmacological treatments and diseases. They act as sensors regulating/modulating transcription of the cells as needed. In nutrition research, transcriptomics can assist in providing information about the mechanisms or underlying cellular effects of a particular nutrient or diet. It can also help in identifying genes, proteins or metabolites that change in the state of prediseases, as well as assisting on recognizing and characterizing the pathways regulated by nutrients or bioactive compounds in foods. Understanding the transcriptome is essential for interpreting the functional elements of the genome and understanding of growth, health, physiological well-being and flesh quality.

(ii) Proteomics

Proteins are an important class of molecules that are found in all living cells. They play a variety of roles in the cell such as structural, mechanical, biochemical, cell signalling, transport and storage. They are also an essential part of the diet. Proteomics is the science that studies the complete set of proteins involved in the biological processes of a certain species. These proteins act in the cell, tissue or organ in its normal state, but in different physiological or pathological situations, they may change their expression level or even their activity, likewise in transcriptomics. Number of proteins produced by an organism is much larger than the number of genes that it possesses. This happens due to the numerous post transcriptional/translational modifications. The proteome represents the protein equivalent of the genome, which is determined by the sequence, the type and number of its nucleotides. In contrast to static nature of the genome, the proteome represents a tremendously dynamic object, which is influenced by a variety of

parameters. However, arraying of proteins is more difficult than the arraying of DNA, because they have to maintain their correctly folded conformations. There are two types of alterations in the proteome by nutritional intervention- the quantitative or functional change. The tools for detecting these changes are also developed differently. However, there are common preliminary steps of protein isolation and purification. The tools used in quantitative proteomics are Mass spectroscopy, digestion of peptides and isotope labeling. The tools used in functional proteomics are 2D PAGE, mass spectroscopy and antibody based techniques. In functional proteomics, the protein structure and function have to be detected. In this field, the proteins are immobilized on antibodies and available after separation by 2D PAGE. It enables the efficient characterization of microheterogenety of proteins. Real time protein kinetics and protein sequencing can also shed light of functional proteomics. Hence, proteomics is a primordial resource for nutrigenomics.

(iii) Metabolomics

Metabolome consists of a set of small primary/secondary metabolites and body fluids of an organism or species. Metabolomics is the area of functional genomics that studies the changes in metabolites, whose goal is to isolate and characterize them. The metabolites are dissolved in the cell cytosol and are small organic molecules that interact directly with the proteins and other macromolecules. They are divided into primary and secondary metabolites. Primary metabolites are directly involved with the routes of synthesis and degradation of macromolecules, while secondary metabolites act as structural components and their defence. The metabolites in living beings can act as substrates, such as inhibitors or activators of an enzyme, molecular precursors, wastes of synthesis, or degradation of macromolecules. Research advances in this area may facilitate the understanding of how the genotype is related to the phenotype of an individual. Nutritionally, metabolomics allows the understanding of metabolic arrangements and instabilities due to interferences from the diet and how these changes may affect the health. This contributes to the knowledge of how the excess or lack of some nutrients or compounds (secondary metabolites) present in food can affect the health/illness of an organism. These compounds interact in several ways within the body, changing the metabolome pathways.

Hence, metabolomics also studies the metabolism under environmental and genetic perturbations, which can be analyzed and interpreted with the help of bioinformatics and statistical tools.

Applications of nutrigenomics

1. Personalized nutritionIdentification of sensitive genotypes.
2. Accurate prediction of effects of nutrients on health

3. Understanding of how nutrition influences metabolic pathways and homeostatic control
4. Prevention of chronic diseases that are partly mediated by chronic exposure to certain food components.

NUTRIENTS/ METABOLITES INVOLVED IN GENE REGULATION

Genes are influenced by the nutrients in feed. The cellular sensor systems detect the nutrients as signals that influence gene and protein expression and subsequently the metabolites. Hence the overall manifestation of growth of an organism is influenced by the nutrients which regulates the growth linked genes and their expression. Some of the examples are as follows:

Fatty acids and their derivatives are known to directly regulate the activities of a variety of transcription factors whose functions are in the control of genes responsible for fatty acid, cholesterol and carbohydrate metabolism. Fatty acids or their metabolites can regulate the nuclear abundance of SREBP1c, a key transcription factor in the synthesis of fatty acids and triacylglycerols.

During the metabolism of folate, the folic acid found in food sources, is absorbed by the intestine and, through many chemical processes of catabolism and synthesis, it is transformed into 5-methyltetrahydrofolate. This chemical component is necessary for the synthesis of methionine, which in turn is used during the process of DNA mutilation. Thus, a diet poor in folic acid can alter this process and interfere on DNA replication, leading to an increased risk of cancer development.

Vitamin A is involved in gene expression of PEPCK (Phospho Enol Pyruvate Kinase) and IGF (Insulin like growth factor). Vitamin A deficiency condition leads to changes in chromosomal structure of RARE (Retinoic Acid Responsive Element), which further leads to change in co regulator binding and activity. Various minerals work as protectors against cancer development. Among them are (i) selenium, which stimulates the production of glutathione peroxidase enzyme that acts on the reduction of hydrogen peroxide and maintain the integrity of cell membranes and (ii) zinc, which act on processes for the maintenance of genomic stability, genetic expression, and apoptosis modulation.

Prostacyclins decrease the oxidative damage of important molecules, such as DNA, lipids, and lipoproteins.

Molecules present in contaminated food can produce toxic metabolites that may interact with DNA, modifying its structure and inducing mutations. In the case of aflatoxin B1, which forms an adding compound is able to bind to the N-7 position of guanine residue, generating a new product. This new molecule cleaves the interaction between one sugar and one nitrogenous base of a nucleotide, leading to the formation of an apurinic site. The mutation can thus cause severe damages on the liver, including necrosis, cirrhosis and carcinoma.

NUTRIGENOMICS IN AQUACULTURE RESEARCH

The applications of omics technologies in fish nutrition will open new knowledge on the molecular mechanisms of growth and metabolism in fish and a more precise evaluation of the biological properties of feeds (Table 2). The variations of transcriptome expression profile of culture fish can be applied to assess the level of stress due to various factors such as handling, stocking density, salinity, hypoxia, temperature, other contaminants etc. and its mitigation through dietary interventions. The estimation of species specific nutrient requirements can be done more precisely using nutrigenomic tools by identifying the expression of key genes. The effects of dietary nutrients on the expression profile of hepatic transcriptome will help to learn the nutrient requirement and its effects in fish species.

In fish larval nutrition, nutrigenomics finds immense applications by determining the quantitative temporal expression profiles of digestive enzyme genes. Weaning of larvae to artificial diets is a crucial step which determines their survival to a significant extend. Since the digestive system of larvae is primitive, the digestive enzymes in adequate quantity may not be available for the digestion of artificial feeds. This results in the mass mortality of larvae in hatcheries. The data obtained through gene expression profiling will aid in the formulation of suitable weaning diets for each larval stages.

The protein nutrition in fish affects the transcription of number of genes through the regulation of transcription regulators expression. The protein requirement study can be effectively studied by the hepatic transcriptome of fish using cDNA microarray techniques. For example, the rainbow trout fed with low protein levels showed that the over expression of one gene is responsible for the inhibition of growth in terms of cell multiplication or division than the high protein fed fish.

The glucose intolerance phenomenon in fish is another area having scope for nutrigenomic intervention. In general, fish can utilize a limited quantity of carbohydrates as source of energy, which is more prominent in carnivorous fish. Those fed with high level of dietary carbohydrates expresses the lower level of its utilization due to the atypical hepatic regulatory mechanism. But in most of the carnivorous fish, dietary carbohydrate induces the synthesis of first phosphorylation enzyme, glucokinase which is related to higher levels of glucokinase gene expression. The metabolic system in fish prefers gluconeogenesis (from protein sources) as a means of glucose production even though carbohydrate substrates are available, which is attributed to evolutionary significance in which fish were survived in a protein rich environment. The expression of genes and factors responsible for carbohydrate utilization was lowered in due course of time. This resulted in the requirement of higher levels of proteins in fish as

compared to terrestrial animals, which adds up to the feed formulation costs, as protein represents the most expensive component of feed. Studies are in progress which focuses on the increased utilization of dietary carbohydrates in fish.

The freshwater fish in general are capable of expressing all the genes responsible for the synthesis of essential fatty acids. However, marine fish are incapable of producing sufficient levels of DHA due to the lack of expression of one or more enzymes in the fatty acid desaturation and elongation pathway. In the wild, marine fish obtain DHA by consuming the algae which synthesizes them; but under controlled culture conditions, DHA should be supplied through the diet especially in larval stages. If the expression of related genes in the synthetic pathway can be enhanced through nutrigenomic intervention, the costs of dietary DHA supplementation can be avoided in mariculture. Polyunsaturated fatty acids being an essential component in the central nervous system, the dietary supplementation of PUFA leads to brain gene expression through transcriptional modulators. Dietary lipid content influences the body particularly the muscle lipid content in fish. The key enzymes mainly the lipoprotein lipase is necessary for lipid transport and storage. In rainbow trout and red sea bream, this enzyme expression studies revealed that this enzyme works similar to the lipase enzymes of higher vertebrates. In some fish, lipoprotein lipase gene was expressed in a tissue specific manner according to the nutritional status of the fish.

Dietary phosphorous utilization in most of the fish species is around 50% of the original level. This can be tackled by understanding the mechanism of absorption of dietary phosphorous by fish. The genes associated with phosphorous metabolism such as type II sodium phosphate co-transporter, intestinal meprin 1A and cysteine sulphinic acid decarboxylase are essential bio-indicators for the dietary phosphorous optimization.

Conclusion and future perspectives

Nutrigenomics shows a new way of working with nutrition and now, the knowledge of how food interferes with the genetic code and how the organism responds to these interferences and with the phenotype can be clarified.

SUGGESTED READINGS

Panserat S and Kaushik S. J.(2010) Regulation of gene expression by nutritional factors in fish. Aquaculture Research, 41, 751-762.

Geurden I., Aramendi M., Zambonino-Infante J. &PanseratS. (2007) Early feeding of carnivorous rainbow trout*(Oncorhynchus mykiss)* with a hyperglucidic diet duringa short period: effect on dietary glucose utilization injuveniles. American Journal of Physiology 292, R2275-R2283.

ZamboninoInfante J.L. &Cahu C.L. (2001) Ontogeny of thegastrointestinal tract of marine fish larvae. ComparativeBiochemistry and Physiology C Toxicology and Pharmacology130,477-487.

Sugiura S.H., McDaniel N.K. & Ferraris R.P. (2003) In vivofractional Pi absorption and NaPi-II mRNA expression inrainbow trout are upregulated by dietary P restriction. American Journal of Physiology Regulatory Integrative andComparative Physiology 285, R770-R781.

Saera-Vila A., Calduch-Giner J.A., Gomez-Requeni P.,Medale F., Kaushik S. & Perez-Sanchez J. (2005) Molecularcharacterization of gilthead sea bream *(Sparusaurata)* lipoprotein lipase. Transcriptional regulation by seasonand nutritional condition in skeletal muscle and fat storage tissues. Comparative Biochemistry and PhysiologyPart B-Biochemistry and Molecular Biology142, 224-232.

Panserat S., Kolditz C., Richard N., Plagnes-Juan E., Piumi F.,Esquerre D., Medale F., Corraze G. & Kaushik S. (2008b) Hepatic gene expression profiles in juvenile rainbow

trout*(Oncorhynchus mykiss)* fed fishmeal or fish oil-free diets.BritishJournal of Nutrition100,953-967.

Seiliez I., Panserat S., Skiba-Cassy S., Fricot A., Vachot C.,Kaushik S. &Tesseraud S. (2008b) Feeding status regulates the polyubiquitination step of the ubiquitin-proteasomedependent proteolysis in rainbow trout *(Oncorhynchus mykiss)* Muscle. Journal of Nutrition138,487-491.

Michael Müller and Sander Kersten(2003) Nutrigenomics: goals and strategies. Nature Reviews Genetics, 4: 315-322.

Leaver M.J.,Villeneuve L.A., Obach A., Jensen L., Bron J.E.,Tocher D.R. & Taggart J.B. (2008) Functional genomics reveals increases in cholesterol biosynthetic genes andhighly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon *(Salmo salar)*. BMC Genomics 9, doi: 10.1186/1471-2164-9-299.

Seiliez I., Panserat S., Kaushik S. &Bergot P. (2001) Cloning, tissue distribution and nutritional regulation of a delta 6-desaturase-like enzyme in rainbow trout. ComparativeBiochemistry and Physiology, B130,83-93.

Panserat S., Medale F., Blin C., BrequeJ., Vachot C., PlagnesJuan E., Gomes E., Krishnamoorthy R. & Kaushik S.(2000a) Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. American Journal of Physiology RegulatoryIntegrative and Comparative Physiology 278, R1164-R1170.

Panserat S., Medale F., Breque J., Plagnes-Juan E. & Kaushik S. (2000b) Lack of significant long-term effect of dietarycarbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout *(Oncorhynchus mykiss).* Journal ofNutritional Biochemistry11, 22-29.

Gomez-Requeni P., Calduch-Giner J., de Celis S.V.R., MedaleF., Kaushik S.J. & Perez-Sanchez J. (2005) Regulation ofthe somatotropic axis by dietary factors in rainbow trout*(Oncorhynchus mykiss).* British Journal of Nutrition 94,353-361.

Hevroy E.M., El-MowafiA.,Taylor R.G., Olsvik P.A., NorbergB. &Espe M. (2007) Lysine intake affects gene expressionof anabolic hormones in Atlantic salmon, *Salmosalar*.General and Comparative Endocrinology152,39-46.

Table 1 Transcription-factor pathways mediating nutrient–gene interactions

Nutrient	Compound	Transcription factor
Macronutrients		
Fats	Fatty acids Cholesterol	PPARs, SREBPs, LXR, HNF4, ChREBP SREBPs, LXRs, FXR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A Vitamin D Vitamin E	RAR, RXR VDR PXR
Minerals	Calcium Iron Zinc	Calcineurin/NF-ATs IRP1, IRP2 MTF1
Other food components		
	Flavonoids	ER, NFκB, AP1
	Xenobiotics	CAR, PXR

Table 2 Examples of nutritionally regulated genes expressed in fish

Genes	Species	Nutritional regulation	Selected reference (one for each gene)
Digestion			
Amylase (intestine/pancreas)	European seabass	+ by dietary carbohydrate levels	Geurden *et al.* (2007)
Trypsin (intestine/pancreas)	European seabass	+ by dietary protein levels	Zambonino Infante and Cahu (2001)
Nutrient transport			
Sodium-phosphate co-transporter	Rainbow trout	+ by dietary phosphorus levels	Sugiura *et al.* (2003)
Lipoprotein lipase (mesenteric fat tissue)	Gilthead sea bream	– by dietary plant protein inclusion	Saera-Vila *et al.* (2005)
Protein metabolism			
Glutamine synthase (liver)	Rainbow trout	– by dietary plant protein inclusion	Panserat *et al.* (2008b)
Atrogin (muscle)	Rainbow trout	– by refeeding	Seiliez *et al.* (2008b)
Lipid metabolism			
Cholesterol biosynthetic genes (liver)	Atlantic salmon	+ by dietary vegetable oil inclusion	Leaver *et al.* (2008)
Delta-6 desaturase (liver)	Rainbow trout	+ by dietary vegetable oil inclusion	Seiliez *et al.* (2001)
Glucose metabolism			
Glucokinase (liver)	Rainbow trout	+ by dietary carbohydrate levels	Panserat *et al.* (2000a)
Glucose-6-phosphatase (liver)	Rainbow trout	No regulation by dietary carbohydrate levels	Panserat *et al.* (2000b)
Hormonal actions			
Growth hormone receptor (mesenteric fat tissue)	Rainbow trout	+ by dietary protein levels	Gomez-Requeni *et al.* (2005)
Insulin-like growth factor I (liver)	Atlantic salmon	+ by dietary lysine levels	Hevroy *et al.* (2007)

(Source: S Panserat and S J Kaushik, 2010, Aquaculture Research, 41: 751-762)

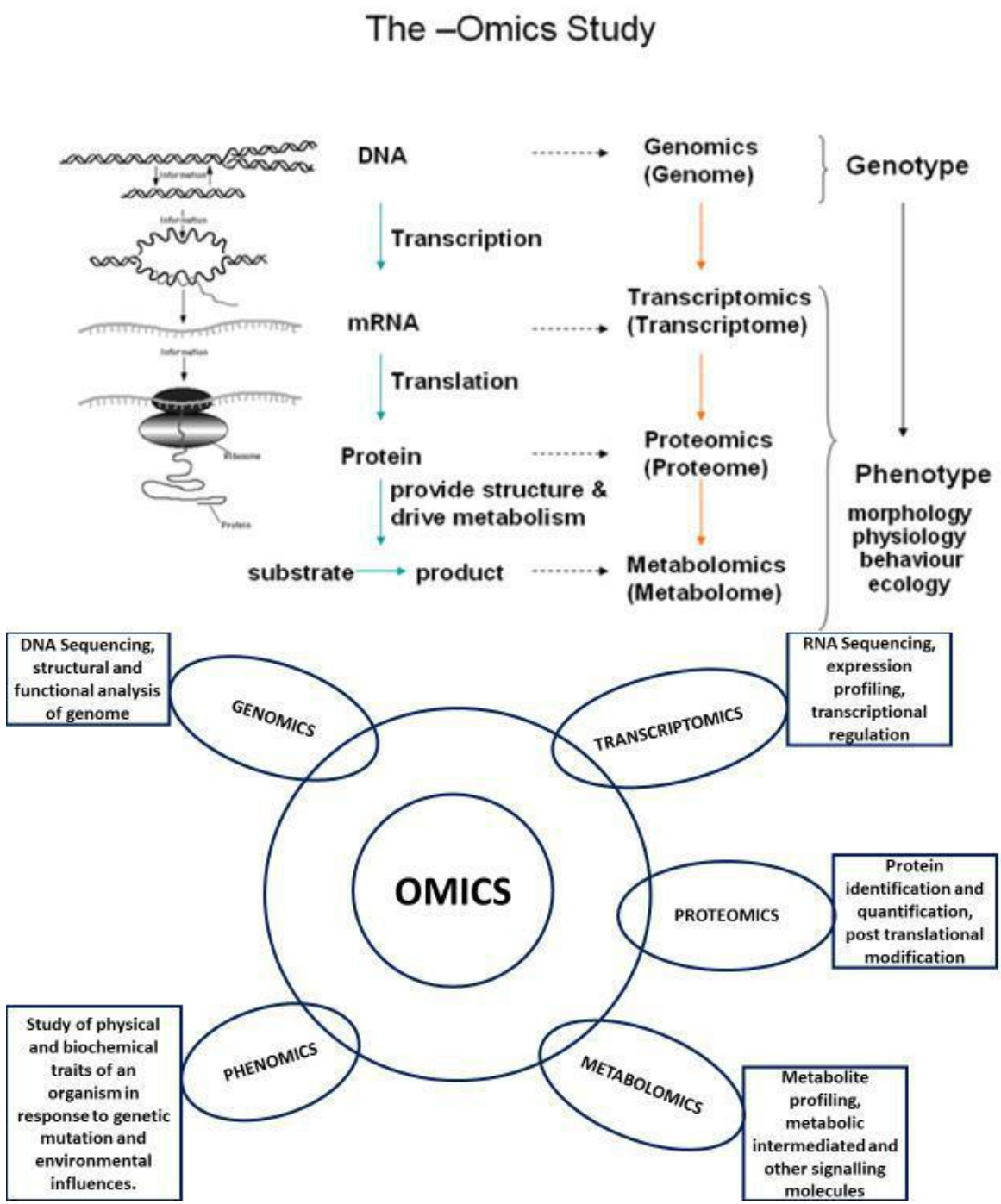


Fig. 1 Major branches and components of Omics study

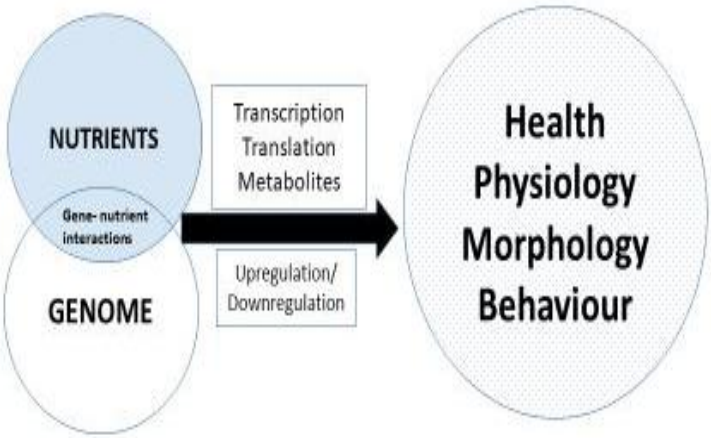


Fig. 2 Nutrient-gene interaction model

Metabolomics applications in aquaculture

P. Sayooj, Sanal Ebeneezar, P. Vijayagopal
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

HATCHERY PRODUCTION

Successful larviculture of fish and marine invertebrates presents one of the foremost challenges in the development of full life cycle rearing practices. Advances in hatchery technology are often incremental and directed by technical managers. Unfortunately, technical developments are poorly captured in the primary scientific literature and often not shared due to concerns over losing commercial advantage (Allan and Burnell 2013). While significant improvements in hatchery production technology have been made over the past two decades, considerable opportunities exist for optimizing the quality of brood stock, feeding regimes and culture systems (Hamre *et al*., 2013). For many species, production yields are greatly hampered by high mortality rates (>90%) during larval rearing (Salze *et al*., 2011; Purcell *et al*., 2012; Sorensen *et al*., 2014). In some cases, this may appears due to inadequate culture practices for new species and a lack of knowledge regarding their fundamental requirements (Calado *et al*., 2009; Manley *et al*., 2014). In other cases, successes have been attributed to environmental (Barton *et al*., 2015), genetic (Islam *et al*., 2015), nutritional (Kousoulaki *et al*., 2015) or disease-related factors (Blindheim *et al*., 2015; Richards *et al*., 2015; Solomieu *et al*., 2015). However, unexpected batch crashes and low production yields are routinely observed in some hatcheries and causation is often not identified (Hardy-Smith and Humphrey, 2010; Hamasaki *et al*., 2011; Beal, 2014). Metabolomics could be utilized within various frameworks to help solve some of these issues by providing mechanistic and functional biochemical information and to support the development of remedial strategies for poor hatchery performance. There are few reports on the application of metabolomics-based approaches to investigate the larval biology of aquatic organisms.

However, recent advances have been made with research on developmental biology using Zebrafish (*Danio rerio*) as a model organism (Papan and Chen, 2009). The Zebrafish larval metabolome is exceptionally novel through embryogenesis and can be used to successfully predict development stage, reveal coordinated gene and protein expressions and identify provisional requirements for energy acquisition and growth (Huang *et al*., 2013; Raterink *et al*., 2013). While the zebrafish is not a commercially cultured species, such research provides an exemplary application and highlights the potential for studying the early life stages of other organisms. Thus far, the only two published papers of specific applications of metabolomics in hatchery production of larvae are that of (Young *et al*.,

2015), who investigated the intraspecific growth variations in mussel larvae and the effects of handling stress and culture conditions. As an exemplar, Young *et al*. (2015) used a metabolomics approach to investigate the physiological condition of mussel (*Perna canaliculus*) larvae during hatchery production to assess larval quality. Gas chromatography–mass spectrometry (GC–MS) was used to obtain metabolite profiles from fast-growing and slow-growing larval samples collected over time from the same cultures. Good candidate biomarkers for the separation of these two larval groups were identified with a variety of univariate and multivariate feature selection methods, including volcano plot analysis, supervised projection to latent squares discriminant analysis (PLS-DA), significant analysis of metabolites (SAM) and empirical Bayesian analysis of metabolites (EBAM). These methods independently and consistently identified metabolite ratios involving levels of succinic acid, glycine, alanine, pyroglutamic acid and myristic acid as biomarkers of mussel larval quality. A closer look at the function of these metabolites suggests the involvement of various biochemical pathways in larval performance. These pathways include energy metabolism, osmotic regulation, immune function and cell-cell communication. This study illustrates not only how metabolomics can be used to assess mollusc larval quality, but signals the potential to generate predictive models of larval performance and provides mechanistic insights of biological pathways for further investigation. In the future, a great benefit to the industry would be the development of an easy-to-use tool kit to evaluate the physiological state of larvae throughout the rearing process. Our limited knowledge of exogenous and endogenous regulation of early development in different marine invertebrate and fish species restricts rapid advancements in larviculture practices. It is crucial that we mature our understanding of the factors that influence developmental timing, energy acquisition and allocation, nutritional requirements and preferences, and immunological response mechanisms. With the imminent likelihood of an expansion in the diversity of farmed marine species, it becomes especially urgent that we develop solutions to address the problem of larval nutrition and disease. Use of metabolomics could also be applied to other areas of hatchery production to help close the loop on full life cycle culture for many species. For example, routine production of high quality gametes for successful fertilization and on-growing could be achieved through better brood stock management and understanding of maternal provisioning, paternal effects and factors associated with high fecundity. While not yet realized within the aquaculture industry, metabolomics has benefitted other areas of developmental biology with respect to investigating reproductive disorders (Courant *et al*., 2013), identifying biomarkers for assessment of sperm fertility (Kumar *et al*., 2015), assessing oocyte quality and predicting embryo viability (Bertoldo *et al*., 2013; Cortezzi *et al*., 2013) and identifying the coordination of metabolic traits during selective breeding for stress resistance and

longevity (Malmendal *et al*., 2013). Metabolomics will undoubtedly be a useful tool in the progression of future hatchery technologies.

NUTRITION AND DIET

Nutritional research in aquaculture aims to improve the health of cultured species through diet and increase growth trajectories and production yields. Providing the appropriate nutrition quality and quantity is likely to have broad ramifications into other cultivation aspects, such as individual performance improvement, disease prevention, and enhancement of brood stock and gamete quality, development of sustainable and high-quality feed alternatives and mitigation of environmental impacts, among others. Optimal nutritional requirements for many new and emerging aquaculture species are unknown. Thus, there is an urgent need to determine peak dietary conditions for these organisms to support sector expansion and diversification. Even for many well-established species, the complex interactions between nutrition, health and environment are poorly understood. Furthermore, efforts to maximize production yields are inhibited by limited knowledge of larval dietary preferences and nutritional requirements for a number of species. Thus, considerable scope exists to boost full life cycle culture productivities. Metabolomics is uniquely suited to assess metabolic responses to nutritional deficiencies or excesses and can provide in-depth mechanistic insights to assist development of optimized feeding regimes. At present, nutritional metabolomics research in aquaculture is an emerging field. So far, metabolomics-based approaches have proved useful for assessing: positive and negative effects of food deprivation in mussels, trout and salmon (Tuffnail *et al*., 2009; Kullgren *et al*., 2010; Baumgarner and Cooper, 2012; Thunathong *et al*., 2012; Cipriano *et al*., 2015; Sheedy *et al*., 2015); influences between diet, environment and disease inabalone and sea bream (Rosenblum *et al*., 2005; Silva *et al*., 2014); effects of nutritional supplementation in sea bream, salmon and carp (Cajka *et al*., 2013; Robles *et al*., 2013; Anderson *et al*., 2014; Wagner *et al*., 2014); the interaction and effects of dietary protein substitution and utilization in salmon and carp (Bankefors *et al*., 2011; Jin *etal*., 2015); feeding effect of charr with contaminated and decontaminated fishmeal tosustainably expand resource use (Cheng *et al*., 2015); effects of using reduced fishmeal based feed alternatives in cobia and charr (Schock *et al*., 2012; Abro *et al*., 2014); effects of replacing fish oil with vegetable oil in sea bass feeds to decrease reliance on wild caught stocks (Castro *et al*., 2015); effects of newly introduced plant-derived contaminants in salmon feeds (Softel *et al*., 2014); and advancement of non-invasive dietary inspection techniques in various finfish species (Asakura *et al*., 2014). To illustrate the use of metabolomics to investigate the dietary performance, Abro *et al*. (2014) tested the use of a protein-rich zygomycetes fungus (*Rhizopus oryzae*) as a substitute for the traditional fish meal protein in Arctic charr (*Salvelinus alpinus*) diets.

The authors produced metabolite profiles of fish fed a commercial diet of unknown composition, a diet with mostly fish meal protein and a diet with mostly zygomycetes protein. Analysis of metabolite profiles from liver samples indicated that the zygomycetes protein diet did not differ from the fish meal protein diet and suggested similar physiological responses to these diets. However, significant metabolite differences were observed between fish fed the commercial diet and fish fed each of the other two protein-based diets with the former being an inferior diet. The study used liver samples (quenched in liquid nitrogen) to extract metabolite signatures since this organ is actively involved in metabolism of absorbed nutrients, such as proteins. The combination of a nuclear magnetic resonance (1H-NMR) analytical platform and statistical analyses, including orthogonal projection to latent squares discriminate analysis (OPLS-DA), provided a powerful pathway to identify the differences and similarities among the metabolite profiles to test for diet effects. In addition, the study highlights the possibility of using alternative protein sources, which may prove to be more sustainable for fish cultivation practices in the future. In another study of fish diets, (Robles *et al*.2013) used the short-chain fatty acid butyrate as a diet supplement to increase body weight and enhance intestinal tract activity in sea bream (*Sparusaurata*). Using a high-performance liquid chromatography–mass spectroscopy (HPLC–MS) platform, the authors measured over 80 metabolites from fish intestine samples before and after feeding with the butyrate supplement diet. Initial samples were taken after a 12h starvation period to obtain a basal metabolite profile with a nonactive intestine. Three hours after the initial feeding, intestine samples were collected from fish that were fed the butyrate supplemented diet and fish fed diets without the supplement. Growth measurements of the remaining fish within each treatment were taken after 8 weeks. Results showed significant improvements with butyrate diets, including weight gains, increases in several essential amino acids and nucleotide derivatives, and potential increases in cell energy provisions through glucose and amino acid oxidation pathways. Based on these results, it appears that butyrate may be a good natural supplement to enhance fish growth and metabolic activity. As an emerging tool in nutrition research, metabolomics offers a unique potential to unravel the complex intertwining mechanisms involved in nutrient utilization, reproduction, growth and disease progression. Through the catabolic breakdown of macromolecules in foods and direct incorporation of smaller components, the metabolome is the receiving depot for the raw materials required by cells to synthesize new products. These materials are essential for the formation and repair of body tissues and the production of energy to support and maintain life. As signaling molecules and enzymatic cofactors, metabolites are involved in the synthesis, degradation and modification of proteins, which regulate gene expression and metabolic pathways. It is the intricate combination of these multifaceted processes which are required for biological systems to maintain homoeostasis. Metabolomics can provide important mechanistic insights to identify how regulation of homoeostatic control is disturbed in the early phases of diet-related diseases. This knowledge could be used to identify the new metabolic biomarkers for health and nutritional status and to develop strategies for the dietary prevention and intervention of diseases.

Future nutrition research in aquaculture will undoubtedly be radically advanced through the application of metabolomics approaches.

SUGGESTED READINGS:

Abro R, Moazzami AA, Lindberg JE, Lundh T (2014) Metabolic insights in Arctic charr (*Salvelinus alpinus*) fed with zygomycetes and fish meal diets as assessed in liver using nuclear magnetic resonance (NMR) spectroscopy. International Aquatic Research 6 (2): 1-11.

Allan G, Burnell (2013) Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited, Oxford, UK

Baumgarner BL, Cooper BR (2012) Evaluation of a tandem gas chromatography/time-of-flight mass spectrometry metabolomics platform as a single method to investigate the effect of starvation on whole-animal metabolism in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 215: 16271632.

Kullgren A, Samuelsson LM, Larsson DG, Bjornsson BT, Bergman EJ (2010) A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 299 (6): R1440-R1448

Schock TB, Newton S, Brenkert K, Leffler J, Bearden DW (2012) An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. Food Chemistry 133 (1): 90-101.

Young T, Alfaro AC, Villas-Boas S (2015) Identification of candidate biomarkers for quality assessment of hatchery-reared mussel larvae *via* GC/MS-based metabolomics. New Zealand Journal of Marine and Freshwater Research 49 (1): 87-95.

Diseases in mariculture–Parasitic Diseases

N.K. Sanil
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The continuous decline in wild catch and the growing demand for quality fish have acted asdrivers forthe rapid growth in aquaculture sector.World over, production from farmed/cultured fish has more than doubled during the past 20 years and the trend is set to continue. Sea-cage culture turned out to be one of the most important factors that have influenced the rapid increase in aquaculture production.

Proper health management is a major problem in all animal production systems. The success of any farming technology is directly correlated with reduction of production costs,and aquaculture is no exception. It has been plagued with numerous diseaseproblems caused by viruses, bacteria, fungi, parasites and diseases of unknown etiologies. Presently, disease is one of the most important constraints that threaten aquaculture and as aquaculture activities intensify and expand, the threat from these diseases also get magnified. Among the pathogens affecting cultured fishes, parasites play an important role, especially in cage culture systems which allow free exchange between wild and caged organisms, paving way for diseases.

Parasites form an integral part of aquatic biodiversity, and under natural conditions have low rates of infection/infestation and inflict minor pathologies on their hosts. Butin intensive rearing systems they can easily spread, causing serious outbreaks and epizootics leading to significant economical losses.Numerous factors includingthe environment influence the outcome of parasitic infections/infestations. Usually, high water temperatures promote faster hatching of eggs and parasite propagation resulting in cyclic peaks of parasitic infestation in sea cages during summer months. Temperature influences the immune defenses in fishes, and may in turn affect the parasite loads. Altered/unfavorable environmental conditions can induce stress in cultured animalsand this can also produce a cascading effect on the physiology and disease resistance/susceptibility of animals thereby influencing the outcome of diseases.Many of the routine aquaculture practices are known to induce stress. High-density living conditions in cages increases host proximity and thereby enhance parasite transmission rates, and parasites having direct lifecycles and low rates of infestation in the wild can easily spread in confined populations in rearing systems. This is true for bivalves also where higher temperature and salinity make them more susceptible, while higher population densities make pathogen transmission easier. Similarly introduction of new species without any sanitary control could also enhance the risk of parasite

transfer.Intensive aquaculture doesplay a role in introducing parasites to new hosts and/or new localities thereby spreading many transboundary diseases. Although, cage farming has many economic advantages, like in any other animal production system, here also diseases act as major limiting factors to the successful production.

IMPORTANT PARASITIC DISEASES IN MARICULTURE

Rapid development of fish culture in marine cages has been associated with an emergence of parasitic diseases and among these, ectoparasitic diseases act as the major threat.

Ciliates: Ciliates comprise of a large group of protozoans inhabiting most of theaquatic habitats. They are mostly free-living, some are commensals while many others areparasitic. In most cases ciliates do not inflict much harm, but some species are severely pathogenic to fish.

C*ryptocaryon irritans*is one of the most virulent parasites of fish.The infective phase is represented by tomites that become pyriform,free swimming theronts, which penetrate skin and gills of fish hosts. Generally the time required to complete its life cycle is approximately 6 days at21-24°C,but may vary. C*ryptocaryon irritans*infests the epithelial tissue of fish causing serious lesionsleading to heavy mortalities.

Brooklynellahostilis feed on epidermal layer of skin and gill tissues as well as blood,causinghemorrhages. In captive conditions and in cages,under favourable conditions it multiply causing outbreaks. Stress and high temperatures favour epizootics.

*Trichodina*spp. arenormally found on the skin and gills offish hosts. Though smallnumbers does not cause any mortalities, massive infestations can cause histopathological and degenerative alterations, such ashyperplasia, increase of mucous cells and inflammatory reactions leading to mortalities.

Dinoflagelates: The most important parasite causing high mortalitiesinmariculturedfish in tropical waters is the dinoflagellate,*Amyloodiniumocellatum* and the disease is known as Amyloodiniosis.The disease is characterized by signs of appetite loss, followed by opacity of skin with whitish areas and erosion of caudal fin. Fish show disturbed swimming patterns, flashing and rubbing against objects/bottom. Numerous parasites are found attached to gills in cases of severe infection causing heavy mortalities. The parasite shows poor host-specificity and outbreaks of *A.ocellatum*have been frequently observed in fish kept in cage/captive conditions.

Monogeneans: Monogeneans or monogenetic trematodes are flatwormsattached tobody, fin & gills of fish.Monogeneans infecting marine fish have a direct life cycle, and are considered dangerous due to theirhigh rate of

transmission among fish in culture systems. Innate susceptibility and stressful environmental conditions can cause massiveinfections.

Capsalidmonogeneans have a flattened, leaf-like body with attachment organs, inhabit the skin and occasionally on the gills and even nostrils. They feed on epithelial cells and mucus causing excessive mucus production, skin lesions, opaque eyes, anorexia and mortality. Many of them have low host-specificity making them dangerous pathogens in fishculture facilities. *Benedenia*, *Neobenedenia*and *Megalocotyloides*are some of the genera that have high pathogenic potential.

Dactylogyrids are common gill parasites of teleost fishes and are characterized by the presence of two pairs of largehooks or hamuli that helps parasite attachment. The attachment and feeding cause severe irritation, excess mucous production, epithelial hyperplasia, erosions, ulcerations, haemorrhage and hyperemia with leukocytic infiltrations.Heavily affected fish may die due to asphyxia as a result of gill pathology and interference with gaseous and ionic exchange mechanisms. Easy multiplication & dispersion coupled with its direct life-cycle make them more dangerous in culture systems. Other important monogenean parasites of cultured fish include Diclidophorids which include *Heterobothrium*and*Neoheterobothrium*an d Microcotylids, capable of causing serious mortalities in cultured fish.

Copepods:Copepods are considered the most important disease-causing parasitesin both wild and cultured fish populations. Aresmall,dioecious crustaceans, with female carrying eggs in egg sacs attached to the genital segment.The egg hatches and develop intonauplius that further develops into copepodids and eventually adults in arather shortened life-cycle. Typical examples include the sea lice (*Lepeophtheirussalmonis*), and thecymothoid isopods,which are notorious parasites of cage reared fish.

Caligids are generally known as sea lice and are common in tropical and warm temperate waters. They are dorso-ventrally flattened andattach to their hosts by acombination of claws and suction. Two important genera parasitizing fishesare *Caligus* and *Lepeophtheirus.* Sea lice infestation isthe biggest problem faced bysalmonid farming industry, where the cost oftreatments alone could be around 6% of the fish production. The presence ofsea lice on non-salmonid production systems has also been associated with mortality anddiseases.Caligidsare considered the most important parasites in marine fish aquaculture.Another important genus of copepods of importance to maricultureinclude the gill parasite *Lernanthropus.*

Isopods: Isopods are marine/brackish parasitic crustaceansinhabiting warmwaters.Majority of the isopods are cymothoids parasitizing marine teleosts.Massive attack by the juveniles of

the parasite can kill fry and fingerlings. The parasites that enter the gill chamber cause anemia and loss of gill filaments due to their movements and feeding habits. The genus *Nerocila*has been found in groupers, seabass and snappers in southeast Asian countries. Isopod menace is considered an emerging problem inMediterranean sea cages causing reduced growth and mortality.The first record of serious mortalities in cage cultured fishes in India was caused by the isopod *Cirolanafluviatilis* in cage cultured seabass in Cochin backwaters. Mortalities appearedone month after stocking and fish were found dead in cages with their flesh eaten away, leaving the remnants of skeleton. *C. fuviatilis*is a voracious, carrion-feeding bottom dwelling isopod,but sometimes colonize the fouled net surrounding the cage and attack the stressed fish causing heavy mortalities. This is an example where parasites/pests that have not been previously considered pathogenic can cause serious mortalities under certain circumstances.

Endoparasites:Common endoparasites including trematodes, cestodes, nematodesand acanthocephalans are metazoans and are not considered as important threats in maricultured fishes. Though protozoans belonging to Myxosporea are capable of causing significant loses in cultured fishthey do not pose a serious threat in cageculture systems. Most of these require an intermediate host to complete their life-cycle and infections are acquired through their natural food including smaller fish and invertebrates. Since artificial feed does not carry any of these infective stages, risks of endoparasitic infections are less, but the situation may change if the feed is replaced with raw trash fish.

Perkinsosis in bivalves: Till recently there have been no reports of pathogens incultured bivalves in India. But mortalities caused by the protozoan,*Perkinsusolseni*, an OIE listed pathogen have started emerging from cultured *Pernaviridis*. Higher temperature and salinity acts as a trigger for infections. The pathogen has been recorded from many wild bivalves which may act as carriers or reservoirs thereby enhancing the transmission potential of the pathogen. *P. beihaiensis* has also been reported from cultured mussels, but information on their pathogenicity is not known.

Foodborne parasitic diseases: The relation between wild fish and thetransmission of foodborne parasitic diseases is well documented, but now transmission of such parasites to humans through aquacultured fish is also a matter of concern, especially in Southeast Asian countries. Most of the fish borne parasites come under digenetic trematodes (Opisthorchiidae and Heterophyidae), nematodes (Anisakidae and Gnathostomatidae), and cestodes, (Diphyllobothriidae). Among these, trematodes are the most important ones when associated morbidities are considered.

Pathogen spread from cages: As there are no impermeable barriers betweenfarm and aquatic environment,net pens or cage

systems involve a higher risk of disease transmission. Thus open cage farms facilitate easy transmission of parasites such as sea lice from wild to farmed fish and vice versa, causing unforeseen consequences in sympatric wild fishes. The importation of feed for aquaculture can also serve as a source of pathogens.Parasites and pathogens play an important role for fish stocks, both cultured and wild in reducing both the yield, increasing mortality and reducing the commercial value of harvested stocks.

Controlling parasitic diseases: Control/prevention of any disease inmariculture is mostly a management issue. Incidence and severity of infection often depend on various factors including the quality of environment and feed theyconsume. While there is scope to manage some of the environmental parameters in land-based aquaculture, in mariculture or cage culture set up, this will not be possible. Environmental quality is almost similar to the sea in mariculture or cage culture facilities.As species diversification is inevitable in aquaculture, characterisation of pathogens, novel diagnostics and understanding the basic epizootiology and host-pathogen interaction is a necessity. Effective quarantine and biosecurity measures need to be implemented at the hatchery level to ensure that the fry/larvae of fish are pathogen-free before being introduced into the net-cages. Practice of using wild-caught fry for stocking should be avoided as far as possible. However, implementing effective health management strategies become difficult in majority of the farms operated by small-scale farmers.

Although cage-farming presently relies on native species, many a time translocation of stocks across different geographical regions needs to be done, bututmost care should be taken. Before introducing any new species for culture in the open cages, even the native species, a thorough profile of its potential pathogens and the possible management measures need to be identified. Culture of diverse species of fishes concentrated in an area will be a serious biosecurity issue, as this would enhance the chances of disease transmission. Maintaining proper hygiene, disinfection and biosecurity is quite challenging in open cage system because of obvious reasons. However, proper cage maintenance by removing excess feed and suspended particulate matter, cleaning of fouling agents, frequent monitoring of farmed animals and removal of dead or moribund animals from cages play a crucial role in better health management.

One of the important sources of disease transmission to cage cultured animals from extraneous sources will be through water. Trash fish are being widely used as feed in most cage-farms. These are a potential source of pathogen transmission and need to be monitored. When live feed is used, it should be ensured that they are free from pathogens. Development of efficient, pathogen-free feed is a requirement for the biosecure production of farmed fish.

Chemotherapy:Various drugs are used in aquaculture to controldiseases/pathogens as well as to correct water quality problems, disinfect eggs and equipment, and control aquatic weeds and molluscs. However, the use of antiparasiticsis very limited in fish and most of the drugs used are based on extrapolation from human and veterinary medicines. Use of chemotherapeutics for controlling parasitic infestation in commercial culture systems is practiced mainly in coldwater species, especially in salmonids and very less information is available on tropical species. The successand failure of treatment depends on correct diagnosis, dose and administration methods. Treatment of parasites inmariculture systemsfaces a lot of practical difficulties and control measures should be "holistic" through proper health management protocols.Methods of treatment can be divided into six different groups - oral, bath, dip, flush, injection and topical application and each has its own advantages and disadvantages and carries potential environmental impacts.

Although, chemotherapy is effective in controlling many parasites and some of the bacterial pathogens, any attempt to apply chemicals or antibiotics in water should be strictly avoided. As in other aquaculture system, problems of drug residue, drug resistance, consumer safety, environmental safety will be great concerns. Further, application of chemicals in open cages will have serious environmental consequences, apart from non-target species safety. As in the case of salmonid aquaculture, the combination of appropriate treatments with other health management and disease prevention strategies, such as vaccination, water quality improvement, alternative treatments etc. will help to ensure successful development of the sector in future.

Bacterial and Viral Diseases of Cultured Marine Fish and Shellfish

S.R.Krupesha Sharma
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The demand for farming of high value fishes and shellfishes through mariculture has been rapidly increasing in the recent years. In India, after the successful breeding and seed production, demand for farming of cobia is also increasing. Other than cobia, Asian seabass is also regarded as a potential marine fish species for sea cage farming in India.

Increased intensification of mariculture production involving high density culture in low volume invite the risk of diseases associated with rearing stress and fish welfare. Rearing of high value fish in high density is also linked with manifestation of altered host-parasite interactions resulting from hosts being reared in new geographic locations. The altered host-parasite association may also result from unrelated hosts being reared in diverse environmental conditions (Kent, 2000). In case of marine cage culture, interactions between wild and cultured fish populations are of greatest disquiet for both aquaculturists and environmentalists. Diseases occur due to intricate interactions between host, pathogen and environment (Snieszko, 1974). Olivier (2002) suggestedpresence of pathogen in both fish and environment (water), presence of susceptible host, viability of the pathogen (number and longevity) inthe environment, and viable infection rout as essential pre-requisites for a disease to spread from either cultured fish to wild fish or vice-versa.

A. Bacterial diseases of cultured marine fish and shell fish

Diseases of farmed fish occurring due to bacterial pathology have been one of the important limiting factors in mariculture practises. Although many pathogenic bacteria have been described in the majority of the existing taxonomic groups, fairly small numbers are responsible for major economic losses in the culture system, globally. Majority of the bacterial pathogens described in the culture systems are normally present in wild fish populations. However, in natural environments, in the absence of stressful conditions, they seldom cause mortality due to disease outbreak. High stocking density and poor environmental parameters act as stressful situations for the occurrence of diseases in culture system.

1. Vibriosis

Vibriosis is one of the most prevalent bacterial diseases of cultured finfish caused by bacteria belonging to the genus

Vibrio. Vibrios are gram negative bacteria, ubiquitous to marine and estuarine environment as well as marine fish farms. There are around 34 recognized species within the genus *Vibrio.* Within the family Vibrionaceae, the species causing the most economically serious diseases in marine fish and shrimp culture include *Vibrio anguillarum, V. alginolyticus, V. harveyi*, *V. parahemolyticus* and *V. spendidus*. Other species in like *V. ponticus* and *V. ordalii* are also seldom reported tocause mortalities in fish culture system. Vibriosis caused by *V. anguillarum* is a major problem in seabass farming. However, *V. alginolyticus* has been suggested to be a pathogen of humans and several marine fish species like like seabream and Asian seabass. This species has been reported to be the causal agent of outbreak of vibriosis in grouper. An outbreak of Vibriosis caused by *V. alginolyticus* in Asian seabass cultured in marine cages in Indian has been reported (Sharma et al., 2013). The outbreak of the disease in this case was associated with increased water temperature. Isolation of *V.ordalii*, which has been established to accommodate strains formerly classified as *V.anguillarum* biotype 2 (Schieve and Crosa, 1981), has been reported predominantlyfrom North America, Japan and Australia affecting salmonid fishes. Mutharia et al (2002) found that cross-reactions subsist between *V. ordalii* and *V. anguillarum* serotype O2 using polyclonal antisera, but immunoblot analysis with absorbed antisera reveal that the LPS of both species do not have identical antigenic properties. Most of the pathogenic *Vibrios* are normal inhabitants of sea water and sediment. Hence, these organisms are opportunistic in nature causing disease when fish are subjected to stress.

Vibrio harveyi, another member of the family Vibrionaceae, is a luminous marinebacterium which is a normal microflora of warm marine environment, body of fish and shellfish, light-emitting organs of marine fish and cephalopods, and intestinal microflora of marine vertebrates and invertebrates. The organism is predominantly responsible for the occurrence of luminous vibriosis, which affects a wide range of marine invertebrates, especially penaeid shrimp and phyllosoma larvae of the rock lobster resulting in severe economic losses. Pathological manifestations of vibriosis caused by *V. harveyi* in the cage farmed mangrove red snappers associated with increased water temperature and handling stress is also reported (Sharma et al., 2014).

In case of vibriosis, the pathogen may enter the host orally, through skin lesion and gill surface consequent to wound caused by ectoparasites and protozoa. Fish affected by classical vibriosis show typical signs of a generalized haemorrhagic septicaemia with the presence of haemorrhagic lesions at the base of fins, ulcerations on the body surface, especially in chronic cases, exophthalmia and corneal opacity. Ailing fish are often anorexic with pale gills due to anaemia arising from haemorrhages. Microscopic lesions in case of vibriosis also reflect the haemorragic nature of the

disease. Histologically, bacteria invading the dermis, subcutaneous adipose tissue, and the underlying musculature are evident. Affected tissues are necrotic and heavily infiltrated by granulocytes. Gill filaments and lamellae are also infiltrated by neutrophills with haemorrhage. Liver shows hypertrophy of the bile ducts, necrosis, haemorrhage and congestion. In myocardium, loss of cross striations and infiltration of polymorphoneuclear cells in to the endocardium is noticed. Kidneys reveal characteristic lesions of acute glomerulonephritis with increased expression of melano-macrophage centres (Fig.1-B). Gastric mucosa contains engorged capillaries and loss of tubular glands. Extensive tissue lesions in vibriosis are primarily due to the release of proteinases and other extra-cellular enzymes produced by the bacteria.

Vibrios are gram negative rods characteristically curved or comma shaped. This morphological appearance may not be always observed when organisms are selected for gram staining from solid media. Specific media like Thiosulfate-citrate-bilesalts- sucrose agar (TCBS) agar may be used for selective growth of Vibrios. Species level identification can be done by biochemical tests, PCR using specific primers and 16S rDNA amplification using universal primers and sequencing.

Treatment and prevention: Eventhogh Vibrios are susceptible to majority of broad-spectrum antibiotics, limitations exist based on the farming system. Since Vibrios are opportunistic pathogens, vibriosis can be best managed by proper husbandry practices. Handling, transportation, overcrowding, low dissolved oxygen and increased water temperature make the farmed fish susceptible to vibriosis. Periodical enumeration of the bacterial load of water and sediment would help in preventing outbreaks.

Due to diversity of V*ibrios* and their serovars, the advancement in vaccine development against vibriosis has been dawdling, and commercial vaccine is not currently available. However, attempts have been made to vaccinate fish against different *Vibrio spp* using oral, killed and sub-unit vaccines. In case of oral vaccination, the vaccine is either mixed with the feed, top dressed on the feed, or bio-encapsulated. Bio-encapsulation is used when fish fry are to be vaccinated. In case of bio-encapsulation, live feed, such as artemia nauplii, copepods or rotifers incubated in a suspension of vaccine are fed to the fish fry. Oral vaccination causes no stress to the fish. However, they have a very short term stability once mixed with the feed. More recently, the outer membrane proteins molecules are used for development of subunit vaccines due to the exposed epitopes on the bacterial surface and conserved nature in different serovars. It has also been demonstrated that outer membrane proteins molecules like OMP -K acts as protective antigen against fish vibriosis caused by *V. alginolyticus*.

2. Photobacteriosis:

Photobacteriosis, also known as fish pasteurellosis, is caused by the halophilic bacteria *Photobacterium damselae* subsp. *piscicida*. Gauthier et al. (1995) included the fishpathogen *Pasteurella piscicida* in the species *P. damselae* according to phylogenetic analyses of 16S rDNA sequences and DNA⁄DNA relatedness, and named as

Photobacterium damselae subsp*. piscicida*. Pasteurellosis has been a serious disease inJapan affecting the aquaculture production considerably. The disease is characterized by the presence of whitish nodules on liver, spleen and kidney. Severe mortalities occur in pasteurellosis when water temperature is above 18– 20 °C. Below this temperature, fish can harbour the pathogen for prolonged periods without causing clinical infection. This disease was first discovered in natural populations of white perch (*Moroneamericanus*) and striped bass. The disease affects various species of fishes like yellowtail juveniles (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis*), black seabream (*Mylio macrocephalus*), red seabream (*Acanthopagrus schlegeli*), oval file fish (*Navodanmodestus*) and red grouper (*Epinephelus okaara*).

Fish pasteurellosis is a septicaemic disease and manifests as an acute or chronic form. Pale gills, dark pigmentation and presence of petechial haemorrhages on the body surface and fin base are normally observed in acute form. Enlarged spleen and mottled liver are seen internally. In case of chronic form, nodules resembling tubercles are seen in spleen and kidney.
Another disease caused by *Photobactrium damselae* subsp. *damselae* is also responsible for mortality in many cultured marine fish species. This disease has been reported from India in cage farmed cobia. The lesions are haemorrhagic in nature resembling the lesions found in vibriosis. The pathological manifestations in both the infections are primarily due to the extra cellular products (ECP) secreted by the bacteria. Both pathogens are normal inhabitants of marine environment.

Disease transmission in case of photobacteriosis occurs through direct contact and ingestion. The bacteria are unable to survive in fresh or brackish water. Predisposing factors for the outbreak normally include rise in water temperature.

The pathogen can be isolated and cultured on marine agar and ordinary media supplemented with sodium chloride. The organism can be confirmed by biochemical tests, 16S rDNA sequencing and slide agglutination. *Photobacterium damselae* subsp. *piscicida* has to be differentiated from *Photobactrium damselae* subsp. *damselae* using a multiplex PCR that combines specific primers for 16S rRNA and urease genes (Osorio et al., 2000).

Several commercial vaccines against *P.damselae* subsp. *piscicida* are available, wherever the disease is more prevalent. Efficacy of these vaccines depends on the species of fish, fish size, etc. Since outbreak of pasteurellosis normally occurs

during larval stages to fingerling stage, a vaccination programme involving dip immunisation during the larval stage with a booster dose when fish reaches a size of 1–2 g is advocated.

3. Flexibacteriosis:

Flexibacteriosis, also known as gliding bacterial disease of sea fish, eroded mouth syndrome, and black patch necrosis, is a disease of marine fish caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*). *T. maritimum* existsexclusively in the marine environment. The disease is normally distributed in cultured and wild fish in North America, Australia, Europe and Japan. Turbot, sole, seabream, seabass, red seabream and black seabream are the cultured fishes affected by *T.maritimum*. The disease occurs in a more severe form in young fish when compared toadults. The severity of the disease increases with increase in water temperature. The predisposing factors in a farming system include injuries and skin abrasions. Stress induced by husbandry practices like inferior water quality, increased water temperature, handling etc., may also predispose the fish to flexibacteriosis.

Affected fish are weak, develop pinkish ulcers on the skin with loss of scales. Affected fish also have eroded and haemorrhagic mouth and frayed fins with tail rot. White necrotic lesions develop on gills of the affected fish. Microscopically, long, thin basophilic bacteria can be seen in sections taken from skin and muscle lesions. Colonization of the bacteria on the scale pockets, loss of scales and dermatitis are also noticed. Congestion and haemorrhage in the superficial dermis is also evident histologically.

The initial presumptive diagnosis of marine flexibacteriosis include microscopic observation of accumulations of long, thin, rod shaped flexing bacteria in wet mounts or Gram-negative stained preparations obtained from gills or lesions. Phase contrast microscopy is advantageous in bacterial identification. The bacteria cannot be easily isolated on conventional media. This pathogen grows only in specific media since it needs an absolute requirement of seawater as well as low concentration of nutrients. Some of the specific media devised for the isolation of this pathogen include Flexibacter Maritimus Medium (FMM). FMM has been regarded as most effective media. The bacteria can be grown at 13 to 34°C. Colonies are rhizoid with uneven edges. A nested PCR which is rapid and sensitive was also developed for an accurate diagnosis (Cepeda et al., 2003).

Hydrogen peroxide used at a dose of 240 ppm would be beneficial in treating the infection. Many of the antiobiotics are also effective in treating the fish infected with *T.maritimum*. However, they have to be used with caution depending on the type ofantibiotic, farming system and environmental factors. A formalin killed vaccine was also tried in Japanese flounder to reduce the effects of infection.

2. **Streptococcosis**

Streprococcosis is a re-emerging disease of both fresh and marine fish caused by gram positive bacteria characterized by central nervous system damage followed by exophthalmia and meningoencephalitis. Streptococcosis is also known as "pop-eye", since one of the most characteristic clinical sign found in this disease is the accumulation of muco-purulent exudates around the eyes. Streptococcosis, a problem in both farmed and wild marine fish, has been reported from USA, Japan, and Spain. In Japan and Spain, this disease forms a majorlimiting factor for the marine fish production of yellowtail turbot. Fish of all the size are susceptible. The sea water and sediment harbours the pathogen which can be isolated from these sources round the year. It has been reported that this pathogen can survive in frozen products for at least 6 months. Warm water streptococcosis which occurs when water temperatures are above 15 °C is normally caused by *L. garvieae, S. iniae, S. agalactiae* and *S. parauberis*. Cold water streptococcosis seen when water temperatures are below 15°C is generally caused by *L. piscium* and *V. salmoninarum*. Pathogens responsible for warm water streptococcosis are of zoonotic importance since they can cause disease in humans. Horizontal transmission can occur through injuries and abrasions.

Streptococci capable of causing disease in marine fish falls into three categories: alpha-haemolytic, beta-haemolytic, and non-haemolytic. The majority of disease epizootics are generally caused by streptococci belonging to alpha-haemolytic group. Among these fish streptococci*, L. garvieae*, *S. iniae* and *S. parauberis* can be regarded as the main aetiological agents causing diseases in marine aquaculture.

L. garvieae infects marine fish like yellowtail in Japan. *S. Iniae* is an important fishpathogen causing disease and mortality in many cultured fish species in both tropical and sub tropical environments. *S. iniae* is the main aetiological agent of streptococcosis in tilapia in USA and rainbow trout in Israel. However, *S. iniae* was also isolated from marine fish including yellowtail, flounder, European seabass, and Asian seabass. There have been no reports from India. *S. parauberis* is reported to be endemic to cultured turbot.

The lesions caused by different streptococcal species in diverse host species are similar. The lesions are of general septecemic in nature affecting liver, spleen, eye, brain and kidney. The eyes show severe exophthalmia with granulametous inflammation. Granulomas with the presence of bacteria may also be seen in pericardium, alimentary tract, peritoneum, brain, ovary, testes and spleen. The most significant clinical signs are exophthalmia, distended abdomen, haemorrhages in the eyes, opercula, fin base, ulceration of the body surface and darkening of the skin. Internally, the abdominal cavity is filled with variable amounts of purulent exudate. A yellowish exudate often covering the peritoneum and the pericardium may be seen. Yellowish exudates may also be seen in

cranial cavity. Haemorrhages are found on all visceral organs.

Fish streptococci can be generally isolated from internal like spleen, liver and brain using brain heart infusion agar or tryptone soya agar supplemented with 1% yeast extract or 0.5% glucose, and growth is enhanced on blood agar. The incubation period is 24 days at 25-30°C. The isolates are then characterized either biochemically or serologically. Significant characteristics are spherical or ovoid colony morphology and formation of pairs or chains. All strains of streptococci are Gram positive, oxidase and catalase negative, non-motile and non-sporulating. Slide agglutination and immunofluorescent techniques are widely used for diagnosis of streptococcosis. PCR based diagnosis including 16S rRNA amplification and sequencing can also be employed.

Good husbandry practises including avoiding over-crowding, excess feeding, handling and the timely removal of diseased or dead fish would help to minimise the economic losses due to streptococcosis. Apart from this, vaccination, use of chemotherapeutics and immunostimulants has also been tried. Many of the isolated strains were sensitive to antibiotics like erythromycin, tetracycline, ampicillin and doxycycline.Attempts have been made to develop vaccines against streptococcosis in fish. Intra-peritoneal route was found to be most effective. β-1-3-glucans used as immunostimulants is also found to be effective.

5. Mycobacteriosis (Fish tuberculosis)

Mycobacteriosis is a chronic wasting granulometous disease of fish caused primarily by *Mycobacterium marinum*. Some species of mycobacterium are found in soil and water. Mycobacteriosis was earlier known as fish tuberculosis or piscine tuberculosisbecause the causative pathogen is taxonomically similar to *Mycobacteriumtuberculosis*, which caused tuberculosis in humans. But other than acid-fast stainingcharacteristics and taxonomic position, the organisms causing tuberculosis in fish and human are dissimilar. In cultured fish, mycobacteriosis was reported in salmon, pejerrey, snakehead fish, turbot, tilapia, European seabass and red drum. Mycobacteriosis caused by *M. marinum* represents a significant threat especially for seabass cultured on the Mediterranean and the Red Sea coast of Israel. Mycobacteriosis is regarded as a serious threat to the turbot culture in Europe. In general mycobacteriosis is more dangerous to marine fishes when compared to fresh water fishes. Incidence of mycobacteriosis in higher in aquarium fish since these fish are kept for long time and also mycobacteriosis being a chronic disease.

Ingestion, trans-ovarian transmission and direct contact from water or infected fish have been suggested as the possible routes of transmission in case of mycobacteriosis. Also, high density of fish in an intensive culture system would be a responsible for an outbreak of

mycobacteriosis due to increased opportunity for transmission through the water column, faecal products or cannibalism (Hedrick et al. 1987).

Clinical signs include emaciation, stunted growth, exophthalmia and slowed swimming. Internal lesions in mycobacteriosis vary depending on the fish species involved but typically consists of greyish-white granulomas in the spleen, kidney and liver. Microscopically, these typical granulomas consist of central area of necrosis surrounded by macrophages, epithelioid cells and fibrous connective tissue. Granulomas are generally seen in spleen, liver and kidney during initial stages of the disease, but later may spread to all internal organs in more advanced cases. Externally, loss of scales and haemorrhages extending to the musculature is seen in advanced cases.

Presumptive diagnosis can be made by clinical signs, gross and microscopic pathology. However, these are many a times are inconsistent and hence definitive diagnosis is not possible base on pathology. Smears from spleen and kidney can be stained with Ziehl-Neelsen stains so as to identify the acid-fast short bacilli. Isolation on specific media can also be helpful. Methods presently under research genetic techniques, high-performance liquid chromatography (HPLC), and capillary gas chromatography for fatty-acid methyl-ester (FAME) analysis.

Since no approved drugs or anti-mycobacterial agents are available, depopulation and proper disinfection is the most commonly adapted policy in case of culture conditions. However, disinfection may not be always successful due to the resistance acquired by the pathogen to many disinfectants (Jacobs et al., 2009).

6. V**ibriosis in shrimp**:

Shrimp vibriosis also known as sea gull syndrome, is caused by numerous etiological agents like *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* causing severe economic losses in shrimp hatcheries and post larvae rearing ponds. Infections of the exoskeleton extend into digestive tract, including the hepatopancreas, and finally septicaemia occurs leading to severe mortality. These bacteria are Gram-negative, motile, rod-shaped bacteria that require supplementation of sodium chloride in the media for their growth. Among the *Vibrio* spp. which cause disease in shrimp, *V.harveyi*, is one of the primary etiologic agents of that causes mass mortalities in *Penaeus monodon* larval rearing ponds. Epizootics of vibriosis occur in all life stages of theshrimp, but are more common and lethal in shrimp hatcheries. Most of the *Vibrio* spp. form part of the natural microflora of wild and cultured shrimps and cause disease when natural defence mechanisms are suppressed due to various stress factors like inferior water quality, deteriorating environmental factors and overcrowding.

Most of the *Vibrio* spp. form part of the natural microflora of wild and cultured shrimps and cause disease when natural

defence mechanisms are suppressed due to various stress factors like inferior water quality, deteriorating environmental factors and overcrowding. Clinical signs of shrimp vibriosis include high mortality, shrimp congregating in surface of pond edge and presence of luminescence which can be appreciated in darkness. Gross lesions include melanosis of the shell which appear as black spots on the cuticle and delayed clotting. Post-larvae infected with *Vibrio* spp. have cloudy hepatopancreas. Gills are mostly brown in colour. Histopathologically, atrophy of the hepatopancreas with multifocal necrosis associated with haemocytic infiltration is evident. Presence of localized haemocytic nodules in the lymphoid organ, heart and connective tissues of the gills, hepatopancreas, antennal gland, telson and muscle are also observed. Vibriosis in *P. monodon* is also associated with the appearance of "spheroids" in the lymphoid organ. Large numbers of gram negative bacteria are present in the hemolymph. Adult shrimps infected with vibriosis are hypoxic, show reddening of the body surface, reduced feed intake and move slowly mostly at the edges and surface of pond. Some of the*Vibrio* spp. also causes red-leg disease which is characterised by red discolouration of the pleopods and gills associated with mortality up to 95% during summer months when the water temperature is high.

Vibrio infection can be readily diagnosed based on clinical signs and demonstration of rod-shaped *Vibrio* bacteria in lesions, nodules or haemolymph. Haemolymph may be inoculated on TCBS agar plate. Luminescent colonies may be observed after 6 to 12 hr when inoculated onto tryptone soya agar.

Luminiscent vibriosis is normally prevented in the hatcheries using appropriate chemicals so that bacterial load of the rearing units or the incoming water can be reduced. In shrimp hatcheries, washing eggs with iodine and formaldehyde and preventing contamination of eggs by excreta is advocated. In case of pond culture, increase in daily water exchanges and reduction in pond biomass by partial harvesting would help in reducing the mortality in case of an outbreak.Probiotics may also be administered through water or feeds.

B. Viral diseases of cultured marine fish and shell fish

Viruses can replicate only inside the living cells of other organisms and can infect types of life forms including animals, plants and microorganisms, including bacteria and archaea. Virus particles or virions normally consist of three components: i) genetic material made up of either DNA or RNA which carries the genetic information; ii) a coat protein that protects these genetic materials; iii) a lipid envelope that surrounds the protein coat which may be absent in some viruses. The shape of viruses may be helical or icosahedral or more complex structures.

The aquaculture industry has expanded globally with an increase in both

productions in terms of biomass and also in number of fish species being cultured. Intensification of aquaculture operations globally has provided new opportunities for the transmission of fish viruses and hence the occurrence of diseases caused by viruses forms a major limiting factor for the sustainable aquaculture production.

Fish viruses have been the subject of research interest in the past two decades. Compared to diseases caused by fresh water fish viruses, there have not been extensive studies on marine fish viruses. Establishment of various fish cell lines lead to path breaking research in fish virology in the recent years. Major viral groups under which fish viruses can be classified include herpes virus, iridovirus, rhabdo virus, reo virus, noda virus and calci virus. However, the most lethal viral disease causing enormous loss to finfish farming is the disease caused by betanodavirus. As for as shrimp farming is concerned, the major viral diseases *of P. monodon* include white spot syndrome and yellow head disease that can also cause serious mortalities in *P. vannamei* farming.

1. Viral nervous necrosis

Betanodavirus is one of the genera making up the family Nodaviridae which is the etiological agent of viral nervous necrosis (VNN) also known as encephalomyelitis and vacuolating encephalopathy and retinopathy. This virus has remained as a major threat for the establishment and expansion of Asian seabass (*Lates calcarifer*) and striped jack (*Pseudocaranx dentex*). The disease was first documented in 1990 in hatchery-reared Japanese parrotfish (*Oplegnathus fasciatus*) in Japan and Asian sea bass in Australia. Later, it was reported in turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), redspotted grouper (*Epinephelus akaara*), striped jack (*Pseudocaranx dentex*) and more recently in cultured warm-water and cold-water marine fish species throughout the world (Munday et al., 2002). In India, mortality caused by betanodavirus infection in hatchery produced larvae of Asian seabass was first reported by Azad et al. (2005). An Indian strain of betanodavirus belonging to RGNNV group was isolated from Asian seabass juveniles reared in a brackish water farm in Bhimavaram in Andhra Pradesh in 2012. Outbreak of mortality due to nodavirus infection in Asian seabass juveniles cultured in fresh water cages in the south west coast of India has also been reported. Mortality of Asian seabass juveniles cultured in indoor cement tanks as well as open sea cages and in cobia cultured in cages in India associated with RGNNV was also recorded (unpublished report).

Betanodaviruses can infect fish species belonging to tropical, sub-tropical, or temperate waters. These viruses can multiply at an optimum temperature depending on the strain of the virus. For RGNNV, the optimum temperature requirement is 25– 30°C while for SJNNV, it is 20–25°C.

Mostly, betanodaviruses are a concern in marine fish species. The species susceptible cobia, sea bass, seabream,

bluefin tuna, grouper, halibut, surgeonfish, lined surgeonfish and tiger puffer. The freshwater fish species susceptible to betanodaviruses include tilapia and the guppy.

In farming system stress factors like high density, transportation, high temperature can act as predisposing factors making the fish susceptible to VNN. Although young fishes are more susceptible, older fishes may also get infected especially when water temperature is high.

During the acute stage of the disease, when the mortality is very high, especially in juveniles, there would be no gross lesions on the body surface or gills. However, affected juveniles and older fish show a abnormal swimming behaviors such as spiral, whirling, floating with inflation of swim bladder, or laying down at rest, circling on their own axis. This erratic swimming behaviour may not be noticed in infected fish larvae. Grossly, the brain is oedematous and in many cases severely congested.

Microscopically, lesions are characterized by severe vacuolation and necrosis of the central nervous system. In general, the anterior brain is more severely affected when compared to the posterior part of the brain and spinal cord. Larvae of the fish are more severely affected by betanodaviruses than juveniles. The most characteristic lesion in the fish larvae is the presence of vacuoles in the grey matter of the brain which are intracytoplasmic. Basophilic, intra cytoplasmic inclusions have been reported in brain cells of Asian seabass.

Lesions in the retina of the infected fish have also been described in all Species. The lesions include vacuolation of the cellular components of the retina especially the bipolar and ganglionic nuclear layers.

Under transmission electron microscopy, fish betanodaviruses appeare icosahedral, non-enveloped with a mean diameter of about 25 nm. The virions may be membrane bound by endoplasmic reticulum or are free in the cytoplasm and may present as paracrystalline arrays. Cells containing virions normally include neurones, astrocytes, oligodendrocytes and microglia cells.

Diagnostic methods for fish nodaviruses have been extensively studied. According theMunday *et al* (2002), VNN can be diagnosed by:

1. Demonstration of characteristic vacuolar lesions in the brain or retina by light microscopy.

2. Detection of virions and viral antigens by electron microscopy and serology

3. Detection of viral nucleotides by molecular techniques including RT PCR, RT Nested PCR, cloning and sequencing by designing the primers for the strain of interest.

4. Tissue culture of virus in a suitable cell line.

Treatment and prevention: Betanodaviruses are highly resistant to variousenvironmental conditions and they can survive for a long time in sea water. The disease can also be reproduced by simple co-habitation of the healthy fish with infected fish. Control measures are including imposing strict bio- security to exclude the virus from the farm premises. The broodstocks should be tested for the presence of viruses in the gonadal tissues and VNN specific antibodies in serum and any positive fish should be culled at first.

2. White spot syndrome:

More than 1100 viruses of invertebrates have been reported so far. Most important groups of viruses reported in Crustacea include Reoviridae, Picornaviridae, Parvoviridae, Togaviridae, Baculoviridae, Paramyxoviridae, Rhabdoviridae and lridoviridae.

White spot syndrome (WSS) is a viral infection of penaeid shrimp caused by a double stranded DNA virus belonging genus Whispovirus within the Nimaviridae family which is a most devastating virus of cultured shrimp characterized by severe mortality and appearance of white spots on the carapace of the infected fish. All decapod crustaceans including prawns, lobsters and crabs from marine, brackish or freshwater environments are susceptible to infection. WSSV is the largest DNA virus of which whole genome sequencing has been done.

WSSV virions are ovoid or ellipsoid to bacilliform in shape and measure 80–120 nm in diameter and 250– 380 nm in length. Most noteworthy feature is the presence of flagella like extension at one end of the virion. Under laboratory conditions, WSS virus is viable for at least 30 days at 30°C in seawater and the virus is viable in ponds for at least 3–4 days.

The virus is normally transmitted by horizontal transmission through water and feed infected. Vertical transmission is also possible brooders to offspring.

Shrimp with acute WSS show a rapid reduction in food consumption, lethargic movements and have a loose cuticle with the presence of white spots which measure 0.5 to 2.0 mm in diameter. The targets organs for the virus are the cells of ectodermal and mesodermal origin, including those of the epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eye-stalk, heart, gonads, haematopoietic cells and cells associated with the nervous system. Hence the death is normally due to multi-organ dysfunction. These white spots are apparent on the inside surface of the carapace. The white spots signify abnormal deposits of calcium salts by the cuticular epidermis. Shrimp showing these signs show high mortality, sometimes up to 100%, in 3 to 10 days of the onset of clinical signs. In a typical shrimp farm, WSSV infected shrimp gather near the pond edge and display clinical signs 1 or 2 days before the first mortalities occur.

White spot disease is histopathologically characterized by the presence of widespread and severe nuclear hypertrophy, chromatin margination and eosinophilic to basophilic large intranuclear inclusions with focal necrosis in most tissues of ectodermal and mesodermal origin including gills, haemocytes and haemotopoietic tissue, lymphoid organ, connective tissues, subcuticular epidermis, stomach, foregut and hindgut epithelium, heart, striated muscle, midgut and ovary walls, antennal gland and the nervous tissues. These inclusion bodies are strikingly distinct and bigger than the Cowdry A-type inclusions which are seen in infectious hypodermal and haematopoietic necrosis virus infection.

The disease can be diagnosed and confirmed by using DNA probes, PCR using WSSV primers, nested PCR, monoclonal antibody based kits which can be used at field level.

Even though research results are available on immunity against WSSV in shrimp injected with inactivated WSSV virions or recombinant structural protein, or by using

RNA interference (RNAi), or by administering orally bacterially expressed VP28 dsRNA, there are still no field data for either the vaccination or the RNAi approach.

3. **Yellow Head Disease**:Yellow head diseases is a viral disease of shrimp causedby most virulent shrimp virus, yellow head virus genotype-1 characterized by yellowish discolouration of the cephalothorax and mass mortality. Yellow head virus (YHV) can remain viable in aerated seawater for up to 72 hours. YHV is an enveloped, rod-shaped, ssRNA virus with a helical nucleocapsid and prominent glycoprotein projections on the virion surface.

Yellow head disease (YHD) outbreaks have been reported in the black tiger prawn (*P.monodon*) and the white Pacific shrimp (*P. vannamei*) and pacific blue prawn (*P. stylirostris*) and few other shrimp species. *P. monodon* which are beyond PL15 aresusceptible to YHV. Like WSSV, YHV also targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia. YHV infection can be transmitted horizontally by injection, ingestion of infected tissue, or by co-habitation of healthy shrimp with infected shrimp. Homologous genetic recombination is also an attribute of the yellow head virus complex. The prevalence and geographic distribution of these recombinant viruses indicate that the reason of swiftly increasing genetic diversity of the virus is primarily due to international trade in live shrimp.

YHV genotype-1 can cause upto 100% mortality in *P. monodon* within 3–5 days of the first appearance of clinical signs. Stress induced by sudden changes in pH or dissolved oxygen levels can be the predisposing factor for an outbreak of the

disease in shrimp farms. Moribund shrimp may congregate at pond edges near the surface In shrimp farms, infection can result in mass mortality especially in early to late juvenile stages. Clinical symptoms of the disease may include cessation of feeding, aggregation of infected shrimp at pond edges and bleached appearance. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax. This is caused by the underlying yellow hepatopancreas, which may be remarkably soft when compared with the brown hepatopancreas of a healthy shrimp. Unusually high feeding activity by the shrimp followed by a sudden cessation of feeding may occur within 2 to 4 days of the appearance of gross clinical signs of disease and mortality.

Microscopically moderate to large numbers of deeply basophilic, evenly stained, spherical, intra-cytoplasmic inclusions of approximately 2 µm in diameter can be seen in tissues of the lymphoid organ, stomach, and gills. YHD can be diagnosed by gross and microscopic lesions and RT-PCR using YHV specific primers.

SUGGESTED READINGS

Azad I.S., Shekhar M.S., Thirunavukkarasu A.R., Poornima M., Kailasam M., Rajan J.J.S., Ali S.A., Abraham M. & Ravichandran P. (2005) Nodavirus infection causes mortalities in hatchery produced larvae of *Lates calcarifer*, first report from India. *Dis. Aqua. Org*. 63: 113–118.

Munday B.L., Kwang J. and Moody, N. (2002) Betanodavirus infections of teleost fish:a review. *J. Fish Dis*., 25:127-142.

Cepeda, C., Garcia-Marquez, S. and Santos, Y. (2003). Detection of *Flexibactermaritimus* in fish tissue using nested PCR amplification. *J. Fish Dis*., 26: 65-70.

Gauthier, G., Lafay, B., Ruimy, R., Breittmeyer, V., Nicolas, J.L., Gauthier, M. and Christen, R. (1995) Small-subunit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Sniezko et al.) Janssen and Surgalla to the genus Photobacterium as *Photobacterium damselae subsp. piscicida*. *IntJ Syst Bacteriol*., 45: 139–144.

Hedrick R.P., McDowell T. & Groff J. (1987) Mycobacteriosis in cultured striped bass from California. *J. Wildlife Dis*. 23: 391–395

Kent, M.L. (2000) Marine netpen farming leads to infections with some unusual parasites. *Int. J. Parasitol*., 30:321–326.

Mutharia, L.W., Raymond, B.T., Dekievit, T.R., Stevenson, R.M.W. (1992) Antibody specificities of polyclonal rabbit and rainbow trout antisera against *Vibrio ordalii* and serotype O2 strains of Vibrio anguillarum. *Can. J. Microbiol*. 39: 492–499.

Olivier, G. (2002) Disease interactions between wild and cultured fish—perspectives from the American Northeast (Atlantic provinces). *Bull. Eur. Assoc. Fish Pathol*., 22: 103–109.

Osorio, C.R., Toranzo, A.E., Romalde, J.L., Barja, J.L. (2000) Multiplex PCR assay for ureC and 16s rRNA genes clearly discriminates between both subspecies of *Photobacterium damselae*. *Dis. Aquat. Organ*., 40, 177-183.

Schieve, M.H., Crosa, J.H. (1981) Molecular characterization of *Vibrio anguillarum* biotype 2. *Can. J. Microbiol*., 27: 1011– 1018.

Sharma, S.R.K., Gaurav Rathore, Dev K Verma, Sadhu, N and Philipose, K.K. (2013*)Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in opensea floating cages in India. *Aquacult. Res*., 44: 86–92.

Sharma, S.R.K., Pradeep M.A., Loka, J., Sadhu, N., Dube, P.N. and Philipose, K.K. (2014)Association of *Vibrio harveyi* in mortality of mangrove red snapper (*Lutjanusargentimaculatus*, Forsskål, 1775) cultured in open sea cages. *Indian J. Fish.,* 44: 8692.

Snieszko, S.F. (1974) The effects of environmental stress on the outbreaks of infectious diseases of fish. *J. Fish Biol*., 6: 197–208

eDNA based estimation of marine biodiversity

P. Jayasankar
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The continuous decline in Earth's biodiversity represents a major crisis and challenge in the 21st century, and there is international political agreement to slow down or halt this decline. The challenge is in large part impeded by the lack of knowledge on the state and distribution of biodiversity – especially since the majority of species on Earth are un-described by science. The currently existing biodiversity is a result of several million years of evolution of life on earth. Biodiversity is a key component of ecosystem and plays a major role in proper functioning of the ecosystem. Several factors like climate change, habitat loss and invasive species are disturbing the ecosystem biotic components thereby adversely affecting the function and services of ecosystem. The effective management measures for restoring the degraded ecosystem can be taken if the information (data) about indicator species, its abundance and pattern of biological diversity (Species) in that ecosystem is available. Marine biodiversity components are largely benthic habitats, plankton, fish, cephalopods, seals, seabirds, cetaceans and ecosystem processes and their functions. All conservation efforts to save biodiversity essentially depend on the monitoring of species and populations to obtain reliable distribution patterns and population size estimates.

Biodiversity monitoring

Monitoring of biodiversity has traditionally relied on physical identification of species by visual surveys and counting of individuals. However, traditional monitoring techniques remain problematic due to difficulties associated with correct identification of cryptic species or juvenile life stages, a continuous decline in taxonomic expertise, non-standardized sampling, and the invasive nature of some survey techniques. Hence, there is urgent need for alternative and efficient techniques for large-scale biodiversity monitoring. With the advent of molecular biology, DNA based species identification methods have been devised using molecular markers (mitochondrial and nuclear). Since the last decade, taxonomically informative genes have been tested over large groups of organisms (animals: mitochondrial cytochrome c oxidase subunit I; Fungi: Nuclear ribosomal internal transcribed spacer; Plants: two chloroplast genes, rbcL & matK; Bacteria: 16S rRNA & protein coding Chaperonin-60, cpn60 for their efficiency to delimit the species and designated them as barcode genes for respective groups). The success in this approach resulted in creation of huge reference databases that include species taxonomic details along with DNA barcode gene sequences (Fish-BOL, BOLD, MarBOL, QBOL etc.). These reference barcode

sequence databases are useful in assigning taxon to unknown specimen by comparing the sequence similarity of specimen barcode gene with reference database. Until recently, most of the barcoding studies were aimed at developing reference databases by generating species specific DNA barcodes from individual specimens. However, it is also important to characterize/assess the species diversity and abundance within an ecosystem as a whole to understand the spatial and temporal changes in species diversity. Normal DNA barcoding approach using Sanger sequencing method can identify only one specimen at a time and cannot identify multiple species if the sample contains a mixture of different species. With the advancements in sequencing technology, it is now possible to assess the species composition of ecosystems including environmental samples such as soil, sediment and water at a stretch than screening individual specimens at a time.

Biodiversity studies

In this decade of biodiversity (2011-2020), fund allocation has been increased for biodiversity characterization and these efforts resulted in creation / strengthening of existing taxonomic reference databases such as Global Biodiversity Information Facility (GBIF) and Barcode of Life database (BOLD). These databases have cured reference taxonomic information for about 144,357 Species (BOLD www. boldsystems.org.in) species and are being constantly updating with new taxon information to include all the species on earth. Once the comprehensive database is prepared, DNA metabarcoding approach can be used to analyse the species composition of different types of samples, from soils to sediments, faeces, air and water. Metabarcoding of soil / sediment samples collected from nuclear power plant areas or any other industrial area can be used to compare temporal and spatial species assemblages to assess human impacts on biodiversity. Likewise, water samples can be used to detect the presence of invasive species. Next generation sequencing technology has been used to analyse species composition of sensitive ecosystem like Coral reefs, and extreme habitats like acid mines. DNA metabarcoding approach has been successfully used to characterize soil microbial diversity, fungal diversity and plant diversity using 16S rRNA, ITS and P6 loop of the plastid DNA trnL intron amplicons, respectively.

Environmental DNA

Environmental DNA (eDNA) is defined as the genetic material obtained from a water sample containing no distinguishing signs of source macro-organisms. The method utilizes DNA which is continuously excreted by organisms into the surrounding environment through mucus, gametes, faeces, blood and other cells, and captures, analyses and obtains the nucleotide sequence of this DNA based on an environmental sample. eDNA analysis has emerged as a potentially powerful tool to access aquatic community structures. The inherent drawbacks in traditional approaches to monitor aquatic biodiversity can be overcome by

employing eDNA techniques. Analysis of this eDNA can give us information on the organisms, their abundance and biomass through two approaches – eDNA barcoding and eDNA metabarcoding. In the former, specific species are targeted in samples using standard or quantitative PCR, and using traditional Sanger sequencing method. In the latter, the whole community is screened using multiple conserved primers and Next Gen Sequencing (NGS) is done. Studies suggest that eDNA metabarcoding outperforms traditional survey methods in terms of non-invasive sampling, sensitivity and cost incurred. There is now increased interest in using eDNA to supplement existing survey methods.

Status of research

Since 2012 there has been a plethora of studies on eDNA metabarcoding as applied in biodiversity conservation, fish community identification, fisheries management, invasive species, as well as in fish biomass/abundance estimations. A total 25 research papers related to eDNA metabarcoding/metagenomics by Indian authors are predominantly pertaining to the study of microbial biodiversity from food, soil and deep sea sediments. Not a single publication related to such study in fish has been cited from the Indian context.

Metabarcoding

Metabarcoding is constrained by factors like PCR efficiency, primer tags and sequencing efficacy. Another limitation is lack of comprehensively cured reference databases for certain metazoans for assigning taxon to the OTUS (Operational Taxonomic Units). Future studies are needed to improve sampling strategies (selection of season, sampling location within habitat, etc.) and to understand the relationship between sequence reads and species density. Gaps in knowledge about the dynamic mechanisms relating to shedding of tissue into the environment and metabolism related processes which could also affect quantity of DNA released by an organism into the water have to be filled. Dynamics of eDNA under field conditions, such as patterns of release, degradation, and diffusion will have to be taken into consideration to get estimates of fish distribution and biomass/abundance based on eDNA.

Methodology includes seawater filtration, quantitative real-time PCR, library preparation, Next Gen Sequencing (NGS) and statistical analysis. High throughput sequencing data analysis using the state-of-the art tools could throw light on family level abundance in general and species level abundance of fish in particular. However, the strength of the relationship depends on environmental parameters, such as water temperature, and technical parameters, such as the filter being used for capturing eDNA. Species biology, environment and filtration methods and other factors (e.g. extraction and fish ecology and spatial distribution) likely to interact and significantly influence eDNA concentration variation. Caution is needed when interpreting the patterns of eDNA concentration in practical contexts.

Parameters such as detection limits in water samples, influence of microbial activities on eDNA degradation, sampling design, seasonal conditions, nature of eDNA and fish ecology should be considered in future studies before predicting fish abundance from eDNA in natural conditions.

Potential advantages of eDNA over conventional approaches

Continuous ship-borne monitoring surveys are time-consuming and expensive. Generally they are invasive, selective and rely on some degree of subjectivity related to the taxonomic expertise of the monitoring personnel; further, problematic due to a general decline in taxonomic expertise and related difficulties associated with correct species identification especially across egg and juvenile life stages. On the other hand, collection and analyses of water samples for eDNA more cost-effective, sensitive and non-invasive for presence/absence surveys of species, in contrast to established monitoring techniques relying on catching whole organisms. As all organisms continuously shed DNA through their metabolic waste products (and gametes), the method has the potential to objectively identify either individual species using qPCR; or entire biological communities across taxonomic groups using NGS platforms.

Factors which control eDNA presence in a given environmental sample

Environmental effects on the production, persistence and transport of eDNA, especially in marine ecosystems, are keys to establish robust and reliable temporal and spatial relationships between recorded DNA and qualitative/quantitative monitoring data. With reference to body surface area and metabolism small adult/juvenile fish are likely to produce more eDNA than large adult fish. Temporal persistence of eDNA particles in water depends on whether it's in free state or encapsulated and the external biotic and abiotic factors. Persistence time of eDNA can be highly variable, such as from 1 day to 58 days; being shorter in marine and brackish environments when compared to freshwater, presumably due to difference in environmental factors or osmoregulation between freshand marine species. In sea eDNA particles are estimated to travel more than 600 km in a week, and are less affected in the near coastal areas than in high seas.

Main challenges

Five principal challenges which affect eDNA concentration and its applications include:
(i) to find what we are looking for, (ii) spatial origin, (iii) relationship between eDNA and biomass/numbers, (iv) application in fisheries management and (v) other sources of eDNA. Concern on 'false negative' and 'false positive' is common. 'False positive' can occur from empty fishing nets, bottom sediments, discards and fish carcases. Low fish density in marine environment compared to freshwater poses challenge in

presence/absence detections in the former; this entails relatively larger volume of seawater to be sampled for eDNA analyses. In marine environment currents dissipate eDNA from the source; hence chances of detection diminish depending on the distance from the fish sampling was done. Faster degradation of DNA and dilution further blockade effective utility.

Improving eDNA analyses

Fish metabolism and eDNA production could be correlated, though such studies are lacking. Influence of physical, chemical and biological environment on eDNA of source organism need to be studied to improve quantitative aspects of eDNA-based monitoring. Oceanographic modelling of eDNA transport and detection is a promising area of research to improve our understanding of the complex interactions and dynamics of eDNA in marine systems. Best estimates of eDNA dynamics are currently from freshwater environment. Research focus should be more on understanding the basic processes of eDNA in marine environments, rather than the present focus on direct application.

Novel applications

eDNA analysis can be applied in ecosystem monitoring, assessment of life history and migration patterns, stock structure analysis, and diet and processed fish product analysis.

Way forward

There has been amazing advancement in technology from quantitative real time PCR to smart phone-powered sequencer, which would minimize many of the classical logistical and practical challenges of handling, storing and transport of environmental samples. Finally technological advancement has reached in automated real time DNA measurements as in Environmental Sample Processor (ESP), which is set to monitor a specific geographic location ranging from coastal to deep sea, and does everything right from regular water sampling and storing to real-time molecular analysis. ESP may be costly, but cost-competitive compared to extensive ship time for visual monitoring or to continuous collection of water samples. eDNA is under the influence of many physical, chemical and biological parameters, which need to be analysed. Its role in direct quantitative assessment is still challenging. Current focus of research in this field should be around relative strengths on detection of presence/absence, migration patterns and life history events, broad ecological understanding, taxonomic coverage and providing basis for ecosystem-based management. Despite the caveats, eDNA-based monitoring will continue to develop to have profound impact on futuristic biodiversity research, monitoring and management.

Bacterial identification: A glance on molecular approaches

Anusree V. Nair, T.G. Sumithra, P.V. Amala
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Identification of bacteria which are either beneficial or pathogenic is one of the key tasks in microbiology. Innovative advancements in molecular systematics motivated the integrated use of genotypic and phenotypic information for microbial classification. In order to completely characterize the bacteria, a combinatorial approach of diverse phenotypic and genotypic methods is required. The limitations of conventional phenotypic techniques in terms of sensitivity, specificity and time, are more apparent for slow growing bacteria and non-cultivable bacteria. Furthermore, phenotypic methods are not always advisable to identify the bacteria up to species level or strain level. These disadvantages associated with conventional techniques could be overcome by employing alternate DNA based detection methods which are generally faster, specific and more reliable. From the past a few decades, molecular techniques have been developed as a major means for the identification of bacteria. They are less laborious, rapid, sensitive, specific and resourceful compared to conventional methods. However, various molecular methods vary in their discriminatory power, reproducibility, ease of use, and ease of interpretation.Recently molecular approaches have been taken up by industries and analytical laboratories for rapid identification of bacteria up to species or strain level.

Microbial Identification of bacteria can be done by:

16S rRNA gene sequencing and analysis.

DNA-DNA hybridization studies with related organisms.

Detection based methods: Identification is done by specific PCR assays targeting marker genes. These detection methods can be done through different variants of PCR including single PCR, multiplex PCR, nested PCR and real time PCR

Typing methods: Include Pulse Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST), Random Amplified Polymorphic Deoxyribonucleic acid (RAPD) analysis, Amplified Fragment Length Polymorphism (RFLP), Repetitive sequence-based PCR, Plasmid profile analysis, Multilocus variable number of tandem repeats analysis, Ribotyping, DNA Microarray, DNA Sequencing, Whole Genome Sequencing *etc*.

DETECTION BASED METHODS

Polymerase Chain Reaction (PCR) is an *in-vitro* method for the amplification of desired fragment of DNA using the specific

oligonucleotide primers. PCR are commonly used to identify bacteria, especially for morphologically similar bacteria and those bacteria which cannot be cultured in lab. This tool is highly specific, sensitive and rapid. The identification can be achieved through any of the following variants of PCR.

Single polymerase chain reaction (**Single PCR):** Single set of primers is usedto target a specific gene (commonly species specific gene) and amplify species specific region.

Multiplex polymerase chain reaction (Multiplex PCR): In multiplex PCR, more than one DNA sequences are simultaneously amplified using multiple sets of primers. The primers pairs have to be optimized in such a way that all primer pairs amplify the corresponding target gene specifically, at same annealing temperature during PCR. It produces amplicon of varying sizes which are specific to each DNA targets.Multiplex PCR can be used to identify and characterize multiple bacterial species simultaneously. As the desired amplicon of each primer set differ, the presence of specific bacteria can be confirmed by looking for specific product length. This method allows the rapid detection or identification of multiple strains/ species through single reaction, which will be more economical and less time consuming.

Nested polymerase chain reaction (Nested PCR): Nested PCR helps toenhance the specificity of amplification. Two primer sets are used in two consecutive reactions. In the first step, one pair of primers is used to amplify DNA products, which may contain products amplified from non-target areas. The products from the first PCR is then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located within (nested) the first set, thus increasing specificity. Nested PCR necessitates more thorough information of the sequence of the target. This method is more successful in specifically amplifying long DNA products.

Quantitative PCR/ Real time PCR: This method can not only help to identifythe bacteria but also, helps to quantify the target DNA (or RNA) in a sample.

TYPING BASED METHODS

Genotyping is the method of determining the changes in genetic make-up of a bacteriaby examining DNA sequence. Genotyping can be done through various methods as follows.

Pulse field gel electrophoresis (PFGE): Pulsed field gel electrophoresis is anagarose gel electrophoresis technique, used for separating DNA restriction fragments by applying electric field which periodically changes its direction in a gel matrix. After digestion of genomic DNA using specific restriction enzymes the product is allowed to run in an electric field so that a highly discriminatory genetic fingerprint will be generated.It is considered as a ‘gold standard’ typing method for pathogen and epidemiological studies. In PFGE the voltage is periodically

switched in three directions than conventional gel electrophoresis. It has high reproducibility and discriminatory power which depends on the choice of restriction enzymes. PFGE is time consuming as it requires minimum of three days to get the result and not cost effective too. PFGE is used to detect large scale genomic polymorphisms and used as a diagnostic strain typing method for pathogenic microbes.

Multilocus Sequence Typing (MLST): MLST is a nucleotide-based techniquefor typing bacteria using the DNA sequence variations of internal fragments of several house-keeping genes. MLST helps to detect small variations within a species and characterize strains by their unique allelic profiles. It can be highly discriminatory if the genes are correctly chosen even though it is costly and time consuming. In MLST, bacteria species with different sequences are considered as distinct alleles for each house-keeping gene and the alleles at each end of the seven loci define the allelic profile or sequence type for each isolate. Bacteria has variation in the 450-500 bp internal fragments of each house-keeping genes to provide many alleles per locus thus allowing millions of distinct allelic profiles to be differentiated utilizing the seven house-keeping loci. MLST data can be used to investigate evolutionary relationships among bacteria and also helps in epidemiological studies. The relatedness of isolates can be visualized with a dendrogram generated using pairwise differences between allelic profiles. PubMLST, a website sustained by Department of Zoology, Oxford University maintains a growing collection of over 50 MLST databases for molecular typing of various microbes.

Random amplified polymorphic DNA (RAPD): This is a PCR-based techniquein which arbitrary primers (typically short primers of 8-12 nucleotides) are used to randomly amplify segments of target DNA under low-stringency PCR condition. This process leads to the amplification of one or more DNA sequences using single primer and give a semi-unique profile which helps to generate a set of finger printing patterns of different sizes specific to each strain of bacteria. This method needs large, intact DNA template sequence. But the detailed information of the DNA sequence of the targeted genome is not a requisite, as the primers will bind randomly in template genomic DNA. The technique has also been applied in gene mapping studies to fill gaps not covered by other markers Many scientific journals do not accept experiments merely based on RAPDs anymore as its reproducibility is less.

Amplified fragment length polymorphism (AFLP): Involves digestion oftotal genome DNA with two or more restriction enzymes and ligation of adaptors to the sticky ends of the restriction fragments and amplification of selected restriction fragments using radioactive and fluorescent labelled primers. This technique has a good resolution, reproducibility, highly sensitive and 100% typability but time consuming. AFLP technology has the capability to detect

various polymorphisms in different genomic regions concurrently. AFLP is extensively used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria. Moreover, this tool is also used in criminal and paternity tests, also to determine slight differences within populations, and in linkage studies to generate maps for quantitative trait locus (QTL) analysis.

Repetitive sequence-based PCR (rep-PCR): Bacterial and fungal genomescontain numerous non-coding, repetitive DNA sequences and their arrangement varies between strains. The rep-PCR technique helps to amplify the repetitive sequences to produce amplicons of varying length that can be separated by electrophoresis giving a fingerprint comprised of bands which fluoresce at varying intensities after binding with an intercalating dye. The method is widely used for typing human pathogens.

Plasmid Profile Analysis: Plasmids are circular deoxyribonucleic acidmolecules that exist independent of the chromosome in bacteria, usually encodes genes for antibiotic resistance or virulence factors. Plasmids can also serve as markers of various bacterial strains in the typing methods. In these methods partially purified plasmid DNA are separated according to molecular size by agarose gel electrophoresis or fragmented plasmid DNA using restriction endonucleases can be separated by agarose gel electrophoresis and the resulting pattern of fragments are used to verify the identity of bacterial isolates. Plasmid profile typing has been used to investigate outbreaks of many bacterial diseases and to trace *inter*- and *intra*-species spread of antibiotic resistance as most of bacteria contain plasmids. Plasmid profile analysis is easy to perform and interpret the results. The disadvantage of this method is that, since plasmids are mobile elements that can spontaneously be lost or readily get transferred between bacteria, there is always a chance that epidemiologically related isolates can easily show dissimilar plasmid profiles. Additionally, the diverse spatial conformations of plasmids (linear, nicked and supercoiled) can affect the repeatability of this technique.

Multi-locus variable number of tandem repeats analysis (MLVA): MLVA isinterrelated to MLST based on PCR amplification and sequencing of rapidly mutating repetitive DNA sequences called tandem repeats. The result is an MLVA pattern to characterize the bacterial strain. MLVA is faster and easier to perform than MLST, but less reproducibility and validation.

Ribotyping / Restriction-Fragment Length Polymorphism (RFLP): Ribotyping is used for bacterial identification and characterization This technique relies on the relative stability of the *16S* and *23S rRNA* genes coding for ribosomal RNA. Ribotyping involves use of restriction enzymes to digest genomic DNA into small fragments. Restriction enzymes were used to cut the genes and the subsequent DNA fragments were separated by electrophoresis. The resulting fingerprint

is visualized using fluorescent probes. DNA fragments are moved onto nylon membranes and hybridized with a labelled 16S or 23S rRNAprobe. Ribotyping has higher discriminatory power at the species and subspecies level compared to strain level. As *16S rRNA* gene is the most conserved rRNA genes and *16S rRNA* gene sequencing has been established as the "gold standard" for identification and taxonomic classification of bacterial species. Knowledge of intraspecies conservation of the *16S rRNA* gene sequence and basic 16S-23S-5S ribosomal operon structure helps to give the first insight into its usefulness in developing ribotyping for bacterial classification.

DNA Microarrays: A microarray is a collection of DNA probes attached to solidsurface in a pattern, which can be used to detect the presence of complementary sequences in bacterial isolates. It helps to detect marker genes in specific bacterial strains. DNA microarray also called DNA chip as it allows the rapid and simultaneous screening of many thousands of genes. The main principle behind microarrays is the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. DNA microarray technology helps in disease diagnosis, drug discovery and toxicological research also.

DNA Sequencing: DNA sequencing is widely and routinely used in theidentification, typing, characterization and taxonomic classification of unknown or novel microbial isolates. *16S rRNA* is a universal marker gene for prokaryotes and it is amplified using universal primers followed by the sequencing of the amplicon for the definite identification of bacterial species or genera.

Whole Genome Sequencing: Genotyping methods mentioned has their ownlimitations and ideally one would sequence the entire genome of a microbial isolate to provide definitive typing. Quite recently, the dawn of next-generation sequencing (NGS) technology, make whole genome sequencing (WGS) possible through high-throughput bench-top NGS instruments like Life Technologies'

Ion PGM™ and the Illumina NextSeq 500. As the costs of NGS are declining, the entire bacterial genome can now be sequenced for about the same price as MLST typing using conventional PCR/Sanger sequencing. A set back with whole genome sequencing is the difficulty in understanding the huge amount of data generated and identifying and extracting the genetic information which is vital for typing. Bioinformatics disciplines assures to provide the solution to this problem by developing computer software to analyze sequencing data and building interactive databases where data can be stored and accessed. Whole genome sequencing has already been used to investigate the genetics of human pathogens involved in outbreaks and promises to revolutionize the process in the near future. The potential meritsof WGS may make it as the gold standard for pathogen typing

(replacing PFGE) in the near future itself, as the costs of sequencing are declining.

Conclusions

The molecular approaches discussed here, helps to characterize and identify the microorganisms in a rapid and specific manner. Moreover, it aids to gain knowledge in microbial diversity, taxonomy research and in its various application too. Polyphasic approach using various techniques will considerably increase the discriminatory power of the detection or typing technique employed which makes the identification easier.

SUGGESTED READINGS

Adzitey F., Huda N., Ali GRR. 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. Biotech, 3(2): 97-107.

Babalola OO. 2003. Molecular techniques: An overview of methods for the detection of bacteria. African Journal of Biotechnology 2 (12): 710-713.

Metagenomics and its application to fisheries science- an overview

T.G. Sumithra, K.J. Reshma
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Microbes, the most metabolically versatile organisms on earth are indispensable for every part of all life and every process in the biosphere. Microbiology, the study of microbial world, stems on the isolation of microbes and almost all the current knowledge in this science is based on microbes raised in pure form. However, the discrepancy between the numbers of bacterial populations estimated by culture based methods and by microscopy (Staley and Konopka, 1985; Grimes *et al*., 1986; Torsvik *etal*., 1990; Torsvik and Ovreas, 2002) indicated that cultured microorganismsrepresented only a minor portion of the actual microbial world. In soil, it was demonstrated that only 0.1 to 1% of the total microbes are only culturable on common media under standard conditions (Torsvik *et al*., 1990; Torsvik and Ovreas, 2002). This realization of the non-cultivable nature of the majority of microbes led to the awareness that microbiologists have reached only up-to the tip of iceberg. Traditional microbiological approaches have already shown microbes are essential for every part of all life and can be very useful source of numerous technologies such as commercial production of many clinical drugs, remediation of wastes and pollutants in environments, enhancement of crop productivity, production of biofuels, fermentation of foods, and to provide unique signatures for disease diagnosis and forensic analysis *etc*. Therefore, unraveling the non-culturable microbes will definitely extend andencourage scientists' ability to discover and benefit from microbial capabilities.

Metagenomics - a cultureindependent approach

Metagenomics is the branch of science dealing with the direct investigation of microbial genomes contained with an environmental sample (Thomas *et al*., 2012) and the sum of the microbial genetic material with in the sample is known as metagenome or microbiome. Metagenomics is also known as community genomics, environmental genomics or population genomics. The word metagenomics was coined (Handelsman *et al*., 1998) to designate the analysis of a collection of similar but not identical items,as in a meta-analysis, which is the analysis of analyses (Glass, 1976). In all metagenomics studies the first step is the direct extraction of DNA from all the microbes inhabiting a particular environment. The extracted DNA samples are then examined directly, or after cloning and transformation into

easily maintainable laboratory bacteria so that a library comprising the genomes of all microbes found in that environment will be generated. The library is then studied either by sequencing the cloned DNA or by defining the function of the cloned genes when they are expressed as proteins. However, the metagenomics library consists of millions of clones, each comprising a random fragment of DNA. It is like thousands of jigsaw puzzles jumbled into one box, so that putting the puzzles into an orderly form is one of the great challenges for metagenomics based studies. However, the availability of economical, high-throughput DNA sequencing methods and the innovative computing competencies needed to make sense of the millions of random sequences contained in the libraries greatly helped in the progress of metagenome based studies.

Approaches to metagenomics analysis

As described earlier, after the generation of metagenomics library, the transformants are screened depending on the target of the study. There are two screening methodologies namely, sequence based metagenomics and functional metagenomics. Even though each approach has its own qualities and drawbacks; together these have improved our awareness of uncultured microbes.

Sequence based metagenomics

In sequence based analysis, there are two methods; in the first, clones are screened for phylogenetic anchors, such as *16SrRNA*, *recA,* internal transcribed spacer (ITS) or for other conserved genes (by hybridization or PCR amplicon sequencing). Afterwards, complete sequencing of the clones containing phylogenetic markers is done. Alternative to this first method is random sequencing and once a gene of interest is identified, phylogenetic anchors is sought in the flanking DNA to identify the phylogeny. Sequence based metagenomics have made new linkages between phylogeny and function and reconstructed the genomes of organisms that have not been cultured (Tyson *et al*., 2004; Venter *et al*., 2004). Use of phylogenetic anchors in sequence based analysis approach either as the initial identifiers or as indicators for phylogeny identification of genes of interest is limited by the small number of reliable phylogenetic anchors. This means that if the DNA fragment of interest does not contain a phylogenetic marker, its origin remains unknown. However, the anticipating progress in the phylogenetic markers in coming decades will increase the power of this approach.

Functional metagenomics: In functional metagenomics approach, the clonesare screened for expression of specific traits. Success in functional metagenomics requires precise transcription and translation of our gene of interest and final secretion of product (if the screening is done through extracellular assays). Functional metagenomics analysis has identified many novel antibiotics (Wang *et al*., 2000; MacNeil *et al*., 2001; Gillespie *et al*., 2002; Venter *et al*., 2004), antibiotic resistance genes (Riesenfeld *et al*., 2004),

Na+/H+ transporters (Majernik *et al*., 2001), and enzymes (Healy *et al*., 1995; Henne *et al*., 1999; Henne *et al*., 2000). This method has the potential to recognize completely novel classes of genes for novel or well-known functions. However, many of the genes will not be expressed in the particular bacterial host selected for transformation even though DNA from a large diversity of organisms has been successfully expressed in *E. coli*. The insufficiency of active clones stresses the necessity for the further progress of efficient screening assays for detection of activities or molecules. The complementation strategy for selection of Na+/H+ antiporters Majernik *et al*. (2001) and biotin producers (Entcheva *et al*., 2001) were successful in some experiments. Selection strategy using antibiotic resistance led to the isolation of novel antibiotic resistant determinants (Diaz-Torres *et al*., 2003; Riesenfeld *et al*., 2004). Utilization of specific media that can provide the selection or identification of active clones is another strategy.

Additional evolving direction for functional metagenomics is the use of functional anchors (functions which can be assessed quickly in all the clones of a library). This is a hybrid approach, in which once the collection of clones with a common function is assembled, they are sequenced to find phylogenetic anchors in the flanking DNA. Such an analysis can determine the diversity of genomes that contain a particular function (Handelsman, 2004).

Sample preparation and processing in metagenomics

It is the most crucial step in metagenomics as the isolated DNA should be representative of all cells present in the sample andshould contain sufficient amounts of high-quality nucleic acids. Processing requires specific protocols for each type of samples, and many literatures are available for different sample types (Venter *et al*., 2004; Burke *et al*., 2009: Delmont *et al*., 2011). Further, many initiatives to discover the microbial diversity from various sample types using single DNA isolation technology are under way (Knight *et al*., 2007). If the target community is associated with a host having large genome size, either fractionation or selective lysis will be suitable to avoid the host DNA mixing with the target DNA (Burke *et al*., 2009; Thomas *et al*., 2010). Physical fractionation by selective filtration or centrifugation or even flow cytometry is used for the analysis of viruses in seawater (Angly *et al*., 2006). Hence, a comparison of multiple methods has to be done before finalization for new type of samples so as to ensure the representative extraction of DNA.

Cloning strategy and metagenomic library preparation

Choice of cloning strategy largely depends on the study target. Shotgun sequencing is generally done on small-insert clones, while functional studies may be conducted on small or large-insert clones. If the target is to obtain information of single gene, small DNA fragments (<10 kb) can be obtained and cloned in *E. coli* into

standard cloning vectors (e.g., pUC derivatives, pBluescript SK(þ), pTOPO-XL, and pCF430). Various enzymes, such as amidases, hydrolases, cellulases and antibiotic resistance determinants, have been identified through these approach. Conversely, to obtain targets encoded by multiple genes, large DNA fragments (>20 kb) must be cloned into fosmids, cosmids, or bacterial artificial chromosomes (BACs), all of which can stably maintain large DNA fragments. Two vectors, *viz*. pCC1FOS and pWE15, have been explored for cloning large DNA fragments from diverse microbial communities. Furthermore, occurrence of considerable flanking DNA on fosmid or BAC clone inserts enables phylogenetic interpretation about the fragment. Small-insert libraries made in plasmids (stably maintain up to 10 kb of DNA) need 3-20 times extra clones compared to libraries built in fosmids (30–40 kb inserts) or BACs (up to 200 kb inserts) to get similar coverage of the identical microbial community. The clones are then selected and transformed into suitable host, with most work being conducted in *E.coli.*Electroporation is the primary and most efficient method for introducingmetagenomics DNA, especially large-insert libraries, into *E. coli*. *E. coli* based metagenomic libraries are then stored in liquid culture supplemented with vector-selective antibiotics and 10-15% glycerol at -800C (Sabree *et al*., 2009).

Sequencing platforms

Over the past 15 years, the revolutionary advance made in sequencing technologies helped in the transformation of traditional microbiology into new generation research of metagenomics. Among the various NGS (new generation sequencing) technologies, two namely, 454 Life Sciences/Roche and the Illumina/Solexa systems are now being extensively applied in metagenomics. Among these, Roche 454 has the longest read length so that it leads to the simplest bioinformatics. The platform also has the lowest throughput and higher cost per unit of data. The other sequencing platform have short-read lengths, high throughput, and lower costs. However, the shorter reads lead to relatively more complicated bioinformatics and the inability to answer some biological questions. The selection of sequencing platforms for any metagenomics project depends on the research purpose and application. Therefore, it is good practice to compare the features of different sequencing platforms to find out which sequencer has the right features for the required experimental outcome. The major considerations are

GC bias: As some phyla, classes or species of bacteria are reported to contain very low or high GC content, the lack or minimal GC bias for the sequencing analysis platform is necessary in metagenomics studies targeting the microbial diversity.

Error rate: As metagenomes are highly variable with tremendous amount of unknown sequences, a low error rate of sequencing platform is mandatory for unambiguous results.

Throughput (number of reads): A large number of reads will lead to a better characterization of the rare organisms in a complex microbial environment. So larger throughput sequencing platform is required for diversity studies. However, it is not required in case of experiments targeting the difference before and after an environmental trials-such as microbiome in the gut before and after antibiotic treatment, where impact on the major organisms is only required in the analysis. Greater throughput by NGS allows for a more detailed and deeper view of the microbial component,

Read length: Longer read length leads to improved resolution in a number of important experimental metrics (i.e., species for long-read length and phyla for short-read lengths). It has been reported that read lengths of 500–700 bp are only required to discriminate between *16SrRNA* sequences at species level (Clarridge, 2004; Paster *et al*., 2001). However, for discovering new genes or proteins of bacteria, minimum 188 amino acids have to be covered so that more read length is necessary.

Methods to infer different levels of information about microbiome

Include 16S ribosomal RNA (rRNA) analysis, whole-genome shotgun (WGS; metagenome) analysis and whole-transcriptome shotgun (metatranscriptome) analysis and whole metabolite analysis. Analysis of *16SrRNA* uses *16SrRNA* gene information to identify the microbes. WGS analysis uses information of all genes to interpret microbial identities down to species or strain level. The meta-transcriptome analysis uses gene expression patterns and functionality of microbial communities. Whole-metabolite analysis provides a comprehensive list of chemicals in the environment of interest and permits correlating richness of microbes to chemicals.

***16S rRNA* data analysis:**The*16S rRNA*gene sequence of bacteria is about 1,550bp long and consist of nine hypervariable regions that are separated by nine highly conserved regions. However, it remains costly to assay the full 1500+ bp in high throughput sequencing and there has been no consensus on which of the nine variable regions of *16S rRNA* should be targeted. Biases in detection of bacteria for a given region are known to be caused by choice of primers (Brooks *et al*., 2015) and estimates of species richness have been shown to vary between 16S regions (Youssef *et al*., 2009). The study by Yang *et al*. (2016) suggested that V4-V6 will be the most reliable regions for the design of universal primers with superior phylogenetic resolution for bacteria, while V2 and V8 are the least reliable regions. Five bioinformatics tools are generally used for *16SrRNA* analysis such as, QIIME, UPARSE, MOTHUR, DADA2 and minimum entropy decomposition (MED).

WGS:provides an integrated understanding of community structure, geneticpopulation heterogeneity and potential metabolism pathways with relatively low costs, improved time

requirements and higher quantities of data. Conversely, the pre-processing of the WGS data can be a challenging task not only for much larger volume of data being processed, but also due to the lack of the reference whole genomic sequences within the available databases. The commonly used tools are MetaPhlAn2, Kraken, CLARK, FOCUS, SUPERFOCUS, MG-RAST, StrainPhlAn, PanPhlan, Constrains, Sigma and LSA. After sequencing WGS includes following steps.

Sequence assembly:In this step, assembly of short read fragments is performedto obtain longer genomic contigs using either reference-based assembly (co-assembly) (software packages such as Newbler (Roche), AMOS or MIRA are used) or *de-novo* assembly (Thomas *et al*., 2012). *De-novo* assembly requires larger computational resources having hundreds of gigabytes of memory in a single machine and run times frequently being days. Fundamentally, during assembly it is important to remember that single reads have usually lesser quality and henceforth lesser confidence in precision than multiple reads that cover the identical fragment of genetic information. In metagenomics data of a complex community with low sequencing depth, it is unlikely to get many reads that cover the same fragment of DNA. Hence assembly may be of limited value for metagenomics. However, longer and more complex genetic elements (e.g., CRISPRS) cannot be analysed without assembly. Therefore, there is a necessity for metagenomic assembly to get high-confidence contigs and none of the existing assembly tools is bias-free.

Binning: Refers to the process of sorting DNA sequences into clusters that mightdenote a discrete genome or genomes from closely related organisms. In other words, it is the phylogenetic analysis of taxonomic groups. Several algorithms have been developed, which employ two types of information contained within a given DNA sequence. Thus, there are two binning methods namely, compositional binning (based on the fact that genomes have conserved nucleotide composition such as certain GC or particular abundance distribution of k-mers) and similarity-based binning (the unidentified DNA segment might encode for a gene and the identity of this gene with identified genes in reference database is used for binning the sequence). Examples of compositional-based binning algorithms are Phylopythia, PCAHIER, S-GSOM and TACAO, whereas similarity-based binning software are IMG/M, MEGAN, SOrt-ITEMS, CARMA, MG-RAST, and MetaPhyler. Binning algorithms that use both composition and similarity are PhymmBL and MetaCluster. Overall, composition-based binning is not dependable for short reads, since they do not cover enough information.If the metagenomics dataset, however, contains two or more genomes, then “chimeric” bins might be produced by similarity based binning. In this case, the two genomes might be separated by additional binning based on compositional features (Thomas *et al*., 2012).

Annotation: Annotation has two steps; first, features of interest (genes) areidentified (feature/gene prediction) and, second, putative gene functions and taxonomic neighbours are assigned (functional annotation). Various tools were specifically designed to handle metagenomic gene prediction of CDS and non-protein coding regions. Functional annotation represents the most computational challenge for most metagenomic projects. Many reference databases such as KEGG, eggNOG, COG/KOG, PFAM, and TIGRFAM are available to functionally annotate the metagenomic datasets. However, as the available reference databases do not cover all biological functions, capacity to merge the interpretations of all database searches within the same framework is executed in the most recent versions of IMG/M and MG-RAST. As per the current estimates, only 20 to 50% of metagenomic sequences can be annotated (Gilbert *et al*., 2010).

Experimental design and statistical analysis: Experimental design shouldbe driven by the question asked.Forstatistical analysis ofmetagenomics data, Primer-E package and a web-based tool called Metastats are well-established tools, allowing for a range of multivariate statistical analyses. In addition, Shotgun-FunctionalizeR package provides several statistical procedures for metagenomics data analysis using the popular R statistical package (Thomas *et al*., 2012).

Sharing and storage of data: For metagenomics data, sharing of findingsdemands an entire new level of organization and collaboration to deliver metadata and centralized services (e.g., IMG/M, CAMERA and MG-RAST) as well as to share both data and computational results. A suite of standard languages for metadata is currently provided by the Minimum Information about any (x) Sequence checklists (MIxS). US National Center for Biotechnology Information (NCBI) is mandated to store all metagenomic data (Thomas *et al*., 2012).

Application of metagenomics to fisheries science

As metagenomics is a relatively new branch of science, the application of metagenomics in fisheries science is not yet common despite of its potential applications. The prospective uses of this molecular tool in fisheries science includes

1 To study the microbial diversitythat thrives within animals and aquaculture facilities: Documenting the bacteria present in healthy animal/itsenvironments is the first step to understand the impacts of microbial manipulation in aquaculture systems (Tarnecki *et al*., 2016). The knowledge on bacterial abundance and diversity can provide insight to the health status of individuals, as there will be an increase in the abundance of the opportunistic pathogens with considerable decrement in bacterial diversity during the incidents of stress and disease (Boutin *et al*., 2013). Most of metagenomics studies in aquaculture have concentrated on the microbial diversity (especially gut) of commercially important aquaculture species with the aim of manipulating the

microbes to increase feed efficiency and decrease disease susceptibility (Llewellyn *et al*., 2014; Ringo *et al*. 2016). These studies are necessary to unravel the intricate host-microbe symbioses, early disease diagnosis and to use the benefits of a healthy microbiome in fish culture. Similarly, quantitativecharacterization of the relative effects of factors affecting these microbial community structure will help in understanding pathogenesis of various diseases.

A few studies on the application of metagenomics in aquaculture environments have been conducted (Krishnani *et al*., 2010). Characterization of the microbial communities of bio-filters in recirculating aquaculture systems to increase the understanding of applied microbial ecology and to provide the basis for managing such communities to enhance their function and reliability has been done by metagenomics approach (Huang *et al*., 2016). Understanding the microbialcommunity profiling in aquaculture environments, quantitative characterization of factors affecting these microbial community structure and function, its changes over time, determining whether and how community diversity changes when culturing the same species under different densities or organics loads *etc*. will help in management of various culture facilities to maximize the yield and sustainability of operation.

2 Identification of core microbiome functions: Apart from the mere communityanalysis, it is important to understand how these commensal microbes influence digestion, immune function, behavior and overall fish health. These studies will advance our knowledge on fish health and help in the microbial manipulation for disease diagnosis, treatment and prevention in culture systems and in design of better probiotics (both feed and water probiotics). Most of such investigations have focused on culturable microorganisms (Aguilera-Rivera *et al*. 2014; Martınez-Cordova *et al*. 2015); however, evidence regarding their uncultivable microbial neighbors is still rare. Similarly, determining the metabolic processes performed by microbes in aquaculture systems is a focal point to achieve a better understanding and increase the possibility of manipulating the microcosms created by aquaculture in order to understand the biogeochemical cycles of nutrients within and outside of ponds, the modification of bacterial communities and the disruption of key processes that lead to disease.

3 Detection of novel and potential pathogens and clear picture of disease pathogenesis: Aquaculture of nearly all taxonomic groups are commonlyimpacted by newly emerging diseases. Microbial profile during disease process through metagenomics helps to elucidate the responsible strains, presence of rare pathogens, synergistic activities of multiple pathogens and potential vectors that favor the proliferation of the pathogen. For example, billions of dollars in aquaculture industry have been lost due to early mortality syndrome or acute hepatopancreatic necrosis disease

(AHPND), still it is unclear that which of the *V.parahaemolyticus* strains are responsible for the disease (Kondo *et al*. 2014), anda metagenomics profile could provide a clear picture during such situations.

4 Detection of novel microbes: The presence of wide diversity of microbesincluding bacteria, viruses and fungi is a common scenario in aquatic and marine environments. Current detection methods are limited in their ability to identify novel microbes especially viruses (Alavandi and Poornima 2012). Therefore, application of metagenomics will help in the detection of novel microbes especially viruses and uncultivable bacteria.

5 Formulation of bioflocs: Presently as the aquaculture has used mostlyartificial feed, 70-80% remains in the water column or sediments, resulting in wastage of feed and money as well as the production of toxic residues limiting the growth rates of the cultured species and intensified culture practices (Avnimelech 2012). In this scenario, biofloc technology has arisen as a successful strategy to take aquaculture intensification and sustainability

(Megahed and Mohamed, 2014; Martınez-Cordova *et al*., 2015). Production of fish and shrimp is shown to be doubled when bioflocs are combined with formulated feed (Kuhn *et al*. 2009; Emerenciano *et al*. 2012). Diversity studies driven by metagenomics are needed in this field for the characterization of the microbes forming these bioflocs. It is assumed that genes involved in the metabolic pathways of nitrogenous and carbonous compounds are present in microbes of bioflocs; however, the full mechanism of the metabolism of such compounds will be clear only after shotgun metagenomics is performed. Recent evidence suggests that bioflocs 'seem' to prevent disease outbreaks

(Avnimelech 2012); In this regard, metagenomics should be used as a tool to detect whether the microbes composing the bioflocs possess gene families encoding for proteins associated with the antagonist activities or whether other mechanisms halt the normal activity of the pathogens. Lastly, for finding better bacteria species for flocculation, determining nutritional composition and identifying the relationships among the microbes contained in bioflocs are still novel research fields in which metagenomics can play a key role (Martınez-Porchas and Vargas-Albores, 2015).

6 Design of probiotics: Metagenomics will assist in the search for novelprobiotics, the analysis of particular microbial communities favoring healthy conditions, and/or the monitoring of effect on commensal microbial communities when probiotics are used.

7 Application in natural aquatic/marine ecosystems for discovery of novel molecules/genes: Identification of enzymes, drugs and other bioactivemolecules from various marine/ aquatic ecosystems are another area

which can be explored through metagenomics. In addition to novel molecules, novel genes encoding for proteins or enzymes with higher activity for any particular biochemical reaction than those already known can be found out through metagenomics. Similarly, novel metabolically and environmentally significant genes in ocean, including genes involved in various biogeochemical cycles, photosynthesis, phosphate stress response, nitrogen stress response, nucleotide scavenging *etc.* can be explored (Duhaime *et al*. (2012).

8 Better understanding of natural scenarios: Application of metagenomics tostudy the imbalances in microbial communities during natural disease outbreaks and natural disasters enable us to reach a better understanding of the scenarios favoring and preventing such scenarios (Hall *et al*. 2014; Hurwitz *et al*. 2014). Similarly, no metagenomics based studies have been conducted onmicrobial diversity present in the effluents that are discharged from aquaculture/mariculture farms to environment and on the changes caused on natural microbiota.

9 For bioremediation and/or recirculation purposes: Aquaculture/Mariculture activities generates millions of tons of waste rich in nitrogenous, sulphurous, phosphorous and carbonous compounds (Martinez-Porchas & Martinez-Cordova 2012; Wang *et al*. 2012); these wastes are sometimes toxic and rebase the nutrient loading capacity of the ecosystems. Thus, investigation of genes and operons encoding for proteins involved in sensing, uptake and neutralization/utilization of specific metabolites usually observed in aquaculture through metagenomics, can unravel the biochemical pathways of a given microbial community to recycle nutrients. Moreover, these genes can be explored for bioremediation and/or recirculation purposes.

10 For improving the knowledge on antibiotic resistance mechanisms in marine environments and aquaculture facilities: Misuse and over-prescription of antibiotics have driven the emergence and spread of antimicrobial resistance in aquaculture facilities (Martinez-Porchas and Martinez-Cordova 2012). Surprisingly nothing is known on the origins, distribution and diversity of resistance genes, particularly those belonging to the vast majority of the unculturable microbes. Aquaculture requires studies on these aspects although, such investigations are rare and this will pay the way to improve the knowledge on antibiotic resistance mechanisms and to detect novel resistance determinants (Port *et al.* 2014).

In conclusion, metagenomics has huge potential to unravel many scientific questions and problems related to fisheries science as well as to discover novel bioactive molecules and potential genes. On contrast, the tool has been underexplored in this field. Metagenomics promises to improve valuation of fisheries science by increasing the productivity and environmental performance of aquaculture and through the discovery of

novel biomolecules and technological outputs.

SUGGESTED READINGS

Thomas T, Gilbert, J and Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis. *Microbial Informatics and Experimentation*, 2:3

Roumpeka DD, Wallace RJ, Escalettes F, Fotheringham I and Watson M (2017) A review of bioinformatics tools for bio-prospecting from metagenomic sequence data. Front. Genet. 8:23.

Tarnecki AM., Burgos FA., Ray CL and Arias CR. (2017). Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. Journal of Applied Microbiology 123, 2-17.

Martınez-Porchas and Vargas-Albores (2017) Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Reviews in Aquaculture (2017) 9, 42–56

Marine fish cell lines: Development and Applications

K. S. Sobhana
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Fish cell cultures have been in use for more than five decades and were originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases. Presently, the number of available fish cell lines has grown tremendously covering a wide variety of species and tissues of origin. While various applications have been reported, their potential role in scientific applications are just beginning to be exploited. In addition to virology, continuous fish cell lines are important for gene expression studies; cytogenetics; as *in vitro* models in toxicology; transgenics as well as in many other basic studies including fish immunology, physiology and endocrinology.

Teleost cell lines are the second most abundant among animal cell lines, mammalian cell lines being the most numerous. The first successful attempt for fish tissue culture was made by Osowski (1914), who cultured explants of trout embryos and fry in Ringer's solution and frog lymph. A major breakthrough in application of fish tissue culture to virology was probably when Schlumberger (1949), used roller tubes and mammalian-type medium in a tissue culture study of a neoplasm from adult goldfish, *Carassius auratus*. Soon after, Sanders and Soret (1954) reported growth of anarthropod-borne virus in intact *Gambusia* embryo grown in mammalian-type medium. Their work marked the first report of a synthetic medium designed for mammalian cells (Medium 199) which was used for teleost tissues.

Grutzner (1958) made a major contribution by trypsinisation of fish tissues from tench *Tinca tinca*, yielding cultivable cells which grew in to a monolayer. Grutzner'smonolayers were successfully subcultured, thus contributing to the establishment of fish cell culture. RTG-2 cell line of rainbow trout, *Salmo gairdneri* gonad origin initiated in 1960 was the first permanent fish cell line to be developed (Wolf and Quimby, 1962). Clem *et al*. (1961) initiated trypsinised blue-striped grunt, *Haemulon flavolineatum* fin cell cultures which provided the GF-1 cell line, the first line of marine fish origin. Subsequently, FHM was initiated in 1962; which was an epithelial-like cell from the fathead minnow, *Pimephales promelas* (Gravell and Malsberger, 1965). Several cell cultures and cell lines from a variety of fishes have been developed since the first cell line from rainbow trout. A comprehensive list of most fish cell lines developed before 1980 has been published by Wolf and Ahne (1982). Fryer and Lannan (1994) reported on 159 cell lines originating from 74 fish species

representing 34 families of fishes. A majority of these cell lines have been established from freshwater or anadromous fish species. The rapid expansion of aquaculture and associated viral diseases in North America, Europe and Japan led to the subsequent development of several fish cell lines for health management purposes. Hightower and Renfro (1988) reviewed current applications of fish cell lines in the study of intracellular movement of organelles, xenobiotic metabolism, thermal stress, ion transport, endocrinology, immunology and cancer biology. They are, however, mostly used in monitoring aquatic pollution and toxicology, and in propagating viruses that are etiologic agents of disease in economically significant species.

Marine fish cell lines

In vitro cell culture systems/cell lines derived from marine fish are necessary forisolation and characterisation of viruses and studies on diversity of fish viruses in marine environment. Unlike other microorganisms, which can be readily grown in artificial nutrient medium, viruses are obligatory intracellular pathogens and their isolation and propagation are totally dependent on the availability of a live host, such as permissive cell cultures. Marine fish cell cultures gained impetus due to the rapidly developing mariculture activities world over which led to outbreak of viral diseases that has become a serious issue causing heavy economic losses. Permissive cell cultures are progressing as viral diagnostic tools, to isolate, propagate and assay infectious viruses, and to prepare and clone attenuated virus strains for antiviral vaccination. Moreover, many new continuous cell cultures are constantly being developed as a result of intensive efforts in several parts of the world, to provide cell cultures from local species utilised in aquaculture. Since cell cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation, cell lines derived from local species are given high priority. The host and tissue specificity of virus underlines the need for developing cell lines from different species in different regions. Cell lines also offer the advantage of allowing problems to be investigated quickly and inexpensively at the molecular and cellular levels in order to gain information that can be used to design the most efficient *in vivo* studies of the problems.

Lakra *et al*. (2010) reported that about 125 teleost fish cell lines have been developed since 1994 with more than 60% of the cell lines being established from the Asian region. The largest number of marine fish cell lines was derived from groupers (Osteichthyes: Serranidae). The species from the genus *Epinephelus* account for roughly 30% of the marine fish cell lines developed since 1994 (Sobhana *et al.,* 2008, 2009, 2011; Lei *et al*., 2012), the highest taxonomic representation overall. The tropical marine food fish, the Asian seabass *Lates calcarifer*, has also been used extensively to develop cell cultures (Chang *et al.,* 2001; Sahul Hameed *et al*., 2006).

Methods in fish cell culture

Tissue/cell culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid medium that supplies the nutrients essential for survival and growth. Stages of cell culture comprises: (1) acquisition of the sample, (2) isolation of the tissue, (3) dissection and/or disaggregation, and (4) culture after seeding into the tissue culture vessel. Cell cultures may be derived from primary explants or dispersed cell suspensions. Because cell proliferation is often found in such cultures, the propagation of cell lines becomes feasible. A monolayer or cell suspension with a significant growth fraction may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a *passage*, and the daughter cultures so formed are the beginnings of a *cell line*. The formation of a cell line from a primary culture implies (1) an increase in the total number of cells over several generations and (2) the ultimate predominance of cells or cell lineages with the capacity for high growth, resulting in (3) a degree of uniformity in the cell population. The line may be characterised, and the characteristics will apply for most of its finite life span. The derivation of *continuous* (or ‘‘established,’’ as they were once known) cell lines usually implies a phenotypic change, or *transformation.* The alteration in a culture that gives rise to a continuous cell line is commonly called *in vitrotransformation* and may occur spontaneously or be chemically or virally induced.Continuous cell lines are usually *aneuploid* and often have a chromosome number between the diploid and tetraploid values. There is also considerable variation in chromosome number and constitution among cells in the population (*heteroploidy*). It is not clear whether the cells that give rise to continuous lines are present at explantation in very small numbers or arise later as a result of the transformation of one or more cells.

Choice of tissues for primary culture

Teleost fish cell lines have been developed from a broad range of tissues *viz.,* ovary, fin, gills, swim bladder, heart, spleen, kidney, liver, eye muscle, vertebrae, brain as well as skin and most fish cell lines originated from normal tissues. Embryos or fins are most frequently listed as the source of the tissues used in the primary culture. After ovary, the second most common tissue used for cultivation is fin, due to its high regenerative ability.

Techniques for isolation of cells

Explantation: The simplest procedure is the explant method which involves finelychopping the tissue into fragments no larger than 1-2 mm3 and plating them in culture dishes along with tissue culture medium. Cells outgrow from the explant eventually forming a monolayer culture.

Enzymatic dispersion: In this method, tissues are minced and suspended in adigestion mixture, usually trypsin, but other proteolytic enzymes such as collagenase or pronase may also be used. Cells are harvested by neutralising the enzyme with media containing serum, then pelleting the cells by centrifugation and re-suspending in fresh media.

Mechanical dispersion: Another common method of cell isolation is mechanicaldispersion. By forcing the tissue through a sieve or syringe, individual or small clumps of cells are released from the bulk of the tissue. However this procedure causes a great deal of mechanical damage and produces lower yields than any other method. Furthermore it is only recommended for softer tissues like brain, kidney and spleen.

Subculture

The first subculture represents an important transition for a primary culture. The need of subculture implies that the primary culture has increased to occupy all of the available substrate. Primary cultures have variable growth fractions. Serial subculture of cell lines enriches a fraction of any cell population based on growth rate and viability. For all fish species cultured to date, cells are anchorage dependent, attaching and growing as a monolayer on the flask surface. Monolayer cell cultures may be dispersed either mechanically or chemically for subculturing. Fish cells differ in the tenacity of their attachment to each other and to the substrate. In part, this reflects differences between cell lines; but media, culture conditions, and even culture age affect the ease or difficulty with which cells may be dispersed.

Maintenance of cells

Many different kinds of tissues and cells from teleosts can be cultured on a routine basis. The maintenance of *in vitro* culture of fish cells closely follows that used with homoeothermic material; the major differences being, first, in temperature requirements and tolerances, and, second, in osmolarity of salines and media. For the most part, mammalian-type solutions are entirely satisfactory for many teleost species.

Temperature: Fish cells can tolerate an even wider temperature range, which hasbeen defined as the endurance zone. To maximise cell production, an optimal growth temperature should be selected. For cold-water fish, like salmon, this is in the 20–23˚C range; and for warm-water fish, this is 26–30˚C. If the purpose is to just maintain the cultures for later use, lower temperatures allow the cells to slowly proliferate, reducing culture maintenance. Nearly all fish cells can be maintained at room temperature, but the variability of the ambient temperature in laboratories makes this undesirable, and a temperature controlled incubator is preferable. The principal advantage of vertebrate cell culture and notably fish cell culture is their innate ability to metabolise through a wide range of temperatures.

pH: The pH of medium necessary for good growth of fish cells does not appear tobe particularly critical, and most cells seem to fare well in the range of 7.2-7.8. Primary cultures and low densities of cells will usually do better at 7.3-7.4 than at pH 7.8. Routine passage of some cell lines can be made at pH 7.8 or even 8.0, but the lag phase may be extended. At the other end, old cultures can have a pH as low as 6.8, apparently without undue damage to the cells. Such cultures can be dispersed in fresh medium and growth will usually resume.

Osmolality: Osmolalities between 260 mOsm kg-1and 320 mOsm kg-1are quiteacceptable for most fish cells. The addition of HEPES and drugs dissolved in strong acids and bases and their subsequent neutralisation can all markedly affect osmolality.

Cryopreservation

Freezing and long-term storage of cultured cells at ultra-low temperature has been practiced successfully for mammalian cells, which works equally well with fish cells. Cell lines in continuous culture are prone to variation due to selection in early-passage culture, senescence in finite cell lines, and genetic and phenotypic instability in continuous cell lines. Even the best-run laboratory is prone to equipment failure and contamination. Cross-contamination and misidentification continues to occur with an alarming frequency. In addition, it ensures that the lines are safely maintained. Optimal freezing of cells for maximum viable recovery on thawing depends on minimising intracellular ice crystal formation and reducing cryogenic damage from foci of high-concentration solutes formed when intracellular water freezes. Cryoprotectants such as dimethyl sulphoxide (DMSO), reduce the amount of ice present during freezing and reduce solute concentration, thus reducing ionic stress. However, these compounds can themselves cause osmotic injury since they are hypertonic and can cause damage during their addition or removal. In general, cells are suspended in cryopreservation medium having 10% or more serum and 5-10% of either glycerol or DMSO as a protective additive. Freezing in liquid nitrogen or in ultra cold freezers is the method of choice for storage of fish cell lines. The medium used for freezing fish cells generally contains 10% or more serum and either of the two cryoprotectants glycerol or dimethyl sulphoxide (DMSO) added to a final concentration of 5 – 10%.

Applications of fish cell lines

Piscine cell cultures are increasingly being applied to studies of fish, which, as the largest and most diverse group of vertebrates, are important model systems in embryology, neurobiology, endocrinology and environmental biology. Cell lines from teleosts would be invaluable in advancing basic knowledge in comparative endocrinology, physiology, and immunology and in providing practical information that could be used to enhance the health, growth and reproduction of fish in aquaculture.

Virology and vaccine development: DeWitte-Orr (2006) described the utilityof cell lines into five major areas for the understanding of viruses. Cell lines are needed to produce sufficient virus to characterise the biophysical and biochemical properties of a virus. Examples of such characterisation would include describing viral proteins and genome organisation. Cell cultures are also needed to study the single-cell reproductive cycle of virus. This would include understanding the molecular events involved in the entry, replication, assembly and release of viruses. Cell lines can contribute to preventing and controlling viral diseases by being a source of viruses for vaccines and an experimental system for the development of antiviral drugs. In some cases, cell lines can advance an understanding of viral pathogenesis. For example, CHSE-214 was used as a tool for studying the characteristics of IHNV persistent infections found both in the cell lines and in whole fish (Engelking and Leong, 1981). Cell lines are powerful diagnostic tools, for discovering new viruses and identifying viral pathogens from environmental samples. Susceptibility of cell lines to viral infection is the basis for isolating and characterising fish viruses.

Immunology: Cellular and molecular aspects of fish immunology have beeninvestigated using both primary cell cultures and immortalised cell lines (Barnes *et al*., 2006). Three major B cell lineages have been described in teleost, which are those expressing either IgT or IgD, and the most common lineage which coexpresses IgD and IgM. A benefit of cell lines is cell type specificity, which allows researchers the ability to study the responses of one cell type in a controlled environment. RTS11, a rainbow trout macrophage-like cell line, RTS11 was initiated from a long-term haemaopoietic cell culture of a rainbow trout spleen (Ganassin and Bols, 1998) has proven to be a valuable tool for studying immune cell-specific responses *in vitro*. RTS11 cells secrete lysozyme and are able to phagocytose and express major histocompatibility complex (MHC) class

II β at the transcript level. RTS11 is also able to respond to lipopolysaccharide by expressing interleukin (IL)-1 β, tumour necrosis factor (TNF)-α and cyclooxygenase (cox)-2. The role of two retinoid-related orphan receptor (ROR)-g homologues (ROR-gammaa1 and -gammaa2) genes expressed in rainbow trout skin was studied *in vitro*. Studies using trout cell lines demonstrated that ROR-g is induced significantly by LPS and down regulated by the presence of Poly I:C and recombinant interferon (IFN)-g. *Invivo* studies demonstrated that its expression was significantly higher in vaccinated *vs* unvaccinated fishes following bacterial (*Yersinia ruckeri*) challenge, but it was down regulated after a viral (VHSV) infection. This data suggest a potential role of trout ROR-g, a putative TH17 transcription factor, in protection against extracellular bacteria (Monte *et al.*, 2012).

Biomedical research: Hightower and Renfro (1988) reviewed fish cell lines witha perspective on biomedical research

which included epithelial ion transport, endocrinological studies, cellular stress (heat shock) response, thermo-tolerance, cancer biology and environmental toxicology. Rakers *et al*. (2011) demonstrated that it was possible to integrate freshly harvested rainbow trout (*Salmo gairdneri*) scales into fish skin cell cultures, and antibody staining indicated that both cell types proliferated and started to build connections with the other cell types. This may be the first step to generate an "artificial skin" with two different cell types, and, in the future, similar studies could lead to the development of a three-dimensional test systems (Rakers *et al.*, 2011). One of the rarest applications of fish cell culture is the use of Brokmann bodies (islets) from the teleost fish for transportation in to diabetic animals to normalise blood glucose level.

Transgene expression: Fish cell lines have been used to express exogenous DNA fora variety of objectives. Many have focused in identifying promoter or enhancer elements that function in fish cell culture, testing their relative strength for possible use in transgenic fish production. Other objectives include the selection of pluripotent cells, investigation of stress protection mechanisms and functional characterisation of recombinant fish proteins. Promoters obtained from fishes that have been used to achieve transient, stable or inducible expression in fish cell cultures include those derived from the metallothionein, beta actin and alpha-globin genes. The luciferase reporter gene under the control of dioxin-responsive enhancers was introduced into RTH-149 cells to generate the recombinant cell line, designated remodulated lightning trout or RTL 2.0. A gene transfer approach using rainbow trout gonad (RTG-2) cells also has been developed to create an *invitro* assay system for the detection of estrogenic compounds in environmental samples.Similarly, the zebrafish embryo cell line, ZEM2s (zebrafish embryo 2-serum adapted), was transfected with plasmids containing a reporter gene under the control of either aromatic hydrocarbon, heavy metal, or electrophile response elements, so that expression from these transgenes indicated induction upon exposure to compounds belonging to each class of inducer. In another application of this technology, plasmid-based metallothionein expression was investigated in transfected CHSE-214 cells, identifying this system in protecting cells against oxidative stress.

Biotechnology: Fish cell cultures have been used as biotechnological tools inaquaculture. Viral diseases affecting fish have brought into focus the importance of biotechnological products like vaccines and interferons both *in vivo* and *in vitro*. Cloning has also been attempted to address questions concerning the evolution of B cell function. Embryo cell cultures were used to drive stem cells for fish transgenesis and also pluripotent embryonic stem cells for genome manipulation with many applications in marine biotechnology. Zhou *et al.* (2008) developed a tail fin cell line from the Chinese sturgeon, *Acipenser sinensis* a cartilaginous ganoid. The species is

considered to be a living fossil. The developed cell lines could serve as a model 'living fossil' and used to support research on cellular genetics and cell biology. Five single-cell clone lines were developed from normal primary cultures of rainbow trout pituitary glands and passaged for over 150 times. Immunocytochemical analysis with monospecific antisera of Gh and Prl showed that cells of all the five single-cell clones produced both Gh and Prl, pituitary hormones. This observation was further supported by results of RT-PCR analysis that mRNAs of gh, prl, tsh, gth-1, gth-2, sl, pomcb, cr and rtE2Ra genes were detected in each of these five single-cell clones. The expression of gh and prl genes in the single-cell clone lines is responsive to induction by E2, dexamethasone, and o, p'–dichlorodiphenyltrichloroethane (Chen *et al.*, 2010).

Toxicology: There are two types of cellular models,*i.e*. undifferentiated anddifferentiated cells, used in acute toxicity testing. Of these two types, the simple systems measuring basal cytotoxicity are probably the more useful in the sense that a central toxic effect is measured. Four useful parameters to evaluate common cellular responses include cytotoxicity, cell growth, genotoxicity and xenobiotic metabolism. Cell lines are more amenable to toxicogenomic techniques and up or down regulation of genes or proteins. The homogeneity of cell lines makes these responses to toxicants easier to detect and with less variability than whole organisms. Short-term primary cultures and cell lines from fish tissues have provided successful cell culture systems for environmental toxicology studies to evaluate the toxicity of industrial effluents, including petroleum refinery effluents, oil-sands process–affected waters and the toxicity of several compounds. Genotoxicity is assessed using comet assay, which detects DNA strand breaks. Schirmer (2006) reviewed the so far established cell cultures and found that vertebrate cell lines can be compared well with fish lethality in their relative sensitivity towards the toxicity of chemicals. Given strong correlations of r-values for cell lines *vs* fish acute toxicity, cell cultures could provide excellent tools as cell-based biosensors. Ecotoxicological effects following exposure to heavy metals *in vitro* have been reported by a number of researchers. Toxicological investigations using cell culture have been extremely useful for clarification of action mechanisms of toxic substances with specialised cell systems; clarification of the effects on basic cell functions mainly with fibroblasts and epithelioid cells and in metabolism investigations.

As the development of animal cell cultures owed much to the needs of two major branches of medical research *viz*., production of antiviral vaccines and understanding of neoplasia, fish cell cultures were initiated to support the growth of fish viruses for studies on fish viral diseases. Tissue culture is considered as the gold standard for identification of diseases associated with intracellular bacterial pathogens and viruses in fishes and fish cell lines have grown tremendously in number covering a wide

variety of species and tissues of origin. Fish cell lines are also used in monitoring aquatic pollution and toxicology. Cell lines offer the possibility of performing experiments in a controlled environment, independent of the complexity and variability of experiments *in vivo*. Fish cells, like mammalian cells, can be maintained *in vitro* using primary cultures, which are temporary or cell lines which are permanent. Early cultures of primary cells may represent a more appropriate model of tissues *in vivo*. The production of short-term primary cultures however, suffers from a lack of reproducibility in the initiation, and homogeneity of cultures that limit their application. Fish cell lines are advantageous to use because they become fairly homogeneous during early passaging, which makes them reproducible, and they have the ability to be cryopreserved, which provides a convenient source of cells.

Although over 283 fish cell lines have been reported in literature (Fryer and Lannan, 1994; Lakra *et al*., 2010), only 43 are maintained in public repositories (Lakra *etal*., 2010). With 53,000 species and subspecies of fish, a dearth in the availability of celllines can be a limiting factor for isolating live virus, as most viruses are species specific and even tissue specific. Fish cell lines have been developed from a wide variety of edible fish to tackle emerging viral diseases, especially regions in the Asian subcontinent have contributed to over 60% of the total fish cell lines since 1994 (Lakra *et al*., 2010). Several cell banks exist for the secure storage and distribution of validated cell lines. Initial seed stock should always be from a reputed cell bank, where the necessary characterisation and quality control are routinely carried out.

SUGGESTED READINGS

Barnes, D., Dowell, L., Forest, D., Parton, A., Pavicevic, P. and Kazianis, S. 2006.

Characterization of XM a novel *Xiphophorus* melanoma-derived cell line.

Zebrafish, 3(3):371–381.

Chang, S. F., Ngoh, G. H., Kueh, L. F.S., Qin, Q.W., Chen, C.L., Lam, T.J. and Sin, Y.M. 2001. Development of a tropical marine fish cell line from Asian seabass (*Latescalcarifer*) for virus isolation. *Aquaculture*, 192: 133–145.

Chen, M. J., Chiou, P.P, Liao, Y.-H., Lin, C.-M. and Chen, T. T. 2010. Development and characterization of five rainbow trout pituitary single-cell clone lines capable of producing pituitary hormones. *J. Endocrinol.,* 205:69-78. doi:10.1677/JOE-09-0315

Clem, L. W., Moewus, L. and Siegel, M. M. 1961. Studies with cells from marine fish in tissue culture. *Proc. Soc. Exp. Biol. Med.*, 108: 762–766.

DeWitte-Orr, S. J. 2006. *A study of innate antiviral mechanisms using fish cell lines.* Ph. D.

Thesis, University of Waterloo, Canada.Engelking, H. M. and Leong, J. C. 1981. IHNV persistently infected Chinook salmon embryo cells. *Virol*. 109: 47-58.

Fryer, J. L. and Lannan, C. N. 1994. Three decades of fish cell culture: A current listingof cell lines derived from fishes. *J. Tissue Cult. Methods,* 16: 87 – 94.

Fryer, J. L., McCain, B. B. and Leong, J. C. 1981. A cell line derived from rainbow trout (*Salmo gairdneri*) hepatoma. *Fish Pathol.,* 15 (3-4): 193-200.

Ganassin, R. C., Schirmer, K. and Bols, N. C. 2000. Cell and tissue culture. In: Ostrander K. G. (Ed.), *The laboratory fish*. Academic Press, New York, p. 631–651.

Gravell, M. and Malsberger, R. G. 1965. A permanent cell line from the fathead minnow (*Pimephales promelas*). *Ann. N. Y. Acad. Sci.,* 126: 555-565.

Grützner, L. 1958. *In vitro*-Zuchtung des Leber – und Nierengewebes von *Tinca vulgaris* Cuv. (Schleie) in trypsinierten Einschichtgewebekulturen. *Zentr.Bakteriol.Parasitenk. Abt. I. Orig.,* 173: 195-202.

Hightower, L. E. and Renfro, J. L. 1988. Recent applications of fish cell culture to biomedical research. *J. Exp. Zool*., 248(3):290–302.

Lakra, W. S., Swaminathan, T. R. and Joy, K. P. 2010. Development, characterization, conservation and storage of fish cell lines. *Fish Physiol. Biochem.,* 37:1-20.

Lei, X. -Y., Chen, Z. -Y., He, L. -B., Pei, C., Yuan, X. -P. and Zhang, Q. -Y. 2012. Characterization and virus susceptibility of a skin cell line from red-spotted grouper (*Epinephelus akaara*). *Fish Physiol. Biochem.,* 38:1175-1182.

Monte, M. M., Wang, T. H., Costa, M. M., Harun, N. O. and Secombes, C. J. 2012. Cloning and expression analysis of two ROR-γ homologues (ROR-γa1 and ROR-γa2) inrainbow trout *Oncorhynchus mykiss*. http://digital.csic.es/bitstream/10261/64698/3/Cloning_ expression _ analysis.pdf

Osowski, H. 1914. Uber aktive Zellbewegung im explantat von Wirbeltierembryonen.*Arch. Entwicklungsmech*. *Organ,* 38: 547-583.

Rakers, S., Imse, F. and Gebert, M. 2014. Real-time cell analysis: sensitivity of different vertebrate cell cultures to copper sulphate measured by xCELLigence®. *Ecotoxicol.,* 23:1582-1591.

Sahul Hameed, A. S., Parameswaran, V., Shukla, R., Singh, I.S.B, Thirunavukkarasu, A. R. and Bhonde, R. R. 2006. Establishment and characterisation of India's first marine fish cell line (SISK) from the kidney of sea bass (*Lates calcarifer*). *Aquaculture,* 257: 92–103.

Sanders, M. G. and Soret, M. G. 1954. Cultivation of animal viruses in embryonic teleost cells. *Trans. N. Y. Acad. Sci.,* 17: 19-25.

Schirmer, K. 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicol.*, 224:163–183.

Schlumberger, H. G. 1949. Cutaneous leiomyoma of goldfish. I. Morphology and growth in tissue culture. *Am . J. Pathol*., 25: 287-299.

Sobhana, K. S., George, G. A., Sheethal, S. M., Sreedevi, S., Surendran, K. K., Anil, M. K. and Kalidas, C. 2011. Establishment of cell lines from various tissues of the honeycomb grouper, *Epinephelus merra* (Bloch, 1793), NH-P41. In: *Book ofAbstracts*, *Symposium on Renaissance in fisheries: Outlook and strategies 2011,* Ninth Indian Fisheries Forum, 19– 23rd December, 2011, Chennai.

Sobhana, K. S., George, K. C., Ravi, G. V., Ittoop, G. and Paulraj, R. 2008. A cell culture system developed from heart tissue of the greasy grouper, *Epinephelus tauvina* (Forsskal 1775) by enzymatic dissociation. *Indian J. Fish*., 55(3):251-256.

Sobhana, K. S., George, K. C., Ravi, G. V., Ittoop, G. and Paulraj, R. 2009. Development of a cell culture system from gill explants of the grouper, *Epinephelus malabaricus* (Bloch and Schneider). *Asian Fish. Sci.,* 22: 1-6.

Wolf, K. and Ahne, W., 1982. In: Advances in cell culture, Academic Press, New York, p.305–328.

Wolf, K. and Quimby, M. C. 1962. Established eurythermic line of fish cells in *vitro*. Science*,* 135: 1065-1066.

Z., Gui, L., Li, Z. Q., Yuan, P. and Zhang, Y. 2008. Establishment of a Chinese sturgeon *Acipenser sinensis* tail-fin cell line and its susceptibility to frog iridovirus. *J. Fish Biol.,* 73:2058-2067

Good laboratory practices through Standard Operating procedure (SOP)

M. P. Paulton
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The primary aim and idea behind formulating a code of conduct classified as standard operating procedure in a biotechnology laboratory is to ensure the safety and security of personals involved in research in different capacities. Such a protocol would certainly ensure the judicious use of sensitive and expensive chemicals and enzymes in a completely secured manner.One can easily assess the level of understanding and to the extent of practising SOP through a self-valuation procedure. The questions to be answered in this process are the following.

- ✓ *Are you aware about the security and safety rules to be followed in a biological laboratory using sensitive and hazardous chemicals?*
- ✓ *Do you know the procedure for storing and handling hazardous materials?*
- ✓ *Are you practising scientific protocols during handling and disposal of biological and chemical materials?*
- ✓ *What is your level of understanding about the health hazards which could cause by the chemicals in regular use?*
- ✓ *What sort of personal protective equipment is required to protect you from hazardous chemicals?*
- ✓ *How do you respond in the event of causality?*
- ✓ *Are you well aware about the medical attention to be followed during emergency?*

If you are successful in responding properly to the above queries, you could asses yourself as proficient in SOP.

LABORATORY SECURITY

As biotechnology Institutes and Industries use variety of hazardous materials, the proper care and security is to be given to avoid the access of the same in the hands of unwanted social elements. Further proper inventory register and a record of the usage of such materials is to be maintained. Unauthorised handling or removal of hazardous materials is to be restricted in the laboratory. Students who are new to the system are to be trained in the

safety protocols to be followed to avoid the theft of such hazardous materials as the same is important from the angle of National security. The protocols related to security are to be designed and practiced based on the situations in each laboratory with the due approval of certifying agencies. Laboratory group is to be trained and responsibilities are to be entrusted to ensure the error free practice of the protocols related to security. Each personal working in laboratory should be aware about an emergency plan and security protocols for reporting immediately in the event of any kind of incidents leads to causality. Proper training should be given to operate fire extinguishers and other emergency handling devices.

Inventory of equipment, Operational knowledge and Periodical servicing.

The laboratory manager or the personal concerned should be aware of the instruments and other minor equipment available. The new recruits should be trained properly on the operational protocols which should be displayed for speedy reference. A log book is to be maintained to record the use of the instruments with adequate instructions to note remarks. Periodic service of the instruments should be monitored to ensure the break down free service. All documents related such correspondences should be made available in the laboratory as the same may be reviewed in social auditing. Adequate power supply depending on the power requirements of the instruments should be made available in the laboratory.

Storage &handling of chemicals, enzymes and reagents.

There is no universal formula or procedures for handling sensitive materials like chemical and enzymes used in a biotechnology laboratory. Some common methodologies to be followed by one and all include proper labelling of all chemicals so that even a person who is new to the system would find it easy. There should be a Material Safety Data Sheets (MSDS) in file for each chemicals and enzymes available in the laboratory. In many countries MSDS is a legally required technical document which is normally being provided by the suppliers.

Specific parameters to be included in the MSDS sheet are as follows

- ✓ Identity of the chemical along with contact information's of manufacturer.
- ✓ The identity of hazard ingredients
- ✓ Physical and Chemical characteristic of the product.
- ✓ Nature of reactivity –This is vital information to determine the place of storage
- ✓ Examples- The acids and bases reacts each other and should not be stored in adjacent places). Certain other chemicals with chances to react with water should be stored with desiccants.

- ✓ Health related details- include the toxicological properties like carcinogenicity, lethal dose (LD50).
- ✓ Precautions and how to handle the chemical safely.
- ✓ Details regarding the personal protective equipment (PPE) suggested wearing.

Safety precautions to handle hazardous chemicals.

One should be well aware about the general route through which a toxic chemical can enter into the body. There exist primarily four routes. Skin and eye –avoid with the use of lab coats, gloves and goggles.

- ✓ Ingestion - keep away the habit of eating and drinking inside the laboratory and also avoid leaving the lab with gloves and without washing hands.
- ✓ Inhalation - Use masks and fume hoods.
- ✓ Injection- Ensure the Proper disposal of all broken glasses and needles.
- ✓ Always try to remove gloves while using phones and touching light switches etc.

List of common hazardous chemicals in a biotechnology Lab

Formaldehyde (carcinogen).
Acrylamide (Neurotoxins).
Acetonitrile (Nephrotoxins).
Ethidium Bromide (mutagens).
Formamide (Teratogens).
Chloroform (Hepatotoxins).
Phenol, strong acids and bases (Corrosives).

Handling Biological hazards

As all well aware, biotechnology laboratories are routinely doing experiments with many biological organisms and few of them are harmful to the user as well as to the environment. Bacteria, fungi and viruses isolated in a biotechnological laboratory include both harmless and harmful microorganisms and many of its toxicological and pathogenic properties are unknown to us. Hence as a general rule, extreme precautions are to be followed while handling microorganisms. Stringent protocols have to be designed especially in Indian Scenario as we do not have generalised pattern in connection with the microbial safety to be followed by all laboratories. The recombinant DNA molecules produced in biotechnology laboratories are to be considered as a biohazard as it could bring about dangerous changes in the host. Same is the case with animal and tissue cultured organisms as they can make an impact in the environment .General precautions such as wearing PPE, washing hands, wearing gloves etc.. described above for chemicals are to be followed for handling live organisms as well. One has to be more careful and must avoid touching face wearing glovesand keep away all personal belongings in the designated area. Following are the essential precautions to be followed while dealing with live organisms.

- ✓ Avoid mouth pipetting and use mechanical devices.

- ✓ Splashes and aerosol generation is to be kept to minimum as majority of the incidents of infection is through inhalation.
- ✓ Always try to ensure the shaking or mixing in closed containers to avoid the release of aerosols.
- ✓ Disinfect the glassware and workbench after each work session.
- ✓ Specific microbial disinfectants like formalin are to be used along with common bleaching powder or ethanol.
- ✓ Use laminar flow hood safety cabinet.

Handling of Radioactive materials

It is quite common nowadays to use radioactive isotopes in biotechnology laboratories working with protocols in genomics and proteomics though non isotopic labelling methods are coming up for labelling experiments.. Radioactive safety measures are generally followed by all the laboratories owing to awareness of health hazard associated.Laboratory must have the services of a trained person in radioactive safety as a mandatory requirement. Specific precautions to be practiced while dealing with radioactive materials are as follows.

- ✓ Earmark certain specific area displayed with radioactive safety symbols to carry out radioactive labelling experiments.
- ✓ Always stick to the guidelines prescribed by the suppliers (BARC) in handling the material.
- ✓ Work behind radioactive shields.
- ✓ Radioactive detectors are to be used to ensure the area is free from radiations after each work session.
- ✓ The wastes including the tissue papers are to be stored separately for disposal.

Disposal of biological, chemicaland radioactive waste

One of the most sensitive and complex area with lots of challenges involved is the perfect and speedy disposal of wastes generated from the biotechnology laboratory. Methodologies to be followed depend on the characteristic feature of the waste to be disposed. Chemicals wastes have to be neutralised before disposal through proper treatment. Buffers, acids and reagents have to be properly diluted before disposal. The real challenge in a biotechnology laboratory is the management of the disposal of biological wastes. Wastes from microbial cultures and other biological samples have to be collected in autoclave bags made of high melting point plasticso that they can be killed at high temperature and pressure before disposal.Students have to brief about the proper waste management and disposal before starting each session which involve lengthy experiments like tissue culture and cell line maintenance. Radioactive waste materials including the laboratory wares and filter papers used have to be collected in separate containers following the guidelines. Usually the radioactive wastes are disposed by separate department like

health physics department upon receiving the same from laboratories.

Summary of Good Laboratory Practices

In order to solve the problemsfaced by a researcher, he/she should be aware about the role of the reagent in isolating macromolecules having different physical and chemical properties.You should be able to anticipate the molecular interaction between different buffers while using simultaneously.

Quality of the Reagents

Always try to use good quality enzymes and reagents in molecular biology and even if substitute go for a higher grade than a lower grade.

Quality of the Water

Try to ensure that you are using good quality water suiting to the requirements in molecular biology.Water prepared through reverse osmosis is the best quality preferred nowadays over distilled and deionized.

Storage life

Try to dispose buffers and other reagents beyond storage life and always check the microbial contamination within the stored reagents and buffers. Media and other buffers should be chemically as well as microbiologically sterile to avoid contamination and precipitation.

HANDLING OF EMERGENCY

Physical, chemical and biological agents which can cause causalities are being handled in a

modern biological laboratory. Incidents of fire, explosion, chemical spills and transmission of infectious aerosols causing injuries and discomforts are the probable causes of accidents in a laboratory. Laboratories should be equipped with fire extinguishers, safety showers, first aid kits, emergency shut off system attached to instruments and appropriate spill control kits. Periodical trainings and demonstrations are to be carried out to ensure the skill of laboratory personnel in handling and operating the devices during emergencies. Handling of equipment such as autoclave used for sterilization and safe disposal of biological cultures should be carefully handled.

SUGESTED READING
Molecular Biology Problem Solver, edited by Alan S. Gerstein
ISBN 0-471-37972-7

Principles of Isolation, Purification and Analysis of Nucleic Acids

M .P. Paulton
Marine Biotechnology Division, Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Advanced Biotechnological research is largely depended on the genome analysis and recombinant DNA technology. Good quality nucleic acid is an essential prerequisite for consistent results in most of the downstream applications in the genome analysis and recombinant DNA technology. The general principle underlying the isolation of nucleic acids is common with few modifications depending on the type of nucleic acids being isolated. The type of the nucleic acid intending to isolate is to be made free from the other biological macromolecules and cell debris .This is achieved by properly lysing the cell wall or cell membrane as the case may be and by selectively denaturing the other macromolecules like proteins. Nucleic acids thus recovered in its native form is to be purified by removing the very closely associated molecules. The finely purified molecule is precipitated by alcohol and suspended in sterile buffer or distilled water. Finally, the qualitative integrity of the isolated nucleic acids is to be checked by agarose gel electrophoresis and ethidium bromide staining before proceeding with the further downstream applications. Quantitative estimation of nucleic acids are carried out by spectrophotometric and fluorimetric methods.

The types of nucleic acids usually isolated on a routine basis are:

Total genomic DNA
Total RNA
Plasmid DNA & Mitochondrial DNA

A) TOTAL GENOMIC DNA

Breaking of the bacterial and plant cell walls as well as solubilizing the cell membrane of animal tissue are to be carefully carried out under optimum conditions. Even the rapid stirring of solution can break high molecular weight DNA into shorter fragments. Vigorous shaking will cause nicks and even cut open the covalently closed circular structures of plasmid and mitochondrial DNA. If physical disruption is necessary as is the case with certain types of tissues, it should be kept to the minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Ultra sonic sounds are used to disrupt the tough cell wall of certain bacteria. Care has to be taken to prevent degradation of DNA by deoxyribonucleases. These enzymes are found in most cells, and may also be present in dust which could contaminate laboratory glass wares. Hence all the glass wares, plastic wares and the homogenizing buffer are to made sterile by autoclaving. This enzyme activity can be inhibited by using EDTA in buffers which

will chelate the Mg++ ions needed for DNase activity. Cell disruption and most of the subsequent steps should be performed at 4˚C. The cell wall could be lysed enzymatically as well. The bacterial cell wall is usually lysed by the enzyme Lysozyme. The cell membranes on the other hand are solubilised by including suitable detergent in the homogenizing buffer. Upon lysis the nucleic acids will be released into the cytoplasm and now the target molecule, DNA, is to be made free from RNA and other associated proteins. The RNA molecules can be selectively denatured by enzymatic treatment with RNase. Prior to its use, the RNase is to be heat treated to inactivate any DNase contaminants. RNase is relatively stable to heat as a result of its disulphide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by enzymatic treatment with proteinase K followed by shaking with water saturated phenol or with phenol-chloroform mixture, either of which will denature proteins but not nucleic acids. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. It is advisable to use cut micro tips while proceeding through these steps. The aqueous solution is recovered and deproteinised repeatedly until no material is seen at the interface. Finally the deproteinised DNA preparation is mixed with two volumes of absolute ethanol, and allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA for protection against DNases, and this solution can be stored at 4˚C for at least a month. DNA solutions can be stored frozen, but repeated freezing and thawing tends to damage long molecules by shearing and hence the DNA preparations in frequent use are normally stored at 4˚C.

B) PLASMID & MITOCHONDRIAL DNA

The principle of isolation of plasmid and mitochondrial DNA is based on the structural characteristics. Plasmids are double stranded, Covalently Closed Circular (CCC) or super coiled structures. Similarly mt.DNA is also having the same structural characteristics and hence almost the same isolation procedure can be adapted. Bacterial cell wall is to be broken by enzymatic treatment (lysozyme) in a suitable buffer with a suitable metallic chelator like EDTA before initiating the isolation process. The tissue for the mt.DNA isolation is to be thoroughly homogenized under ice cold conditions.

The classical method is to isolate the plasmid and mitochondrial DNA by Caesium chloride density gradient ultra-centrifugation in the presence of ethidium bromide. Ethidium bromide causes unwinding of DNA as it binds to it, simultaneously producing a decrease in its buoyant density .Since the super coiled plasmid and mt.DNA can unwind to only a very limited extent, it will not bind as much ethidium bromide as with the linear and open circle forms of DNA in the presence of saturating levels of ethidium bromide. Because of this density

difference, plasmid and mt.DNA can be separated from other DNA by ultra-centrifugation.

Another method which is relatively fast is based on alkaline lysis. In this method the property of super coiled DNA to remain intact at pH between 12 and 12.5 is exploited for the isolation. At this pH selective denaturation of linear DNA will occur whereas the super coiled DNA will remain intact. Further reduction of the pH to acidic condition will enhance the formation of a complex network of proteins and linear DNA and the resultant supernatant after centrifugation will contain the intact plasmid or mt.DNA. This can be purified and precipitated as in DNA isolation procedures. For mitochondrial DNA, this method works well with fresh tissues with minimum nicks.

Mitochondrial DNA can also be isolated by differential centrifugation technique. This involves the selective isolation of the mitochondria which is further lysed with suitable detergents to release the mt.DNA. This will be further purified and precipitated by conventional means.

C) RNA

RNA molecules are relatively short, and therefore less affected by shearing. RNA is, however very vulnerable to digestion by RNases which are present abundantly even on fingers. These enzymes are stable and generally require no co-factors. Hence gloves should be worn, and a strong detergent should be included in the isolation medium to denature any RNases immediately. The solutions used are to be treated with nuclease inhibitors like Diethyl pyrocarbonate (DEPC).Care should be taken while using DEPC as it is a suspected carcinogen. Glass wares should be baked at 300˚C for 4 to 5 hours as autoclaving alone may not be sufficient to fully inactivate RNases. The plastic ware can be rinsed with chloroform. Tissue homogenization is to be carried out under ice cold conditions with all the precautions detailed above. As in the case of DNA, RNA is to be made free from DNA and proteins. Proteins are denatured by proteinase K treatment followed by phenol chloroform extraction .This is followed by the ethanol precipitation of RNA in the presence of sodium acetate or sodium chloride. The overnight precipitated pellet is washed with 70% ethanol to remove the salts and finally dissolved in DEPC treated water. Contaminating DNA can be removed by treatment with RNases free DNase. The RNase can be inactivated by RNAsin or vanadylribonucleoside complex.

COMMERCIALLY AVAILABLE KITS

Several readymade kits are available commercially and many laboratories are depending on such products for the isolation of nucleic acids. In most of these kits, the nucleic acids are either trapped by ultra-filtration membranes or allowed to bind with certain resins which have affinity towards nucleic acids. The advantage with these kits is that the process is very fast and devoid of using corrosive organic chemicals like phenol. The main disadvantage is that they are quite expensive and hence unaffordable to many laboratories. Hence it is advisable to use alternative non organic protocols, for

DNA isolation, based on the use of high concentration of salts for removing proteins in place of phenol, which are easy to perform in the laboratories especially while isolating from liquid connective tissues like blood, haemolypmph etc. Meanwhile, the commercial kits are effectively used for the isolation of total RNA and mRNA as the manual isolation is a sensitive process with increased chances of degradation.

QUANTITATIVE ESTIMATION OF NUCLEIC ACIDS

DNA and RNA can be spectrophotometrically estimated by taking optical density (OD) at 260nm, 1 O.D corresponds to 50 micro gram of DNA and 40 micro gram of RNA. Purity of the DNA can also checked spectrophotometrically by taking O.D at 260 &280nms. The ratio of 260 and 280 will result a value of 1.8 with pure nucleic acid preparations.

SUGGESTED READINGS

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning. Cold Spring Harbor, New York.

Old, R.W. and Primrose, S.B. (1985). Principles of Gene manipulation, 3rd edition. Blackwell Scientific Publications, Oxford.

Walker, J. M. and Gaastra, W. (Eds) (1983). Techniques in Molecular biology. Croom Helm, London.

Enzymes used in the Manipulations of DNA

Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Gene/DNA is the heart of molecular genetic techniques. A gene/DNA must be isolated and characterised before it can be used in genetic engineering, expressed to obtained proteins or enzymes. The molecular genetic techniques for isolation, amplification, preparation of recombinant DNA molecule and expression uses many enzymes as tools. They are originally identified and isolated from different bacterial strains and now available commercially as highly optimised and purified recombinant proteins/enzymes. Based on their functions, they can be grouped into 5 classes: a) Nucleases – which cut or degrades DNA molecules b) Polymerases – which copy or synthesise new strands of DNA c) Ligases – which join pieces of DNA fragments together d) Modifying enzymes – which modify the DNA by adding or removing chemical groups attached to it, and e) Topoisomerase – remove or introduce supercoils in circular DNA. Nucleases /Restriction enzymes are known as molecular scissor and DNA ligase as molecular glue because it allows the creation and manipulation of DNA sequences from different sources. DNA fragments from different sources with complimentary sticky or blunt ends, generated by Nucleases/Restriction enzyme can be combined to create new molecules (recombinant DNA).

NUCLEASES

Nucleases enzyme cut or digest the DNA molecule by breaking sugar-phosphate backbone (phosphodiester bond) of DNA molecule. Generaly nucleases are grouped into two types a) Exonucleases and b) Endonucleases. Exonucleases cleave the phosphodiester bond present at the ends of the DNA molecule where as endonucleases cleave the bonds present within DNA molecule.

Exonuclease III and VII (Exo III, Exo VII) digest dsDNA and ssDNA respectively. There are two types of Endonucleases a) which cleave DNA at nonspecific cleavage sites like DNAase I – digest dsDNA (originaly isolated from bovine pancrease) and mung bean nuclease – digest ssDNA (isolated from sprouts of mung bean) b) Restriction endonucleases (RE) - Cleave DNA at sites with specific sequences within the DNA molecule. They generate both blunt end and sticky end after the clevage. Restriction endonucleases are widely known as molecular scissors

POLYMERASES

Polymerases are enzymes that can synthesize new nucleic acid strand which is complimentary to an existing DNA/RNA strand. There are mainly three type of polymerases normally used in molecular genetic techniques. a) DNA polymerase I,

b) Klenow fragment and c) Reverse transcriptase.

DNA polymerase I

DNA polymerase adds free nucleotides to the 3’ end of the new strand and extends it in 5’ – 3’ direction. It requires a primer/short sequence fragment which attaches to the single stranded DNA/template DNA. The single stranded portion of a partially dsDNA/primer attaching to the single stranded DNA is required to initiate synthesis in 5’ to 3’ by adding free nucleotides to its 3’ end according to the template DNA. It has inherent 3’ to 5’ and 5’ to 3’ exonuclease activity, thus they can remove nucleotides attached to the template DNA ahead of the growing DNA strand. It is used mainly in second strand synthesis of cDNA and nick-translation.

Klenow fragment

Klenow fragment is derived from DNA polymerase I and can synthesize DNA strand complementary to the single stranded template DNA region. It is characterised by lack of nuclease activity (3’ to 5’ and 5’ to 3’ exonuclease activity). They are mainly used in DNA sequencing and random priming labeling.

Reverse transcriptase

Reverse transcriptase uses RNA as template and synthesize a DNA strand complementary to it. it is used in cDNA synthesis (synthesizing cDNA from RNA).

LIGASES

Ligase enzymes are known as biological glue as they catalyze the joining of two large molecules by forming a new chemical bond between them.

DNA ligases

DNA ligases catalyze the ligation of the 5’-phosphate nucleic acid donor to a 3'-hydroxyl 3’-OH termini nucleic acid acceptor in a duplex DNA, through the formation of a 3´ → 5´ phosphodiester bond. They have the ability to bind to both blunt end and sticky end produced by restriction endonuclease, adaptors, linkers and homopolymer tailing. DNA ligases are naturaly synthesized in cells to repair single stranded nicks (breaks) that occur during DNA replication. In molecular genetic techniques, they are used in DNA cloning to join two individual pieces of DNA.

T4 RNA ligase

Catalyzes the ATP-dependent covalent bonding of single stranded 5’-phosphate nucleic acid donor of DNA or RNA to single-stranded 3’-OH termini nucleic acid acceptor of DNA or RNA.

TOPOISOMERASES

Topoisomerases induce conformation changes in closed circular DNA such as plasmids by adding or removing supercoils.

DNA/RNA MODIFYING ENZYMES

They modify the DNA and RNA by either adding or removing chemical groups attached to it. The most widely used DNA and RNA modifying Enzymes are: a). Alkaline phosphatase, which removes a phosphate group from the 5’ end of the DNA. It iswidely used to prevent vector re-ligation during cloning. b). Polynucleotide kinase, it reverse the alkaline phosphatase activity by adding a phosphate group (phosphorylation) to the 5’-terminus of DNA. c). Poly Nucleotide Kinase, it is extracted from *E. coli* which is infected with bacteriophage. Poly Nucleotide Kinase has the power to add phosphate group in the 5’ end of the DNA molecule. d). Terminal deoxy-nucleotidyle transferase, has the capacity to add one or more number of nucleotide to the in 3’ end of DNA molecule. This is initialy extracted from calf thymous tissue.

All these enzymes such as endonucleases, ligases, topoisomerases, DNA or RNA polymerases are used in molecular genetic techniques, like gene identification and charecterization, gene manipulation and genetic engineering, DNA sequencing and fingerprinting. Without these enzymes we cannot use molecular genetic techniques to produce resources (plants, animals or others) with improved quality or quantity, for DNA based diagnosis and taxonomy, as a tool for conservation and management of resources.

Polymerase Chain Reaction - PCR

M. P. Paulton
Marine Biotechnology Division, ICAR - Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The concept of PCR was first conceived by Dr. Kerry Mullis in 1983, first reported in1985, while working at the Cetus Corporation in Emeryville, CA, along with other researchers at Cetus Corporation (Molecular Station, 2006). Polymerase Chain Reactionor PCRis a molecular technique which allows *in vitro* synthesis of billions of copies of a target DNA fragment within hours using a simple enzymatic reaction. This is achieved by using a pair oligonucleotide primers that hybridize (anneal) to the complementary sequences on the opposite strands of the target DNA, at positions flanking the region to be amplified. New strands are made through the simultaneous extension of both the primers by addition of nucleotides to the primers by the enzyme DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the enzyme DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR can yield about a million-fold amplification. The method is simple, as the PCR can be

performed in a single tube. It can be performed on relatively crude DNA containing samples. These factors have made the PCR an attractive method for amplification of specific sequences. This method is extremely rapid; it takes only 3 hours to amplify a known sequence of interest.PCR generates sufficient copy numbers of target DNA sequences for their routine visualization through standard procedures such as electrophoresis followed by staining with ethidium bromide. The PCR products may be sequenced to determine the exact sequence of the nucleotides within the amplified product. As a result, the PCRpermits routine analysis of DNA from single egg and larvae, and from non-invasively secured tissues such as fin clips and scales. Even partially degraded DNA from poorly preserved sources can be analyzed if sufficiently small PCR products are identified.

Materials and reagents for PCR

The components required for the PCR are the template (the DNA to be amplified), a pair of primers, thermostablepolymerase, the four types of de-oxynucleotide

triphosphates (dATP, dCTP, dGTP and dTTP) and appropriate reaction buffer containing magnesium ions (KCl, Tris-HCl (pH 8.4), MgCl2 and gelatin). They are assembled in a tube and the amplification reaction is carried out by manipulating the temperature within the reaction tube, in cyclic manner, using a thermal cycler. For any given pair of primers, the optimal concentrations of all the above ingredients and parameters have to be standardized. Even though there is no single set of conditions and concentrations that will be optimal for all reactions, the parameters outlined below defines a common starting point from where modifications can be attempted.

Primers: Primers are the most important components of PCR, and the success of a PCRlargely depends on the primers. Primers are short, single stranded DNA molecules which will bind (anneal) to either ends of region to be amplified (one on each strand) and serve as the starting point for attaching nucleotides on its 3' end, leading to the building of a new complementary nucleic acid strand. Primers are generally made in pairs, called "forward" and "reverse".

The Reaction Buffer: The PCR buffer contains KCl, TrisHCl (pH 8.4), MgCl2and gelatin.The components of PCR buffer, particularly the concentration of MgCl2 have a profound effect on the specificity and yield of an amplification product. Success of PCR is dependent on MgCl2 concentration in the reaction to a great extent. Mg2+ ions form a soluble complex with dNTPs which is essential for dNTP incorporation, stimulate polymerase activity and increase the Tm (melting temperature) of primer / template interaction (i.e. it serves to stabilize the duplex interaction). Concentration of about 1.0 to1.5 mM is usually optimal (when 200uM each of dNTPs are used). Excess of Mg 2+ willresult in the accumulation of non-specific amplification products and insufficient Mg2+ will reduce the yield. Optimization by titration of MgCl2 concentration is recommended to establish an optimum concentration for a particular reaction.

Thermal Cycles for PCR: Amplification of a target DNA is achieved by repeated cyclesof denaturation, primer annealing and extension. These events are controlled by manipulation of temperature. The above three major steps in a PCR are repeated for 35 to 40 cycles. This is done using an automated thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Detection and analysis of PCR product: The PCR product will be DNA fragments(amplicons) of defined length. The simplest way to check the PCR product is to load a portion of it into an agarose gel containing ethidium bromide along with molecular weight markers and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator. Hybridizing the PCR product with suitable DNA probe is also in practice for conformation.

Types / Variants of PCR

PCR has been adapted to fit many different applications and to achieve amplification of other molecules of interest like RNA. Hence, there are many different types and each one is unique to the application for which it is designed. The common types of PCR are: conventional/basic PCR, multiplex PCR, reverse transcription (RT)-PCR, nested PCR, real-time PCR and random primed PCR. And there are many other types for specific purposes.

Conventional/ Basic PCR: Conventional PCR uses a thermostable DNApolymerase to amplify a region of the DNA defined at each end by a specific primer. The exponential replication of the same target sequence produces enough DNA product or amplicons for use in subsequent analyses. PCR typically consists of three basic steps, as mentioned earlier.

Multiplex PCR: Multiplex PCR is a modification of conventional PCR in which twoor more different PCR products are amplified simultaneously within the same reaction. This type of PCR consists of the same steps as conventional PCR, except that multiple sets of primers are used, each one priming a PCR product. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. The advantage is that it requires less time and effort in amplifying multiple target templates or regions than individual reactions and may be a useful screening assay. However, significant optimization is required to obtain all of the products with equal efficiency and sensitivity. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Since its first description in 1988 (Chamberlain *et al*., 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription PCR.

Nested PCR: Nested PCR is a very specific PCR amplification and is a variation of theconventional PCR, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first PCR utilizes a pair of primes flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene, which was amplified in the first PCR. The fragment produced by the first reaction is used as the template for the second PCR. The second set of primers called nested primers (as they lie / are nested within the first fragment) is specific to the DNA sequence found within the initial PCR product. The use of a second amplification step with the "nested" primer set results in a reduced background due to the nested primers' additional specificity to the region. The amount of product produced, which is shorter than the first one, is increased as a result of the second round of amplification. Carrying

out nested PCR can further enhance the reliability ofthe PCR. Therefore, when information on the sequence of specific genes is available, amplification and visualization of that gene using a nested PCR could be carried out for confirmation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): This isbased on the processes of reverse transcription and polymerase chain reaction. RT-PCR is a two-step process. The first step consists of the formation of complementary or copy DNA (cDNA) from RNA (generally mRNA). This is followed by the second step which is a conventional PCR using the cDNA as the template. The first step referred to as the "first strand reaction" uses enzyme reverse transcriptase for the production of the cDNA from the RNA. In the second step, the cDNA sequence is amplified by using primers specific to it. The RT-PCR forms a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing cDNA libraries to be created which contain all sequences of all the genes expressed in a cell. It allows the creation of cDNA constructs for the gene expression studies.

Real Time PCR: Real-time PCR is different from other PCR as it quantifies the initialamount of the template instead of detecting the amount of final amplified product (Freeman *et al*., 1999; Raeymaekers, 2000). Real Time PCR is characterized by the point in time during cycling when amplification of the PCR product of interest is first detected *rather* than the amount of the PCR product which has accumulated at the end point. Real Time PCR does this by using fluorescent dyes such as Sybr Green, or fluorophore-containing DNA probes such as Taq Man which get incorporated into each of the new strand, and monitoring the amount of fluorescence emitted during the PCR. This acts as an indicator of the amount of PCR amplification that occurs during each PCR cycle. Thus, in Real Time PCR machines, one can visually see the progress of the reaction in "real time". Quantification using real-time PCR can be ‘relative or absolute

Tools for Primer Designing

The use of software in biological applications has given a new dimension the field of bioinformatics. There are a numerous web based resources and different programs available for primer designing. There are number of simple standalone programs as well as complex integrated networked versions of the commercial software available. Some important tools are doPrimer, primer 3, web primer, primo pro 3.4, GAP, PCR primer design, the primer generator etc, these all are web- based resources and very essential for molecular biologist. There are different software freely and commercially available for PC installation like PrimerSelect, Primer 3, Primer Premier, NetPrimer, GenomePRIDE 1.0, OLIGO 6, Primer Designer 4, GPRIME, Primer Designer, Primer Premier, Primer Design, Gene Runner, Primer BLAST etc.

In conclusion, the heart of PCR lies in the proper designing of primers. Several basic

parameters including the length of the primer, %GC content and the 3' sequence, Tm value need to be optimized for successful PCR. Certain of these parameters can be easily by hand optimized while others are best done with available primer designing tools. The increasing use of information from the internet and the sequences held in gene databases are practical starting points when designing primers and reaction conditions for the PCR. It is also possible to include more than one set of primers in a PCR for getting desired results.

PCR applications

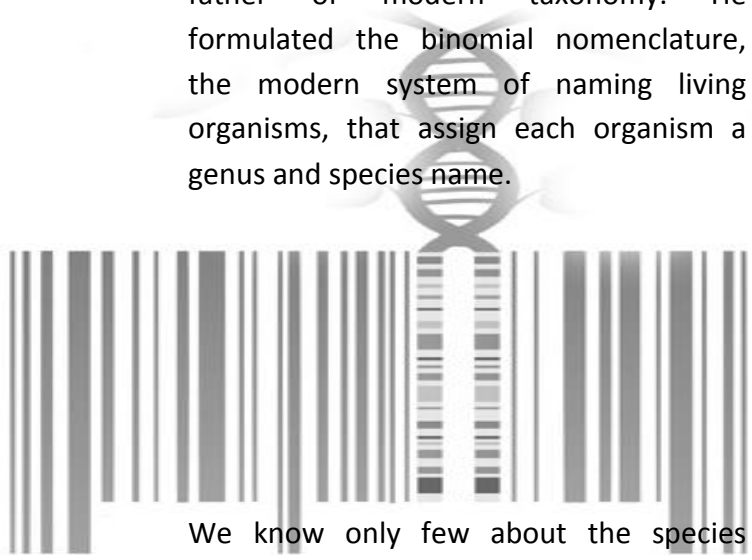
PCR has transformed the way that most studies requiring the manipulation of DNA fragments and DNA cloning may be performed as a result of the simplicity and usefulness of PCR. Cell-free DNA amplification by PCR is able to simplify many of the standard procedures for DNA cloning, DNA analysis, and the modification of DNA. Previous molecular biology techniques for isolating a specific piece of DNA had relied on gene cloning, which is a tedious and slower procedure. An alternative to cloning, PCR, can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. This method of amplifying rare sequencesfrom a mixturehas numerous applications in basic research, human genetics testing and forensics. Some of the PCR applications includesite-specific mutagenesis studies, amplification and detection of DNA*in situ* from cells for rapid diagnosis, genomic subtraction, analysis of protein functions and intermolecular assembly, DNA fingerprinting (RAPD/AFLP/VNTR/) for evaluation of genetic heterogeneity & relationship, paternity verification, forensic application, generation of single chain antibody fragments for immunology, sensitive disease diagnosis, cDNA synthesis from RNA for cDNA library construction, production of clones for sequencing, molecular epidemiology, molecular taxonomy and many more.

DNA Barcoding – Using nucleotide sequence to identify and classify living organisms

Wilson Sebastian, Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

DNA barcoding refers to a taxonomic method involving the use of DNA sequence to identify species based on comparisons with the same DNA/gene sequences in other species. Taxonomy is the science of classifying living things based on their shared characters. Carl Linnaeus, a Swedish botanist is known as father of modern taxonomy. He formulated the binomial nomenclature, the modern system of naming living organisms, that assign each organism a genus and species name.



We know only few about the species diversity of animal, plant and microbes living in the diverse habitat on earth. Less than few millions of the richest species diversity of animals and plants on earth has been identified. Thousands of animals, plants and microbes die out every year, most of them have not yet been identified and the rate of extinction has increased by the effect of global climate change and habitat destruction. Identifying and classifying living organisms is gaining more attention as a basic need for conserving species diversity in the face of accelerating habitat destruction by global climate change and human disturbances.

Oceans have the richest biodiversity on the earth with the presence of diverse and unique habitats. As the most productive ecosystems, marine resources are very important to food security and potential source of many secondary metabolites with economical and medical value. Thus identifying and classifying living things in ocean is very important.

For traditional taxonomy, the species must be carefully collected, handled and preserved without losing its biological features. A trained expert is necessary for the identification of taxonomically relevant anatomical features of the specimen.

The DNA barcoding solved this problem partially, which allow a non-expert to identify species, even from small tissue fragments, damaged, processed and digested materials. Similar to the unique pattern of black and white bars (universal product code-UPC) in consumer products, a DNA barcode is a unique order of nucleotides in a DNA sequence that potentially identify a living organism. The DNA barcodes are usually about 700

nucleotide sequence (base pairs-bp) in length, can be processed easily from thousands of individuals and analysed with computer programs.

OVERVIEW OF EXPERIMENTAL METHODS

I. Collect, document, and identify specimens

Identify specimen.

Document the specimen's necessary details.

Collect tissue sample.

Store in 95%ethanol/-20^{0}c, short-term at 4° c.

II. ISOLATE DNA

Use appropriate DNA isolation method for Animal, Plant, Fungal and Microbial Samples.

Check the quantity and quality of isolated DNA.

Store at -20 °c.

III. AMPLIFY DNA BY PCR

Using appropriate primer pairs.

Store at -20° C.

IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

V. SEQUENCE PCR PRODUCT AND ANALYZE RESULTS

Send sample for sequencing.

Analyze results using bioinformatics tools.

Table 1. The following primers were commonly used in this barcoding purpose in animals, plants and fungi.

Vertebrate (fish) COI gene	
vF2_t1 (forward primer)	5'-CAACCAACCACAAAGACATTGGCAC-3'
FishR2_t1 (reverse primer)	5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'
FishF1 (forward primer)	5’TCAACCAACCACAAAGACATTGGCAC3’
FishR1 (forward primer)	5’TAGACTTCTGGGTGGCCAAAGAATCA3’
FishF2 (reverse primer)	5’TCGACTAATCATAAAGATATCGGCAC3’
FishR2 (reverse primer)	5’ACTTCAGGGTGACCGAAGAATCAGAA3’
vertebrate (non-fish) COI gene	
vF1_t1 (forward primer)	5'-TCTCAACCAACCACAAAGACATTGG-3'
vR1d_t1 (reverse primer)	5'-TAGACTTCTGGGTGGCCRAARAAYCA-3'
Invertebrate COI gene	
LCO1490_F (forward primer)	5’-GGTCAACAAATCATAAAGATATTGG-3’
HC02198_R (reverse primer)	5’-TAAACTTCAGGGTGACCAAAAAATCA-3’
Plant rbcL gene	
rbcLa f (forward primer)	5’- ATGTCACCACAAACAGAGACTAAAGC-3’
rbcLa rev (reverse primer)	5’- GTAAAATCAAGTCCACCRCG-3’
Plant matK gene	
matk-3F (forward primer)	5’- CGTACAGTACTTTTGTGTTTACGAG-3’
matk-1R (reverse primer)	5’- ACCCAGTCCATCTGGAAATCTTGGTTC-3’
Plant ITS region	
nrITS2-S2F (forward primer)	5’- ATGCGATACTTGGTGTGAAT-3’
nrITS2-S3R (reverse primer)	5’- GACGCTTCTCCAGACTACAAT-3’
Plant tufA gene	
tufA_F (forward primer)	5’- TGAAACAGAAmAwCGTCATTATGC-3’
tufA_R (reverse primer)	5’- CCTTCNCGAATmGCRAAwCGC-3’
Fungi ITS region	
ITS1 F (forward primer)	5’-TCCGTAGGTGAACCTGCGG-3’
ITS4 R (reverse primer)	5’-TCCTCCGCTTATTGATATGC-3’
Fungi (lichen-specific) ITS region	
ITS1F_(Gad) (forward primer)	5’-CTTGGTCATTTAGAGGAAGTA-3’
ITS4 R (reverse primer)	5’-TCCTCCGCTTATTGATATGC-3’

The international barcode of life was a collaboratory campaign by participants from more than 150 countries with the aim of assessing diversity among plants, fungi and animal groups from different ecosystems like sea, forest and polar regions. The census of marine life has been completed in 2010 with first extensive list of about 20,0000 marine species, among that more than 6000 are new species. It shows that amazing level

of biodiversity exists in our ecosystems with immense potential to harness them.

DNA barcodes have been used to detect presence of banned species in processed food items from marine resources. Many of the sea food items available in our market are mislabeled as expensive species. It is also used to detect bacterial, fungal and viral diseases in marine organisms or as a tool for disease diagnosis. In aquaculture, DNA barcoding can be utilized to screen larvae for bacterial and viral diseases and thus ensure disease free larvae for stocking. Now DNA barcoding is widely used to identify the products seized by customs.

Generally DNA barcoding depend on highly variable regions of genomes. Even though there is some regions, which are widely used by scientific community (known as universal barcode regions), the growing list of genomic region/resources can be used from different taxonomic groups. Mitochondrial and chloroplast genome can be readily amplified by polymerase chain reaction (PCR), even from a fragment of tissue or degraded tissue, because of its abundance (thousands of copies in a cell) in cells. The region of mitochondrial genome used for DNA barcoding including CO1 (cytochrome c oxidase subunit 1), 12s (12s ribosomal RNA), 16s ribosomal RNA (16s rRNA), and Cytochrome b (Cyt B) involved in electron transport systems of respiration. In plants the region of chloroplast genome include rbcL-RuBisco (ribulose 1,5 bis phosphatase) and mat K ( ) are used for barcoding. The genes used in barcoding are associated with key life function so the same could be present in all species. The CO1 and other mitochondrial genesare difficult to amplify, having variability in fungi and lichens and some fungi groups lack mitochondria. In such situations, nuclear internal transcribed spacer (ITS, a variable region surrounded by ribosomal RNA gene) can be used. Like the organelle genes, there are many copies of ITS in a genome and hence this is also widely used for barcoding of small multicellular eukaryotic organisms, fungi, lichen and some plants (difficult to identify with rbcL and matK). DNA barcoding at species level is sometimes difficult with single barcodes, due to identities within and between species. For them, use of more taxa specific and multiple barcode regions can help.

DNA barcodes can be used to identify animals, plants, fungi and microbes or products made from them and infected by bacteria and viruses. The procedures include sample tissue collection, storing the specimen in alcohol/freezer whenever possible and collecting its geographical and local climatic information. A small muscle tissue fragment or leaf disc is suitable for DNA extraction. The DNA is extracted and the barcode region (CO1/rbcL/ITS gene) is amplified and sequenced in both directions. The sequence results are then error corrected, trimmed and used to search DNA/barcode database, the search algorithm identifies the matched sequences of species already in the database. Sometimes the sequences/barcodes will be new, then

identification can be done by performing phylogenetic tree generation by including the barcode of unknown species with other related species. Novel barcode sequences can be submitted to databases like GenBank and BOLD.

DNA barcoding - Steps

Collect specimens under sterile conditions

Use sterile scalpel or scissors to collect a small sample of tissue.

Freeze your sample at -20°C/ in 95% ethanol until you are ready to begin with DNA isolation.

Photograph specimen in its natural environment, or where it was obtained by using a smart phone or digital camera. Take wide, medium, and close-up views. Include a ruler or coin for scale in close-up photos, or place specimen on graph paper.If possible collect longitude and altitude coordinates along with other metadata for each specimen. Record this information along with other metadata on the sample documentation sheet.

Use a field guide or taxonomic key to identify your specimen as accurately as possible: kingdom > phylum > class > order > family > genus > species.

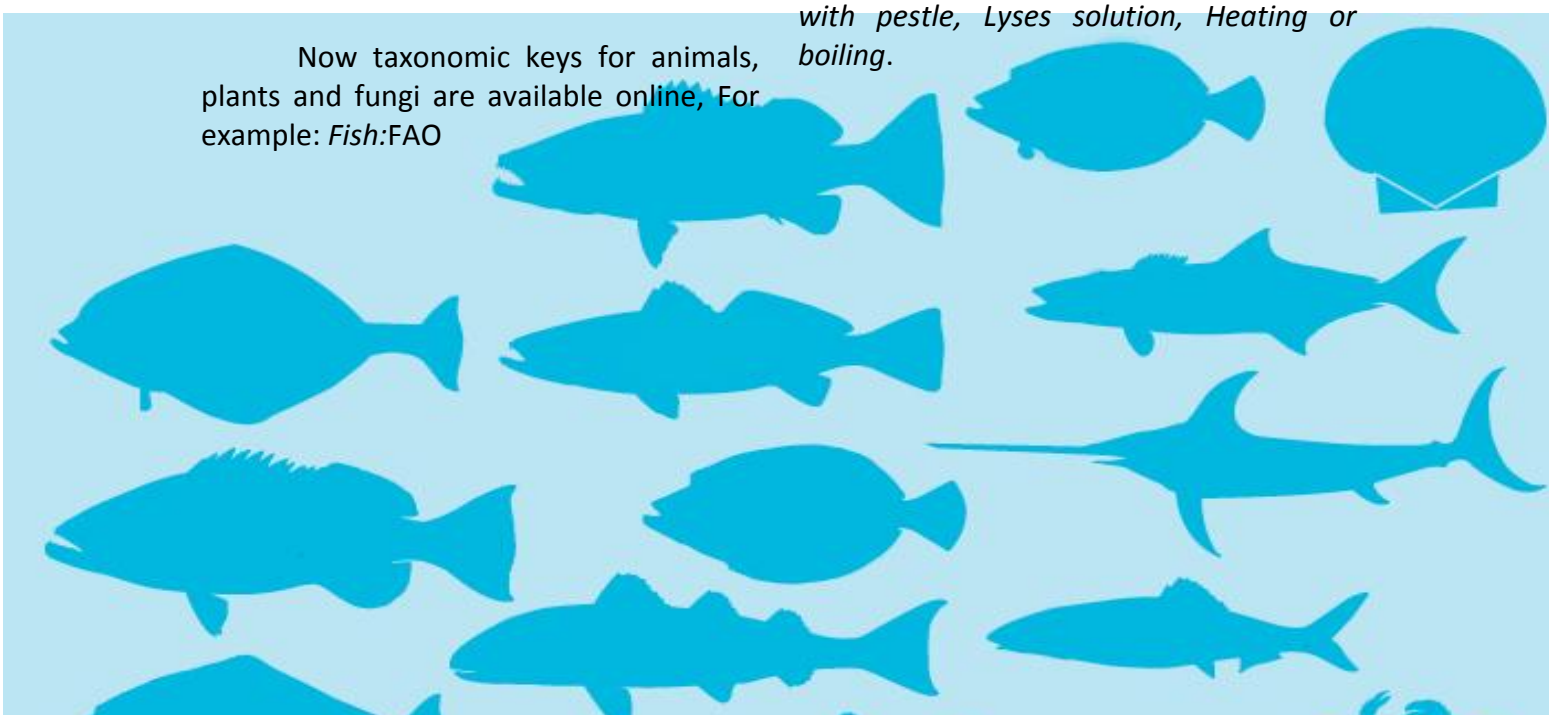
Now taxonomic keys for animals, plants and fungi are available online, For example: *Fish:*FAO *Animals:* Integrated Taxonomic Information System (http://www.itis.gov) *Plants:*Tropicos (http://tropicos.org)

Check to see if your specimen is listed in the GenBank, (www.ncbi.nlm.nih.gov) or Barcode of Life Database, BOLD (www.boldsystems.org)
In GenBank, use search bar by entering genus and species names in it. If the species is listed in the database, the "Taxonomy Browser" will list the number and collection sources other details of specimen.

We can download the public barcode/sequences from various databases as FASTA file or other DNA sequence formats.
The "Taxonomy Browser" in GenBank helps to explore barcode data by taxonomic groups.

To amplify a DNA barcode region, choose the most appropriate set of primer pairs for eachof your samples. The table above (Table 1) lists commonly used primer pairs and the type of target organisms.

The following steps or reagents used in DNA isolation determine the quality of DNA obtained after procedure; *Collecting fresh or dried specimens, Using only a small amount of tissue, Grinding tissue with pestle, Lyses solution, Heating or boiling*.



Genomic DNA isolation using Salting out method - practical guidelines

Lakshmi P. Mukundan, K.A.Sajeela
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

One of the obstacles encountered when extracting DNA from a large number of samples is the cumbersome method of deproteinizing cell digests with the hazardous organic solvents phenol and chloroform. Several other non-toxic extraction procedures have been published, but require either extensive dialysis or the use of filters. Salting out method is one of the simplest of all the published methods which involves salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution. Following this procedure, it takes anywhere from 3 to 4 hrs to isolate DNA for large number of samples with the yield ranging (6 to10µg) good quality of DNA.

REAGENTS REQUIRED

1. Solution 1:

Tris-HCl (pH8.0	- 50 mM
EDTA (pH8.0)	- 20 mM
SDS	- 2 %

Prepare in double distilled water. Autoclave and store at 4^0C

2. Solution 2:
NaCl solution (saturated) - (6 M)
Prepare in double distilled water. Autoclave and store at 4^0C
3. Proteinase K: 20mg/ml
4. Ethanol
5. TE buffer

PROCEDURE

Wash alcohol preserved tissue with TRIS buffer (pH 8.0) by spinning and then follow the steps listed below.

Place the tissue sample in 1.5 ml tube & add 500µl Solution 1.

Homogenize tissue sample with sterile homogeniser or melted filter tip.

Add 5µl of Proteinase K (20 mg/ml)

Incubate at 55^{o}C in water bath for 2 hours (with occasional mixing)

Chill on ice for 10 minutes.

Add 250µl Solution 2 and invert several times for through mixing.

Chill on ice for 5 minutes.

Centrifuge at 8000 rpm for 15 minutes.

Carefully collect clear supernatant (~500 µl) with wide-bore filter tip into a newly labeled 1.5 ml tube.

Add twice the volume (~1 ml) of ice cold 100 % molecular biology grade Ethanol to precipitate the DNA.

Incubate overnight at -20^{0}C.

Next day, centrifug at 11000 rpm for 15 minutes and removed supernatant.

Rinse DNA pellet in 250 µl of ice-cold 70% ethanol.

Centrifuge at 11000 rpm for 5 minutes.

Carefully remove supernatant and partially dry with lid off at room temperature.

Resuspend partially dried DNA in 50-200(depending on size of pellet) of TE buffer (pH-8) by gently pipetting the sample with wide-bore filter tip until dissolved.

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

Genomic DNA isolation using Phenol chloroform method

K.Nisha, Anjaly Jose, Neenu Raj, Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Phenol chloroform method is a liquid – liquid extraction technique in molecular biology used to separate nucleic acids from proteins and lipids. This method involves the serial addition of several chemicals.First; Lysis buffer and SDS are added to the tissue to be lysed. Lysis buffer helps for breaking open cells and contain salts to regulate the acidity and osmolarity of the lysate. SDS can solubilize the proteins and lipids that form the membrane and help to release DNA from histones and other DNA binding proteins by denaturing them. Further addition of Proteinase K helps for the destruction of proteins in cell lysate and for release of DNA. Next phenol chloroform mixture is added to separate the proteins from DNA. Since DNA has net negative charge it is more soluble in the aqueous portion of the organic aqueous mixture. When centrifuged, the unwanted proteins are separated away from the aqueous phase due to its hydrophobic and hydrophilic domains and will be present in the interphase. Finally, isolation of nucleic acid (DNA) accomplished using alcohol precipitation of the collected aqueous portion and centrifugation.

MATERIALS AND REAGENTS REQUIRED

1. Forceps
2. Centrifuge
3. Vials
4. Micropipette and Pipette tips
5. Water bath

1. Lysis buffer
   - -NaCl - 23.36g (400mM)
   - -Tris - 1.2114g (10mM)
   - -EDTA - 0.37224g (1mM)
   - -DW - 1000ml
2. SDS
3. Proteinase K (10mg/ml)
4. Phenol Chloroform Isoamyl Alcohol (25:24:1)
5. Chloroform Isoamyl Alcohol (24:1)
6. Isopropanol
7. 70% alcohol
8. TE buffer

PROCEDURE

1. Dissect 10-20 mg of tissue
2. Mince the tissue finely with razor blade
3. Transfer the minced tissue to a microfuge tube
4. Add 400µL of Lysis buffer and 100µL of 10% SDS to the tissue
5. Add 10µL of Proteinase K to the lysate to increase the yield of genomic DNA

6. Mix the contents of the tube by vortexing
7. Incubate the solution in water bath for $1\frac{1}{2}$ – *2* hrs at 550C until a clear homogeneous solution is obtained.
8. Add equal volume of Phenol Chloroform Isoamyl Alcohol (25:24:1) and mix the organic and aqueous phase by vortexing.
9. Centrifuge the tubes at 10,000 rpm for 10min at 40C.
10. Centrifugation of the mixture yield two phases, upper aqueous phase and lower organic phase
11. Use a wide bore pipette to transfer upper aqueous to a fresh centrifuge tube without disturbing protein interphase.
12. Add equal volume of Chloroform Isoamyl Alcohol(24:1) and mix it thoroughly by vortexing
Since chloroform has higher density and is immiscible with phenol, it prevents retention of phenol in aqueous phase and thus improves DNA yield
13. Centrifuge the tube at 10,000 rpm for 10min at 40C
14. Transfer the upper aqueous phase to a fresh centrifuge tube
15. Add equal volume of chilled isopropanol to increase the precipitation rate of DNA.
16. Mix the solution well and incubate at -200C for 2hrs
17. Centrifuge the tube for 10min at 10,000rpm at 40C.
18. Remove the supernatant, Wash the DNA precipitate with 70% alcohol(500µL)
19. Collect the DNA by centrifugation at 10,000 rpm for 5-10min at 40C
20. Remove the supernatant and allow the pellet to dry in the air for 15min.
21. Redissolve the pellet in 50µL of 1X TE buffer
22. Store the DNA solution at 40C.

NB: Wear gloves while handling the reagents and make sure that the vials and tips are DNAase and RNAase free

Genomic DNA isolation using Qiagen DNeasy Blood and Tissue kits

Introduction

DNeasy Blood & Tissue Kits are designed for rapid purification of total DNA (e.g., genomic, mitochondrial, and pathogen) from a variety of sample sources including fresh or frozen animal tissues and cells, blood, or bacteria. DNeasy purified DNA is free of contaminants and enzyme inhibitors and is highly suited for PCR, Southern blotting, RAPD, AFLP, and RFLP applications.

Purification requires no phenol or chloroform extraction or alcohol precipitation, and involves minimal handling. This makes DNeasy Blood & Tissue Kits highly suited for simultaneous processing of multiple samples. For higher-throughput applications, the DNeasy 96 Blood & Tissue Kit enables simultaneous processing of 96 or 192 samples.

The buffer system is optimized to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. After lysis, the DNeasy Blood & Tissue spin-column procedure can be completed in as little as 20 minutes. Using the DNeasy 96 Blood & Tissue Kit, 96 or 192 samples can be processed in just 1 hour after lysis.

Simple centrifugation processing completely removes contaminants and enzyme inhibitors such as proteins and divalent cations, and allows simultaneous processing of multiple samples in parallel. In addition, DNeasy Blood & Tissue procedures are suitable for a wide range of sample sizes.

Purified DNA is eluted in low-salt buffer or water, ready for use in downstream applications. DNeasy purified DNA typically has an A260/A280 ratio between 1.7 and 1.9, and is up to 50 kb in size, with fragments of 30 kb predominating. The DNeasy procedure also efficiently recovers DNA fragments as small as 100 bp.

Procedure

DNeasy Blood & Tissue procedures are simple. Samples are first lysed using proteinase K.* Buffering conditions are adjusted to provide optimal DNA binding conditions and the lysate is loaded onto the DNeasy Mini spin column or the DNeasy 96 plate. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water or buffer, ready for use. DNeasy purified DNA has A260/A280 ratios of 1.7–1.9, and absorbance scans show a symmetric peak at 260 nm confirming high purity.

The DNeasy membrane combines the binding properties of a silica-based membrane with simple microspin technology or with the QIAGEN 96-Well-Plate Centrifugation System. DNA adsorbs to the DNeasy membrane in the presence of high concentrations of chaotropic salt,

which remove water from hydrated molecules in solution. Buffer conditions in DNeasy Blood & Tissue procedures are designed to enable specific adsorption of DNA to the silica membrane and optimal removal of contaminants and enzyme inhibitors.

Cut tissue into small pieces and place in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 560C until completely lysed. Vortex occassionally during incubation. Vortex 15s directly before proceeding to step 2.

Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 560C for 10 min.

Add 200 µl ethanol(96-100%). Mix thoroughly by vortexing.

Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g(8000 rpm) for 1 min. Discard the flow through and collection tube.

1.

Place the spin column in a new 2 ml collection tube. Add 500 µl buffer AW1. Centrifuge for 1min at ≥6000 x g. Discard the flow through and collection tube.

Place the spin column in a new 2 ml collection tube, add 500 µl buffer AW2 and centrifuge for 3 min at 20,000 x g(14,000 rpm). Discard the flow through and collection tube.

Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

Elute the DNA by adding 200 µl buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature(15-250C). Centrifuge for 1 min at ≥6000 x g.

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

Genomic DNA isolation using XpressDNA Tissue/Cell line Mini Kit

Introduction

The XpressDNA Tissue/Cell line Mini Kit allows rapid and efficient purification of genomic DNA from both fresh and frozen tissues (animal or human) and cell lines. The genomic DNA is extracted using the XpressDNA magnetic nanoparticles-based technology. This approach facilitates easy and inexpensive DNA isolation and avoids time consuming steps like centrifugation and column separation. The extracted DNA is suitable for use in downstream applications including PCR, restriction enzyme digestion, etc.

The XpressDNA Technology

The XpressDNA magnetic nanoparticles-based technology works with a swappable charged solid surface which facilitates nucleic acid purification. Under suitable buffering 5 conditions, the magnetic nanoparticles acquire a positive charge enabling the negatively charged DNA molecules to bind to them leaving the contaminants in the solution.

General Information:

Handling of MagNa Mix

- Store the MagNa mix at room temperature
- Before use mix the MagNa mix on vortex mixer
- When added to the sample mix by inverting the tube. Try to avoid bubbles while mixing
- Donot over dry the the MagNa mix after ethanol wash.
- Do not freeze the MagnaMix.
- Do not reuse the MagnaMix

MagnaStand

The MagnaStand consists of a base station and a tube holder moulded together. The tube holder accommodates 12 microcentrifuge tubes. The base of the MagnaStand contains six rare-earth magnets that come in contact with the microcentrifuge tube when in-use.

Wash Buffers (TI3 and TI4) are provided as concentrate. Before the first use, add 96-100% ethanol as indicated on the bottle. Reconstitute the lyophilized Proteinase K powder in Proteinase K Buffer (volume as mentioned on Proteinase K vial) and store at 4 °C after each use.

Procedure

Cut and weigh appropriate amount of fresh tissue and transfer it to a microcentrifuge tube.

For ethanol preserved tissue, mince approximately 50mg of tissue and transfer it in a DNase-free 1.5 ml microcentrifuge tube containing 500 µl of nuclease free water or PBS. Tap mix the contents and centrifuge the tube for 3min at 8000 rpm and discard the supernatant. Repeat the washing step again. Add another 500 µl of

nuclease free water or PBS Incubate for 30min at room temperature to rehydrate the sample. Centrifuge at 8000 rpm for 3 min and discard the supernatant. Proceed to Step 2 for tissue lysis.

Tissue disruption and homogenization can be performed using a sterile blade. Briefly, place the tissue in a sterile petri dish and cut the tissue into small pieces using a sterile surgical blade to increase the efficiency of tissue lysis. Transfer the minced tissue into a DNase-free 1.5 ml microcentrifuge tube. Proceed to step 3.

1. Add 750µl Lysis Buffer to all the tubes.

2. Add 20µl of RNase A to the tube and vortex the tube for 30 seconds. Incubate the tube at room temperature for 15 minutes.

3. Add 20µl of Proteinase K to the tube and mix by vortexing the tube for 30 seconds.

4. lncubate the sample for lysis at 560C until the lysate appears clear (Approximately 1 hour). Note: The lysis time varies with the type, storage and amount of tissue used. For soft tissues, lysis would be completed within 1 hour and for hard tissues 2-8 hours/ until lysate clears.

5. After lysis, centrifuge the tubes at 6000 rpm for 5 minutes and transfer the supernatant to fresh tubes.

6. Add 350µl of XpressDNA MagNa Mix to the lysate and mix by inverting the tube 6-8 times. Incubate at room temperature for 5 minutes.

7. Place the tube on the XpressDNA MagnaStand for 1- 2 minutes or until the solution appears clear.

8. Carefully discard the supernatant with the tube on the XpressDNA MagnaStand. Make sure that the pellet is not disturbed.

9. Add 250µl of XpressDNA Wash Buffer I to the tube and remove it from the stand.

10. Resuspend the pellet by pipette mixing for about 5-8 times for complete dispersion of the particles.

11. Place the tubes back on the XpressDNA MagnaStand and keep it for 30seconds or until the solution becomes clear.

12. Carefully discard the supernatant with the tube on the XpressDNA MagnaStand. Make sure that the pellet is not disturbed.

13. Add 500µl of Wash Buffer 2, invert the tube placed on the XpressDNA MagnaStand for about 5-6 times. 14. Discard the supernatant without disturbing the pellet.

14. Discard the supernatant without disturbing the pellet.

15. Repeat steps 15-16.

16. Keep the tubes open on the XpressDNA MagnaStand for 10 minutes for drying. Note: Do not over dry the pellet.

17. After drying, remove the tubes from XpressDNA MagnaStand.

18. Add 50µl of nuclease-free water/ 10mM Tris (pH 8) to the tube and gently pipette mix 5-8 times to resuspend the magnetic nanoparticles.

19. Incubate the tube at 560C for 5 minutes with intermittent tapping.

20. Place the tube on the XpressDNA MagnaStand for 5 minutes or until the solution appears clear.

21. Carefully transfer the supernatant containing the DNA to a sterile 1.5ml microcentrifuge tube, with the tube on the XpressDNA MagnaStand. Make sure that the pellet is not disturbed.

22. Discard the magnetic nanoparticles.

Note: In the elution step, if the magnetic nanoparticles take more than 4-5 minutes forclearing, then spin the tubes at 12,000 rpm for 5 minutes and collect the supernatant for pure DNA. Protocol 2: Extraction of Genomic DNA from cell lines

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

RNA Isolation - Practical aspects

Lakshmi P. Mukundan, Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Ribonucleic acid or RNA, a polymericcompoundfound in all living cells and several viruses, contains a long single-stranded chain of phosphate and ribose sugarand nitrogen bases like adenine, guanine, cytosine, and uracil, bonded to the ribose unit. RNA functions in cellular protein synthesis in all living cells and carry genetic information for many viruses by replacing DNA.

TRIZOL METHOD

The isolation of RNA with high quality is a crucial step essential to carry out various molecular biology experiments. TRIzol Reagent is a ready-to-use reagent used for isolation of RNA from various cells and tissues. It works by maintaining the RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. The partitioning of DNA and RNA between the organic phase and the aqueous phase is determined by the pH of phenol. Due to acidic phenol, RNA is retained in the aqueous phase, whereas DNA is moved into the phenol phase because the phosphate groups on the DNA are more easily neutralized than those in RNA. RNase activity is also minimized by an acidic pH. Chloroform addition, after the centrifugation, separates the solution into aqueous and organic phases. RNA is retained in the aqueous phase.

The aqueous phase is transferred, and the RNA can be recovered by precipitation with isopropyl alcohol. But the DNA and proteins can be recovered by sequential separation after the removal of aqueous phase. DNA can be obtained from the interphase by precipitation with ethanol, and an additional precipitation with isopropyl alcohol requires proteins from the organic phase. Total RNA extracted by TRIzol Reagent is pure and free from the contamination of protein and DNA. This high quality RNA can be used in Northern blot analysis, in vitro translation, poly (A) selection, RNase protection assay, and molecular cloning.

- DEPC-treated water (Ambion)
- TRIzol Reagent (Invitrogen)
- Ice cold PBS
- Cell scraper
- 70% ethanol
- Isopropyl alcohol

PROCEDURE

- Homogenize tissue samples in 1ml of TRIZOL reagent per 50 to 100mg of tissue using a homogenizer. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization.
- Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

- Add 0.2ml of chloroform per 1ml of TRIZOL Reagent. Cap sample tubes securely.
- Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes.
- Centrifuge the samples at no more than 12,000 x g for 15 minutes at 4^0C.
- The mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase.
- Transfer the aqueous phase containing the RNA into a new tube without disturbing the interphase.
- Add 0.5 ml of isopropyl alcohol per 1ml of TRIZOL Reagent used for the initial homogenization.
- Incubate samples at 15 to 30 ^{o}C for 10 minutes and centrifuge at not more than 12,000 x g for 10 minutes at 4 ^{o}C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
- Remove the supernatant completely. Wash the RNA pellet with 75% ethanol, adding at least 1ml of 75% ethanol per 1ml of TRIZOL Reagent used for the initial homogenization. Vortex the sample briefly, then centrifuge at 7,500 x g for 5 minutes 4 ^{o}C.
- Discard the supernatant and air dry the pellet for 5-10 minutes.
- Resuspend the pellet in 20-50µl of DEPC treated water.
- Proceed to downstream applications or store the RNA at -70^0C.

NOTES

Perform RNA isolation immediately after sample collection or quick freeze samples immediately after collection and store at -80^0C or in RNAlater until RNA isolation.

Always use gloves and avoid the contact of reagents with skin or clothing.

RNA isolation using QIAGEN RNeasy® Mini Kit

The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water.

With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides enrichment for mRNA since most RNAs <200nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15-20%of total RNA) are selectively excluded.

Fast procedure delivering high-quality total RNA in minutes

Ready-to-use RNA for high performance in any downstream application

Consistent RNA yields from small amounts of starting material

No phenol/chloroform extraction, no CsCl gradients, no LiCl or ethanol precipitation

PROCEDURE

1.Add 350µl of buffer RLT to less than 20mg of tissue and homogenize the lysate according to step 2a, 2b, or 2c.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with a rotor– stator or QIAshredder homogenizer generally results in higher RNA yields than with a syringe and needle.

2a. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 3.

2b. Homogenize the lysate for 30 s using a rotor–stator homogenizer. Proceed to step

2c. Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 3.

3. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

4. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.

5. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

Discard the flow-through.

6. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

8. **Optional:** Place the RNeasy spin column in a new 2 ml collection tube, and discardthe old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

9. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

10. If the expected RNA yield is >30 µg, repeat step 9 using another 30–50 µl RNasefree water, or using the eluate from step 9 (if high RNA concentration is required). Reuse the collection tube from step 9.

Notes: Perform RNA isolation immediately after sample collection or quick freeze samples immediately after collection and store at -800C or in RNAlater until RNA isolation. Always use gloves and avoid the contact of reagents with skin or clothing. Make sure that the vials and tips are DNAase and RNAase free

SGGESTED READINGS

Simms, Domenica, Paul E. Cizdziel, and Piotr Chomczynski. "TRIzol: A new reagent for optimal single-step isolation of RNA." *Focus* 15.4 (1993): 532-535. RNeasy® MiniHandbok, www.qiagen.com.

Quantitative estimation of nucleic acid

Lakshmi P Mukundan, Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Any experiment requiring manipulation of a nucleic acid most likely also requires its accurate quantitation to ensure optimal and reproducible results. One of the most commonly used practice to quantitate DNA or RNA is the use of spectrophotometric analysis. A spectrophotometer can determine the average concentrations of the nucleic acids (DNA or RNA) present in a mixture, as well as their purity based on absorbance.

UV-Visible spectrophotometer has a monochromator, light source, sample holder and detector. Light from the source are converted to a monochromatic light of particular wavelength and allow it pass through the sample and amount of light that emerges is detected by a detector. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. The resulting effect is that less light will strike the photodetector and this will produce a higher optical density (OD).

Both DNA and RNA exhibit strong absorbance of UV due to the presence of conjugated double bonds of the constant purine and pyramidine bases and these have characteristics of OD (optical density) of absorbance maximum at 260nm(A260) which is linearly related with the concentration of the DNA in the solution up to the OD value of 2 . The spectroscopic method is used to check the purity of DNA. Proteins are the major contaminants in the nucleic acid extracts and these have the maximum absorbance at 280nm.

Absorbance maximum at 260nm (A260)as quantity measurement nucleic acids

The "A260 unit" is used as a quantity measure for nucleic acids. A solution of DNA with a concentration of 50 µg/ml will have an absorbance at 260nm equal to 1.0. The same conversion factors apply, and therefore, in such contexts:

1 A260 unit dsDNA = 50 µg
1 A260 unit ssDNA = 33 µg
1 A260 unit ssRNA = 40 µg

Nucleic acids purity (260:280 / 260:230 Ratios)

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein (since aromatic aminoacids absorbs light at 280nm), phenol or other contaminants that absorb strongly at or near 280 nm.

260/230 ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than

expected, it may indicate the presence of contaminants which absorb at 230 nm (salts).

NANODROP

This system allows scientists to quickly and easily quantify and assess purity of samples such as proteins and nucleic acids. Relevant for virtually every lab that deals with biological samples, NanoDrop instruments are often a favorite in the lab due to their speed, simplicity and robust performance. NanoDrop allows for the analysis of 0.5–2.0µl samples, without the need for cuvettes or capillaries.

QUBIT

The Invitrogen Qubit Fluorometer is the next generation of the popular benchtop fluorometer designed to accurately measure DNA, RNA, and protein quantity. It easily measures RNA integrity and quality. The easy-to-use touch screen menus make it easy to select and run the assays you need, with results displayed in just a few seconds. It is more sensitive than UV absorbance–based quantification, making it ideal for precious samples.

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

Gel Electrophoresis- Practical aspects

Neenu Raj, K.Nisha, Anjaly Jose
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

All molecules carry a certain net charge, and this charge can be used to separate out molecules (DNA, RNA, Proteins). Some have a positive charge when suspended in a solution, making them cations, while others have a negative charge, making them anions. Under the influence of an electrical field, these molecules migrate at a rate that depends on their net charge, size and shape, the field strength, and the nature of the medium in which the molecules are moving.

Using a technique called gel electrophoresis, these molecules can be separated based on their charge and size. Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins such as sequencing, RFLP analysis, marker analysis, DNA fingerprinting and DNA purification.

In biology, electrophoresis uses porous gels as the media. After the sample mixture is loaded into a gel, the electric field is applied, and the molecules migrate through the gel matrix. Thus, molecules separate based on both the molecular sieve effect and on the electrophoretic mobility.

Principle

Electrophoresis is a method used to separate charged molecules from one another based on difference in its charge and size in an applied electric field. Under the influence of electric field,the charged molecules migrate towards oppositely charged electrodes. Those molecules with a positive charge move towards the cathode and negatively charged molecules move towards Anode. The migration takesplace due to charge on the molecules and potential applied across the electrodes. The fundamental driving force of electrophoresis is voltage applied to the system.

TYPES OF ELECTROPHORESIS

Two compounds are dominantly used for gel electrophoresis namely polyacrylamide and agarose. Polyacrylamide, which makes a small-pore gel, is used to separate most proteins, ranging in molecular weight from< 5000 to >200 000, and polynucleotides from<5 bases up to ~2 000 base pairs in size. Polyacrylamide gels are physically tougher than agarose gels. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins, and protein complexes. Agarose gels are relatively fragile and should be handled carefully.

AGAROSE GEL ELECTROPHORESIS (AGE)

Agarose gel electrophoresis is an effective way to separate and analyse DNA. It is also used to separate other charged molecule such as RNA. Agarose is a linear polysaccharide consists of D-galactose and L-galactose purified from seaweed genera*Gelidium* and *Gracilaria.* Agarose gel is used to conduct heat evenly and provide an extra sieving effect. Agarose gels are cast by melting the agarose in the desired buffer until a clear, transparent solution is achieved. Chains of agarose forms helical fibers that aggregate into supercoiled structures which provide sieve like effect. This electrophoresis is used as a diagnostic tool to separate and visualize fragments based on its size. It is based on two key principles: 1) DNA has an overall negative charge (due to the phosphate backbone) and thuswill migrate towards a positive charge. 2) A gel acts as a sort of microscopic sieve; smaller *DNA* fragments will travel through the gel more rapidly than larger fragments. Thus DNA can be separated based on the length (size) of the DNA segment.

Gel concentration

The concentration of agarose used depends upon the product size. The greater the concentration of agarose, the smaller the linear DNA that can be resolved. The greater the agarose concentration, smaller the pores created in the gel matrix and the more difficult for the large linear DNA molecules to move through the matrix. Changing the agarose concentration changes the size of the sieve matrix of the gel. When concentration of agarose is increases then the rate of migration of the DNA fragments decrease, i.e. lower the concentration, faster the DNA migration rate and vice versa. Generally used agarose concentration is 0.8% to separate DNA fragments of range 2- 10 kb and 1.2% agarose for separation of small fragments such as 0.1- 1 kb.

Table 1. Percentage of Agarose recomentod for Optimum Resolution for Linear DNA

Recommended % Agarose	DNA
0.5	1,000–30,000bp
0.7	800–12,000bp
1	500–10,000bp
1.2	400–7,000bp
1.5	200–3,000bp
2	50–2,000bp

Electrophoresis buffers

For electrophoresis, several different buffers can be used. Most commonly used buffers are TAE (Tris-acetate EDTA) and TBE (Tris-borate EDTA).A buffer not only provides ions to support the conductivity, but also establish a pH. Electrophoresis buffers are usually made as concentrated solutions and stored at room temperature. The working concentration of the buffer is 1X and stock is prepared for 10X.

Table 2. 10X TBE

Tris	108g
Boric acid	58g
EDTA	8.5g
Distilled water	1000ml

Table 3. 10X TAE

Tris	48.4 g
Acetic acid	11.4ml
EDTA	3.7 g
Distilled water	1000ml

REAGENTS, SUPPLIES, & EQUIPMENT

1. Agarose
2. 1X TBE Buffer
3. DNA ladder standard
4. Microwave oven
5. Electrophoresis chamber
6. Power supply
7. Gel casting tray and combs
8. DNA Loading dye
9. Gloves
10. Pipette and tips
11. DNASamples/PCR producs

Staining of DNA

Ethidium bromide (EtBr) is the most convenient and commonly used staining dye to visualize DNA in agarose gel. It is a fluorescent dye that intercalates between stacked bases of DNA. As it is florescent it emits light when subjected to UV light. Ethidium bromide is a potent mutagen and become moderately toxic after an acute exposure. Therefore, it is highly recommended to handle with considerable caution. Ethidium bromide is prepared as a stock solution of 10mg/ml in distilled water which is stored in amber coloured bottles or bottles wrapped in aluminium foil. Make 0.5µg/ml working solution according to the volume required.

NB : Ethidium bromide is a potent carcinogen

Gel loading buffer

Gel loading buffer are mixed with the samples before loading into the well. These buffers are used to increase the density of the sample, ensuring that the DNA sinks evenly into the well and they add colour to the sample, which enable tracking the progress of the electrophoresis. It consists of Bromophenol blue and glycerol in 1X TBE buffer.

PROCEDURE

To prepare 0.8% agarose gel, weigh about 0.32g agarose and dissolve in 1X TBE buffer by boiling it in water bath or microwave oven. Melt agarose until it becomes clear.

Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly. When molten gel has cooled, add ethidium bromide to a final volume of 0.5µg/ml. Mix the gel solution thoroughly by gentle swirling.

Seal the ends of the casting tray with two layers of tape.

Place the combs in the gel casting tray.

Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).

Carefully pull out the combs and remove the tape.

Place the gel in the electrophoresis chamber. Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Mix the sample of DNA with desired gel loading buffer.

Slowly load the sample mixture into the well using a disposable micropipette. Load DNA ladder into the well on either left or right side of the gel to calibrate.

Close the lid of the gel tank and attach the electrodes. Connect the electrode wires to the power supply and make sure that the positive (red) and negative (black) are correctly connected.

Turn on the power supply to about 80-150 volts. Depending on the size of the electrophoresis chamber,maximum allowed voltage will vary. Check to make sure that the current is running through the buffer by looking for bubbles forming on each electrode.

Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye.

Let the power run until the blue dye approaches the end of the gel.

Turn off the power. Disconnect the wires from the power supply. Remove the lid of the electrophoresis chamber.

Using gloves, carefully remove the tray and gel.

The gel is gently pushed onto the UV Transilluminator. Now the UV light is switched on and the DNA bands are seen as orange bands.

SUGGESTED READINGS

Lee, Pei Yun et al. "Agarose gel electrophoresis for the separation of DNA fragments" *Journal of visualized experiments :JoVE* ,62 3923. 20 Apr. 2012, doi:10.3791/3923.

Mu, Plummer, and David T. Plummer. *Introduction to practical biochemistry*. Tata McGraw-Hill Education, 1988.

Sambrook,J., Russell DW. (2001). Molecular cloning: a laboratory manual (3rd edition) Cold Spring Harbor Lab Press ,Cold Spring Harbor,New York.

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

Polyacrylamide Gel Electrophoresis (PAGE)

Neenu Raj, Anjaly Jose
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is used for the separation and analysis of proteins and relatively small nucleic acid molecules. It has a clearer resolution than agar and is suitable for quantitative analysis. Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of molecules of typical size.

The resolution of the PAGE is so high that, in the size range of about 10-1000 nucleotide units, it is capable of separating DNA molecules that differ in length only by a single monomer unit. In the case of single-stranded DNA, individual molecules are separated only based on their length.

Polyacrylamide electrophoresis separates macromolecules based on a combination of charge, size and shape. Size (shape) separation takes place due to the molecular sieving property of the gel. The size range in which molecules can be separated depends on the average pore size of the gel. In the case of polyacrylamide gels, the concentration of the acrylamide monomer and the proportion of the cross-linking*N*,*N*′-methylenebisacrylamide can control the size of the pore. The pore size of the gel may be varied to produce different molecular seiving effects for separating proteins of different sizes. So the percentage of polyacrylamide can be controlled in a given gel in this way. Polyacrylamide gels offer both greater flexibility and more sharply defined banding than agarose gels. Polyacrylamide act as a solid support for electrophoresis when polypeptides, RNA, or DNA fragments are analyzed. Acrylamide plus N,N'-methylene-bis-acrylamide in a given percentage and ratio are polymerized in the presence of catalysts such as ammonium persulfate and TEMED (N,N,N',N'-tetra-methyl-ethylene-diamine).

Table 2. Gel Concentration for Optimum Resolution for DAA

Concentration of Acrylamide(%)	Range of separation (bp)
3.5	1000-2000
5	80-500
8	60-400
12	40-200
15	25-150
20	6-100

MATERIALS REQUIRED

Electrophoresis Buffers

TBE buffer is used for polyacrylamide gel electrophoresis. Polyacrylamide gels are run in 1X TBE to prevent denaturation of small fragments of DNA. Other electrophoresis buffers can be used, but they are not as good as TBE. TBE provide high buffering capacity than TAE.

Table 3. TBE buffer 10X (pH 8.0)

TBE buffer 10X (pH 8.0)	
Tris buffer	10.8g
Boric acid	5.5g
EDTA	0.75g

Autoclaved and stored at room temperature.

Gel Loading Buffer

Bromophenol blue 0.5%

Glycerol 30%

Prepared in 10XTBE

Stored at $4^{o}C$

10% Ammonium persulfate (AP)

TEMED(N,N,N,N–etramethylethylenediamine)

Electrophoresis buffer

Pipettes and tips

Distilled water

Electrophoresis tank

Gel combs, glass plates, spacers

Ethanol

Binder clips

Power supply

DNA samples

Syringe

Acrylamide:bisacrylamide

Polyacrylamide Gel (%)	29%acrylamide plus 1% N,N-methylenebisacrylamide (ml)	H2O	5XTBE	10% Ammonium persulfate(ml)
3.5	11.6	67.7	20	0.7
5	16.6	62.7	20	0.7
8	26.6	52.7	20	0.7
12	40	39.3	20	0.7
20	66.6	12.7	20	0.7

PROCEDURE

Wash the glass plates and spacers in warm detergent solution and rinse them well, first in tap water and then in distilled water. Hold the plates by the edges or wear gloves, so that oil from the hands do not get deposited on the working surfaces of the plates. Rinse the plate with ethanol and set them aside to dry.

Assemble the glass plates with spacers in gel caster

NB: Extra band of tape around the bottom of the plate can prevent the leaks

According to size of the glass plates and the thickness of the spacers, calculate the volume of gel required.

Prepare the gel solution with the desired polyacrylamide percentage.

NB: Special attention should be paid while using acrylamide(since it is aneurotoxin

Place the required quantity of acrylamide : bisacrylamide solution in a clean flask.

Add required amount of TEMED to acrylamide : bisacrylamide solution and mix the solution by gentle swirling.

NB: Work immediately after the addition of TEMED

Draw the solution using syringe by inserting its nozzle into the space between the glass plates. Fill the space almost to the top with acrylamide solution without air bubbles.

Immediately insert the appropriate comb into the gel, with utmost care to avoid air bubbles. While inserting, top of the teeth should be slightly higher than the top of the glass plate.
Allow the acrylamide to polymerize for 30-60 min at room temperature.

After polymerization is complete, place the glass plate along with the gel in the electrophoresis chamber filled with 1X TBE. Carefully remove the comb from the polymerized gel. Using a syringe rinse out the wells with 1X TBE. Remove the gel sealing tape from the bottom of the gel with scalpel.

NB : Use same electrophoresis buffer in both the reservoir and in the gel.

Mix the DNA samples with the appropriate amount of gel loading buffer. Load the mixture into the wells using pipette with drawn out plastic tip.

Connect the electrodes to a power pack, turn on the power and run the gel until the marker dye reaches desired distance.

Detach the glass plates and remove the gel using spatula.

Stain gels with silver nitrate to visualize the bands.

STAINING OF DNA

Reagents required
Fixing solution (5X)
3.0% Benzenesulfonic acid in 24% ethanol

Staining solution (5X)
1.0% Silver nitrate
0.35% Benzene sulphonic acid

Developing solution (5X)
12.5% Sodium carbonate

37% Formaldehyde in water
2% Sodium thiosulphate in water

Stopping and Preserving solution (5X)

5% Acetic acid
25% Sodium acetate
50% Glycerol

PROCEDURE

Put your gel in a large glass tray.
Fix the gel using 1X fixing solution for 20 minutes.
Wash in distilled water for one minute.
Stain with Silver Stain for 20 minutes.
Wash the gel in distilled water for about one minute.
Develop in 1X Developing solution for 10 minutes. When the bands were dark enough, developing solution was poured out.
Stopping and preserving solution (1X) was immediately added and the gel was documented.

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

SUGGESTED READING

Bassam, Brant J., Gustavo Caetano-Anollés, and Peter M. Gresshoff. "Fast and sensitive silver staining of DNA in polyacrylamide gels." Analytical biochemistry 196.1 (1991): 80-83.

Mu, Plummer, and David T. Plummer. Introduction to practical biochemistry. Tata McGraw-Hill Education, 1988.

Sambrook,J., Russell DW. (2001). Molecular cloning: a laboratory manual (3rd edition) Cold Spring Harbor Lab Press ,Cold Spring Harbor,New York.

Polymerase Chain Reaction (PCR) – Practical aspects

Anjaly Jose, Neenu Raj, K. Nisha, Lakshmi P. Mukundan, Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

PCR tool are fundamental for genome analysis. Due to its simplicity, sensitivity, specificity and reliability PCR is an integral part of almost every genomic studies.It has a wide range of applications in molecular cloning including DNA sequencing, in vitro mutagenesis, mutation detection, cloning of cDNA and genomic DNA and allelotyping. Using PCR we can amplify a defind target sequence of DNA rapidly and selectively to generate millions of copies out of complex mixture of genomic sequences.

This technique was invented in 1985 by Kary B Mullis.The enzyme first used was klenow fragment of E.coli DNA polymerase I and the reaction was carried out in a series of watrebaths. Since this enzyme is heat liable, it had to be replenished at each cycle of amplification. Further advances in this technology leads to the replacement of E.coli polymerase by thermostable Taq (*Thermophilus aquaticuus*) DNA polymerase together with the development of thermal cyclers for accurate temperature control during amplification provided the greatest improvement in PCR methodology.

Principle

PCR is an in vitro method of nucleic acid synthesis by which particular segment of DNA can be specifically replicated. Majority of PCR methods rely on thermal cycling. During thermal cycling the reactants undergo cycles of repeated heating and cooling to permit different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication. The quantity of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.

Essential components for a basic PCR set-up

DNA template that includes the target region to amplify

A thermo stable DNA polymerase to catalyze template dependent synthesisof DNA. Heat resistant Taq polymerase is commonly used , as it is more likely to remain intact during the high-temperature DNA denaturation process

A pair of synthetic oligonucleotide to prime DNA synthesis.

Deoxynucleoside triphosphates, or dNTPs ,the building blocks from whichthe DNA polymerase synthesizes a new DNA strand

Divalent cations: All thermostable DNA polymerase require divalent cations,usually $Mg2^{+}$ for activity. some polymerase also work with buffers containing $Mn2^{+}$. $Mg2^{+}$ has two function in PCR -Reacting with dNTPs to form complexes that are the substrate for Taq

polymerase and stabilize the primer template junction.

A **buffer** to maintain pH by providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Monovalent cations, typically potassium (K^+) ions.

PCR is a cyclic process of 3 basic steps of denaturation, primer annealing, and extension that are repeated several times.

Denaturation*:*Template DNA is denatured by providing appropriatetemparature.The applied temparature and time required for separating the two strands may vary based on the G+C content and length of the target sequence. In PCR catalysed by Taq DNA polymerase ,denaturation is performed at 94^0 C- 95^0 C for 45 seconds to 5 minutes.

Annealing: Now, the template is availbalble for binding with the oligonucleotideprimer. Annealimg temparature is related to the meliting temparature(Tm) of the oligonuleotide – DNA complex and it usually3^0C- 5^0C lower than the Tm. The temparature should selectively determined so that it must permit the binding of oligonucleotide, at the same time it should prevent the reannealing of template. Usually the temparature is reduced to 50^0C-65^0C for 20-40 seconds.

Extension: In this step, the DNA polymerase synthesizes a new DNA strandcomplementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction. Extension of oligonucleotide primer is performed at or near the optimum temaprature of the DNA polymerase used. In the case of Taq DNA polymerase it is72^0C-78^0 C. The specific time required for this step depends both on the DNA polymerase used and on the length of the DNA target region to amplify. By successive reaction cycles the target region is exponentially amplified to generate millions of copies.

REAGENTS, SUPPLIES & EQUIPMENT

Taq DNA polymerase (SIGMA-ALDRICH, from *Thermus aquaticus®,* catalog number d1806) *

10× pcr buffer
Appropriate primer (10mm)*
Deoxynucleotide mix /dntp mix (10mm) *
DNA from specimen(s)*
Container with crushed ice
Micropipettes and tips (2–100 µl)
Microcentrifuge tube rack
PCR tubes
PCR tube rack
Permanent marker
Thermal cycler
*Store on ice

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

PROCEDURE

1. Instructions for Taq DNA Polymerase from SIGMA-ALDRICH (from *Thermus aquaticus,* Catalog Number D1806)

Table 1. For each fragment of target DNA to be amplified, combine the following reagents in a PCR tube.

Components	25µl reaction	50 µl reaction	Final concentration
10X standard Taq reaction buffer	2.5µl	5µl	1X
10mM dNTPs	0.5ul	1µl	200µM
10µM forward primer	0.5µl	1µl	200µM
10µM reverse primer	0.5µl	1µl	200µM
Template DNA	Variable	variable	~100ng
Taq polymerase (5 units/µL)	0.125 µl	0.25µl	0.05 units/µL
Nuclease free water	To 25µl	To 50µl	

Use a micropipette with a fresh tip and Ensure that no liquid/DNA remains in the tip after pipetting.
Gently mix the reaction mix and quick spin for few seconds to bring all liquid to the bottom of the tube.
Store the sample on ice until ready to begin thermal cycling.
Place PCR tube in a thermal cycler/PCR machine and perform the thermal cycling under appropriate conditions.

Table 2. Thermo cycling conditions/PCR protocol for a routine PCR

STEP	TEMPARATURE	TIME
Initial step	94.0C	4-5 minutes
32 cycles of the following profile:		
Denaturation	94.0C	30seconds
Annealing	42.0C-68.0C	30 seconds
Extension	72.0C	1minute/kb
Final synthesis step to complete the synthesis of incomplete products		
Final extension	72.0C	7 minutes
Final step to preserve the sample	4-10.0C	Infinitum

The duration and temperature used for each phase of amplification and the total number of cycles required depend on particular aspect of the reaction being performed.
After PCR, store the amplified DNA on ice or at -20° C.

SUGGESTED READING

Birren,B., Green, ED., Klapholz., Myers, RM.,Roskams J. (1997). Analysing DNA: A laboratory manual(vol.1). Cold Spring Harbor Lab Press, Cold Spring Harbor,New York .

Dieffenbach,CD., Dveksler,GS. (2003)PCR Primer: A laboratory manual (2nd edition) Cold Spring Harbor Lab Press ,Cold Spring Harbor,New York.

Sambrook,J.,russell DW. (2001). Molecular cloning: a laboratory manual (3rd edition) Cold Spring Harbor Lab Press ,Cold Spring Harbor,New York

Reverse transcription polymerase chain reaction (RT-PCR) for first strand cDNA synthesis – practical aspects

Wilson Sebastian, Sandhya Sukumaran
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of traditional polymerase chain reaction (PCR) technique which is commonly used in molecular biology to synthesise cDNA (Complementary DNA). Subsequently, the newly synthesized cDNA is amplified using traditional PCR and real-time polymerase chain reaction for detecting RNA and its expression respectively. The discovery of reverse transcriptase in retroviruses by Howard Temin and David Baltimore in 1970 led to the development of RT-PCR. RT-PCR has replaced northern blot technique, which had been widely used as a method for RNA detection and quantification since its introduction. RT-PCR and real-time polymerase chain reaction (qPCR)/quantitative PCR/quantitative real-time PCR/real-time quantitative PCR are two distinct techniques. While RT-PCR is used to reverse transcribe the RNA of interest (expressed genes) into its DNA complement through the use of reverse transcriptase, qPCR is used to quantitatively measure the amplification of DNA. In addition to the identification of genes, RT-PCR can be utilized for quantification of RNA by incorporating qPCR into the technique (called quantitative real-time RT-PCR)

PRINCIPLE

In RT-PCR, the template RNA is first converted to a complementary DNA (cDNA) using a reverse transcriptase enzyme. Subsequently the cDNA synthesised is then used as a template for amplified using traditional PCR.

MATERIALS AND REAGENTS REQUIRED

One-step RT-PCR kit (with a mix with reverse transcriptase and the PCR system such as Taq DNA Polymerase and a proofreading polymerase).
or
Reverse transcriptase and buffer solution.
Taq DNA Polymerase, proofreading polymerase and buffer solution.
dNTPs.
RNase inhibitor.
Nuclease-free water.
Intact high quality template RNA.
Oligo(dT)N/ Random Hexamer.
Sequence-specific primers.
Thermal cycler.
PCR tube.

PROCEDURE

RT-PCR can be carried out by the one-step RT-PCR protocol or the two-step RT-PCR protocol.

One-step RT-PCR

One-step RT-PCR use mRNA as targets and convert them to cDNA by reverse transcription and then PCR amplification in a single test tube with a sequence-specific primer.

1. Prepare a reaction mix (include dNTPs, primers, necessary enzymes and a buffer solution).
2. Add the mix to a PCR tube for each reaction.
3. Add the template RNA.
4. Place PCR tubes in the thermal cycler.
Run the thermal cycler to begin cycling. The first cycle is reverse transcription to synthesize cDNA.
The next 40 to 50 cycles are the amplification program, which consists of three steps:
(a) denaturation, (b) annealing, (c) elongation. During initial denaturation reverse transcriptase is inactivated and subsequently Taq DNA Polymerase and a proofreading polymerase exponentially amplify the cDNA).
5. The RT-PCR products can then be visualized with an agarose gel electrophoresis.

Two-step RT-PCR

Two-step RT-PCR, as the name implies, occurs in two steps. First the reverse transcription and then the PCR. This method is more sensitive than the one-step method. Kits are also useful for two-step RT-PCR. Just as for one-step, use only intact, high quality RNA for the best results. The primer for two-step does not have to be sequence specific. Oligo(dT)N primer anneal with the Endogenous poly(A) tail found at the 3' end of eukaryotic mRNA and Random Hexamer anneal with many sites throughout the length of an RNA (mRNA, tRNA or rRNA).

Step 1

1. 1 Prepare a reaction mix (include dNTPs, appropriate primers , necessary enzymes and a buffer solution).
2. Combine RNase inhibitor and reverse transcriptase to the mix.
3. Add the master mix to a PCR tube for each reaction.
4. Add the template RNA.
5. Place PCR tube in thermal cycler.

Run the thermal cycler to begin cycling (one cycle includes annealing, extending and then inactivating reverse transcriptase enzyme.
Proceed directly to PCR or store on freezer until PCR can be performed.

Step 2

1. Prepare a reaction mix (containing buffer, dNTP mix, $MgCl_2$, Taq polymerase and nuclease-free water).
2. Add the master mix to a PCR tube for each reaction.
3. Add appropriate primer (Sequence-specific primer) to each PCR tube.
4. Place PCR tubes in thermal cycler.
5. Run the thermal cycler for 30-35 cycles of the amplification program

(three steps: (a)denaturation (b) annealing (c) elongation.

The RT-PCR products can then be visualized with an agarose gel electrophoresis.

RAPID AMPLIFICATION OF CDNA ENDS (RACE PCR)

Rapid amplification of cDNA ends (RACE) is a technique used to obtain the full length sequence of an RNA transcript. It can provide unknown sequences at either the 3' (3' RACE-PCR) or the 5' - end (5' RACE-PCR) of a small known sequence within the RNA transcript. RACE results in the production of a cDNA copy 5' end or 3' end of the RNA transcript of interest, by reverse transcription and following exponential amplification of the cDNA by PCR. RACE is commonly followed up by cloning and then sequencing.

PROCEDURE

RACE can be used to amplify and identify unknown 5' (5'-RACE) or 3' (3'-RACE) ends of RNA molecules where the protocols for 5' or 3' RACES are slightly different. RNA/mRNA is the starting material for a first round of cDNA synthesis (by reverse transcription) using an anti-sense (reverse) oligonucleotide primer (gene specific primer –GSP1) that complimentary to a known region in the gene/RNA of interest. The reverse trancriptase extend the 3' end of the primer binds to RNA and generate a specific single-stranded cDNA product that is the reverse complement of the RNA. The enzyme terminal deoxynucleotidyl transferase (TdT) is then used to add a string of identical nucleotides (homopolymeric tail) to the 3' end of the cDNA. To amplify the cDNA product from the 5' end a PCR reaction is then carried out with a second anti-sense gene specific primer (GSP2) that binds to the known sequence and a universal primer (UP) (sense) that binds to the homopolymeric tail added to the 3' ends of the cDNA.

3' RACE-PCR uses advantage of the natural polyA tail in the 3' end of all eukaryotic mRNA as a priming site for PCR amplification during reverse transcription of eukaryotic mRNA. cDNAs are generated using an Oligo-dT primer (that complements the polyA stretch in the 3' end of mRNA. PCR with a sense GSP and an anti-sense primer complementary to the Oligo-dT primer is then used amplify 3' end cDNA.

There are some other ways to add the 3'-terminal sequence for the first strand of the cDNA synthesis and full length cDNA librarie synthesis like SMART™technology (based on template-switching effect) which are much more efficient than homopolymeric tailing.

Quantitative real - time PCR- Concepts, strategies and practical aspects

K. A. Sajeela
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Quantitative real-time PCR or real-time PCR; often abbreviated as qPCR is a method by which the amount of the PCR product can be determined, in real-time, and is very useful for investigating gene expression. The main advantage of qPCR over PCR is that qPCR allows you to determine the initial number of copies of template DNA (the target sequence) with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (the presence or absence of a sequence) or quantitative (copy number). In contrast, PCR is at best semi quantitative. Additionally, real-time qPCR data can be evaluated without gel electrophoresis, resulting in reduced bench time and increased throughput. Finally, because real-time qPCR reactions are run and data are evaluated in a closed system, opportunities for contamination are reduced and the need for post amplification manipulation is eliminated in qPCR analysis. In combination with reverse-transcription PCR (RT-PCR), qPCR assays can be used to precisely quantitate changes in gene expression, for example, an increase or decrease in expression in response to different environmental conditions or drug treatment, by measuring changes in cellular mRNA levels. Quantitative Reverse Transcription PCR (qRT-PCR) is possible either Two-step or One-step. Two-step quantitative reverse transcriptase PCR (qRT-PCR) starts with the reverse transcription of total RNA into cDNA using a reverse transcriptase (RT) and primed using random primers, oligo(dT), or gene-specific primers (GSPs). Next, approximately 10% of the cDNA is transferred to a separate tube for the real-time PCR reaction. One-step qRT-PCR combines the first-strand cDNA synthesis reaction and real-time PCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. Gene-specific primers (GSP) are required because using oligo(dT) or random primers will generate nonspecific products and reduce the amount of product of interest. One of the most important parameters in real-time PCR is to design gene-specific primers according to standard PCR guidelines with an amplicon length of approximately 50–150 bp, since longer products do not amplify as efficiently.

The main Applications of qPCR include:

1. Quantitative mRNA expression studies.
2. DNA copy number measurements in genomic or viral DNAs.
3. Allelic discrimination assays or SNP genotyping.

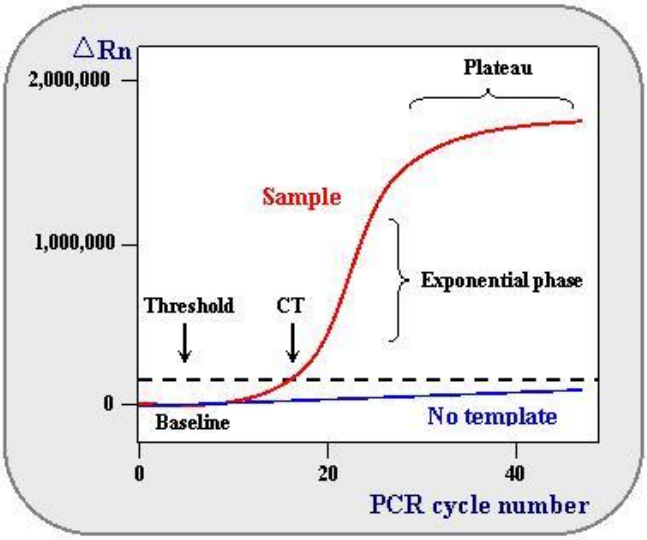
4. Verification of microarray results.
5. Drug therapy efficacy.
6. DNA damage measurement.
7. Cancer phenotyping.

Disadvantages of qPCR include the higher cost of equipment/chemicals/consumables, tedious and lengthy loading times, PCR inhibition, sensitivity to errors and complicated data analysis

HOW IT WORKS.

In qPCR, the amplified DNA is fluorescently labelled and the amount of the fluorescence released during amplification is directly proportional to the amount of amplified DNA. Fluorescence is monitored during the whole PCR process (along all 30 to 45 cycles). Two common methods for the detection of PCR products in qPCR are:
(1) Non-specific fluorescent dyes that intercalate with any double-stranded DNA (SYBR Green)
(2) Sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence.

Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Quantification is possible by measuring the amount of amplified product at each stage during the PCR cycle. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles.



Real-time PCR (qPCR) amplification plot

Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (upto cycles 1–18) even though product accumulates exponentially and this refers to the baseline. Eventually, enough amplified product accumulates to yield a detectable fluorescence signal. The cycle in which fluorescence can be detected is termed threshold cycle (Ct) or quantitation cycle (Cq) and is the basic result of qPCR: lower Ct values mean higher initial copy numbers of the target and vice versa. This is the basic principle of quantitative approach that real-time PCR provides.

ANALYSIS STRATEGIES

Selection of the right quantification method depends on the goals of the experiment.

- **Absolute quantification** determines actual copy numbers of target, in whichsamples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve. Absolute quantitation is often used for determining viral titer.

- **Comparative quantification** determines the relative abundance rather than exactcopy number, in which the expression of a gene of interest in one sample (i.e., treated) is compared to expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease) in expression of the treated in relation to the untreated. A normalizer gene (such as β-actin) is used as a control for experimental variability in this type of quantification. This is the method of choice for gene expression studies and offers two methods for quantification: a) Relative standard curve and b) Comparative Ct (ΔΔCt) method.

Relative Standard Curve Method requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent. This method requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate. This approach gives highly accurate quantitative results because unknown sample quantitative values are interpolated from the standard curve(s). Consider this method when testing low numbers of targets and small numbers of samples and if you are looking for very discrete expression changes.

Comparative Ct Method (ΔΔCt) does not require standard curves to run on each plate and can reduce reagent usage. If you are using custom primers and probes, an initial validation relative standard curve is recommended to validate the PCR efficiencies of the target and endogenous control(s), particularly when you are looking for low-expression-level fold changes. The comparative Ct method is useful when a high number of targets and/or number of samples are tested.

Molecular Cloning of PCR Products

Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Molecular cloning is similar to polymerase chain reaction (PCR) in that the replication or multiplication of DNA sequence occurs. The fundamental difference between the two methods is that molecular cloning involves replication of the DNA in a living micro organism, while PCR replicates DNA in-vitro in a solution, free of living cells. Molecular cloning takes advantage of the fact that the chemical structure of DNA is fundamentally the same in all living systems. Therefore, if a segment of DNA from an organism is inserted into a host DNA containing the sequences required for DNA replication, the resulting molecule is termed as the recombinant DNA. The recombinant DNA containing the foreign DNA will be replicated along with the host cell's DNA in the transgenic organisms. The first recombinant DNA molecule was generated and studied in 1972.

MATERIALS AND REAGENTS REQUIRED

Water bath
Centrifuge
E. coli culture
Cloning vector
Preparation of DNA to be cloned
PCR machine
DNA ligase

PROCEDURE

In standard molecular cloning experiments, the cloning of any DNA fragment generally involves selection of host organism and cloning vector, preparation of DNA to be cloned, creation of recombinant DNA, Introduction of recombinant DNA into host organisms, selection of organisms containing recombinant DNA, screening for clones with desired DNA inserts.

A very large number of host organisms and molecular cloning vectors are available in the market however majority of molecular cloning experiments uses a laboratory strain of bacterium *Escherichia coli* (*E. coli*) and plasmid cloning vectors. *E. coli* is technically sophisticated, versatile, and widely available. A variety of engineered strains of *E. coli* with certain mutations to suit different experiments are available. If the DNA to be cloned is very large, (hundreds of thousands to millions of base pairs) then bacterial artificial chromosome or yeast artificial chromosome vector can be used. For special applications, specialized host vector system is used. For example, if we wish to harvest a particular protein from the recombinant organisms, then an expression vector with appropriate signals for transcription and translation in the host organism is chosen. However most

specialized molecular cloning experiments generally begins with cloning into a bacterial plasmid and subsequent sub cloning into a specialized vector.

The cloning vector generally contains four DNA segments that are critically important to its function and experimental conditions. a) DNA replication origin is necessary for the vector to replicate inside the host organism. b) One or more unique restriction endonuclease recognition sites where foreign DNA is introduced c) A selectable genetic marker gene that is used to allow the survival of cells that have taken up vector sequences d) A tag gene that can be used to screen for cells containing the foreign DNA.

The creation of a recombinant DNA is a simple process. Basically it includes simply mixing together DNA prepared from the vector and foreign DNA at appropriate concentrations and treating them with an enzyme (DNA ligase) that links the ends of DNA together (ligation).

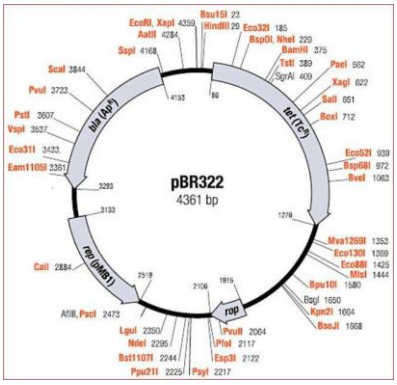


Diagram of cloning plasmid vector pBR322.
pBR322 is the first widely used E. coli cloning vectors. It was created in 1977 in the laboratory of Herbert Boyer at the University of California, San Francisco. It is a circular piece of DNA with 4361 bases. Two antibiotic resistance genes– *bla*, *tet* (providing resistance to ampicillin and tetracycline) an origin of replication (for replicate the plasmid DNA) anddifferent restriction sites are present

The linearized cloning vector is prepared by treating it with a restriction endonuclease (for example EcoRI) which cleaves the DNA at the site where foreign DNA will be inserted. The choice of restriction enzyme is based on its cleavage site, which must be capable of being linked to the ends of the foreign DNA. Generally it is attained by cleaving the vector DNA and foreign DNA with the same restriction enzyme. Before foreign DNA is ligated, the cleaved vector may be treated with an enzyme (alkaline phosphatase) that dephosphorylates the vector ends. The molecules with dephosphorylated ends are unable to replicate and replication can only be restored if foreign DNA is ligated to the cleavage site. Thus it will help to improve the ratio of recombinant to non-recombinant vectors. Most present vectors contain a variety of unique cleavage sites located at convenient sites like within a gene (for example beta-galactosidase) whose inactivation can be used to distinguish recombinant from non-recombinant organisms.

Virtually DNA from any source can be used after purification using simple methods to remove contaminating proteins, RNA and smaller molecules (methods like extraction with phenol chloroform after proteinase K digestion, removal of RNA with ribonuclease, chromatography etc).

PCRs are regularly used for amplification of specific DNA or RNA (RT-PCR, cDNA cloning) or gene sequences proceeding to molecular cloning.

Blunt-end PCR products (by proofreading DNA polymerases) can be directly ligated with the vector with blunt end and PCR products with 3'-dA overhangs (using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerase) can be blunted with DNA blunting enzyme before ligation. If necessary, short double-stranded segments of DNA (linkers) containing desired restriction sites may be added to create end structures that are compatible with the vector. The new TA Cloning method simplifies traditional restriction and ligation cloning with a one-step cloning approach which does not require enzymatic modifications of the PCR product and the use of primers that contain restriction enzyme sites. TA cloning method use advantage of the poly adenine (poly-A) on PCR products amplified using a Taq DNA polymerase which adds a single deoxyadenosine to the 3' end of the product. The vectors (linearised) used in TA cloning method have complimentary 3' deoxythymidine (T) residues which allow the insert to ligate into the vector easily and efficiently.

DNA mixture after ligation results in a complex mixture of DNA molecules with randomly joined ends as DNA ligase recognizes and acts only on the ends of linear DNA molecules. It usually includes vector DNA linked to foreign DNA (desired products) and other sequences like foreign DNA linked to itself, vector DNA linked to itself (and other combinations of vector and foreign DNA).

DNA mixture after ligation containing randomly joined ends is then ready for introduction into the host organism. The methods used to get DNA into host cells are different (e.g. transformation, transduction, transfection, electroporation). Whatever method is used, the introduction of recombinant DNA into the chosen host organism is usually a low efficiency process and the DNA taken up by the cells will be a complex mixture of recombinant and non-recombinant DNA. This problem is solved by a step of artificial genetic selection, in which cells that are unsuccessful in taking up plasmid DNA are selectively killed, and only those cells that can actively replicate vector/plasmid DNA (with selectable marker gene) and express the gene encoded in them are able to survive. For bacterial host organisms, the selectable marker is generally a gene that has resistance to an antibiotic (usually ampicillin). The antibiotic would kill the cells without this gene. Thus the cells harboring the plasmid with selectable marker will survive when exposed to the antibiotic, while those that are unsuccessful in taking up plasmid DNAs will die. Modern bacterial cloning vectors (e.g. pUC19, pGEM, pJET1.2 vectors) use the blue-white screening method to differentiate colonies of transgenic cells (vector DNA with recombinant sequence inserted) from those that contain the vector DNA with no recombinant sequence inserted. In this method foreign DNA is ligated into a gene sequence that encodes beta-galactosidase enzyme (whose activity results in formation of a blue-colored colony) and disables the function of the enzyme, thus the colonies containing transformed DNA remain

colorless (white) on the culture. Therefore, experimentalists can simply eliminate cells without vector DNA and those do not containing recombinant DNA.

Different methods are used to screen clones with the desired DNA construct or recombinant molecule, including the nucleic acid hybridizations, antibody probes, polymerase chain reaction, restriction fragment analysis and DNA sequencing. For example in an experiment with pJET1.2 cloning vector clones with the desired DNA can easily screened by a simple colony PCR with pJET1.2 forward and reverse sequencing primer using plasmid DNA isolated from the cells/ clones as a template and subsequent sequencing.

PREPARATION OF COMPETENT CELL

Bacteria generally take up DNA from their environment by three ways; conjugation, transformation, and transduction. In transformation process, the DNA directly enters to the cell and the uptake of DNA requires the recipient cells to be in a specialized physiological state. Competent cells are bacterial cells with altered cell walls by which foreign DNA can be passed through it more easily. Most bacterial cells cannot take up DNA efficiently unless we make them competent. Competence is distinguished into natural competence, an inherent ability of bacteria to take up DNA from their environment and induced or artificial competence, arising when cells in laboratory cultures are treated to make them temporarily permeable to DNA. Natural competence was first discovered by Frederich Griffith in 1928. The DNA brought into the cell's cytoplasm by transformation may be degraded by the nuclease enzymes or may recombine with the chromosome or plasmid DNA. The standard method for making the ready to use competence cells (make them temporarily permeable to DNA) are a) brief exposure of cells to an electric field (electroporation) b) treatment with calcium ions (cold CaCl2) and following heat shock treatment at 42oC for transformation (Calcium chloride method). The exact mechanisms behind the artificial competence are not yet known well. Ready to use competent cells are available in commercial markets.

It is easier to induce competence more in rapidly growing cells (cells in log phase) than cells in other Growth stages. So it is essential to brought cells into log phase before the procedure is begun because the cells in log phase are healthy, and actively metabolizing.

MATERIALS AND REAGENTS REQUIRED

Luria broth/Lysogeny broth (LB)
0.1M $CaCl_2$ (cold)
0.1M $CaCl_2$/15% Glycerol
LBAmp or LBCarb (100µg/ml) plates
Water bath
Centrifuge
E. coli culture (Competent Cell Strains)

PROCEDURE (*E. coli* Calcium Chloride competent cell)

a) Inoculate a single colony of *E. coli* culture into 5ml LB in 50ml falcon tube. Grow overnight at 37°C. Inoculate 1ml to 100ml of LB in 250ml bottle the next morning. Grow at 37°C in a shaker for 1.5-3hrs or inoculate a single colony into 25ml LB in a 250 ml bottle in the morning. Grow at 37°C in a shaker for 4-6 hrs.

b) Put the cells in ice for 10 mins (keep it cold from now on)

c) Collect the cells by centrifugation at 6krpm for 3 mins, decant supernatant and gently resuspend on 10 ml cold 0.1M CaCl2 (to avoid mechanical disruptions of cells treat them softly), then incubate on ice for 20 mins.

d) Collect the cells by centrifugation at 6krpm for 3 mins, discard supernatant and gently resuspend on 5ml cold 0.1MCaCl/15% Glycerol

e) Distribute in 1.5 ml tubes (300µL/tube) and freeze in -80°C until use.

Transformation of competent cells

a) Take 1µL of circular plasmid or all of a ligation reaction of plasmid DNA in a 1.5 ml microtube, and then gently add ~100µL of competent cells to it.

b) Keep a DNA control tube with cells without DNA.

c) Incubate for 30 mins on ice and heat shock for 2 mins 42°C (by put the tube in 42°C water bath). Then put it back on ice.

d) Add 900 µL of LB to tubes and incubate at 37°C for 30 mins.

e) Directly plate 100-1000 µL of the cells in LBAmp or LBCarb (100µg/ml) plates. (If the ligation efficiency is low or need for a lot of colonies, centrifuge the transformation for 1 min at 6krpm, discard 900 µL of supernatant, resuspend on the 100 µL left on the tube and plate it immediately).

f) Grow overnight at 37°C.

PLASMID DNA EXTRACTION FROM *E. coli* USING ALKALINE LYSIS METHOD

Alkaline lysis is a well-established, quick and efficient way to extract *E. coli* plasmid DNA. This technique was invented by Birnboim and Doly in 1979. This method involves following steps; a high cell density culture of *E. coli* with plasmid/vector is harvested and lysed with SDS/NaOH solution. The detergent cleaves the phospholipid bilayer of cell membrane and the strong base denatures the proteins, both of them are involved in the structure and stability of the cell membrane. Then rapid acidification by concentrated potassium acetate will be used to precipitate protein and chromosomal DNA. The supercoiled plasmid DNA remains in solution which can be precipitated with ethanol/captured on a silica spin column. The various plasmid isolation methods involve three phases: a) Growth of bacterial cells b) Harvesting and lysis of bacterial cells and c) Purification of plasmid DNA

MATERIALS AND REAGENTS

LB medium
1% Tryptone
0.5% yeast extract
200 mM NaCl
Resuspension solution (P1 buffer)
50 mM glucose
10 mM EDTA

25 mM Tris (pH 8.0)
Store at 40 °C
Lysis solution (P2 buffer) (Store at room temperature)
0.2 N NaOH
1% SDS
Neutralizing solution (P3 buffer)
3 M KOAc (pH 6.0)
For 100 ml solution, 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H O) 11.5 ml glacial acetate and 28.5 ml H O, store at room temperature.
TE buffer
1 mM EDTA
10 mM Tris-HCl (pH 8.0) RNAase
Isopropanol (EM Science)
Absolute Ethanol (stores at -20 °C)
Table-top highest speed centrifuges
1.5-ml eppendorf tube
Heat blocker (370c)

PROCEDURE

a) Prepare an overnight culture of bacteria (*E. coli*) in LB medium with suitable antibiotics in a shaking incubator at 37°C. (Usually for a plasmid with >10 copies, 3 ml cell culture is enough.

b) Transfer the culture to a 1.5-ml eppendorf tube, and spin down cells at 6krpm for 1 min.

c) Discard the supernatant and remove the liquid completely by leaving the tube in upside down position on a paper towel for few seconds.

d) Add 100 µl of P1 buffer into each tube and vortex vigorously to resuspend cell pellet completely.

e) Add 100 µl of P2 buffer and mix by inverting the tube 10-15 times (when bacterial lysis has taken place the solution become transparent and more viscous).

f) Add 150 µl of P3 buffer and mix by inverting the tubes 15-20 times (the formation of white precipitate at this point indicate precipitation of bacterial chromosomal DNA).

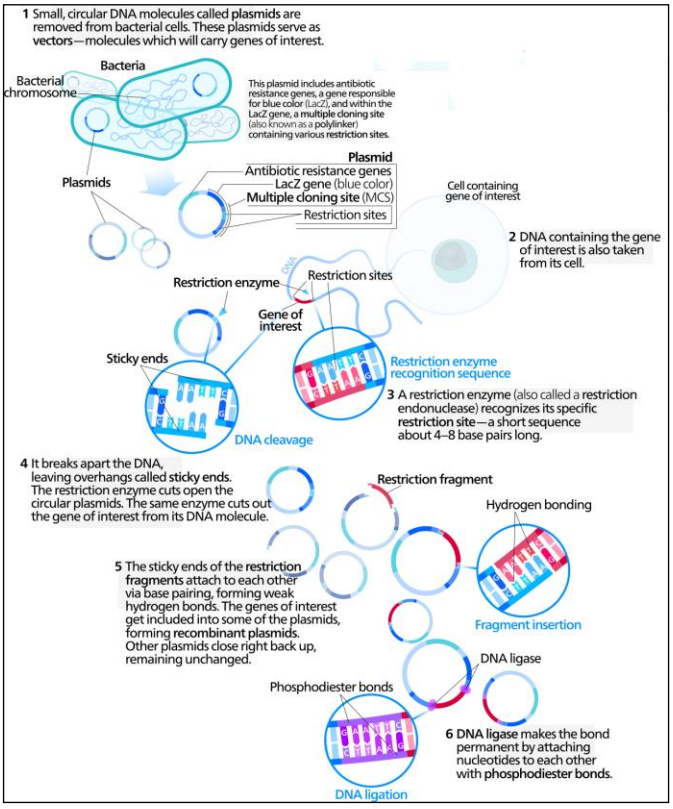
g) Centrifuge the tubes at 10krpm for 10 min.

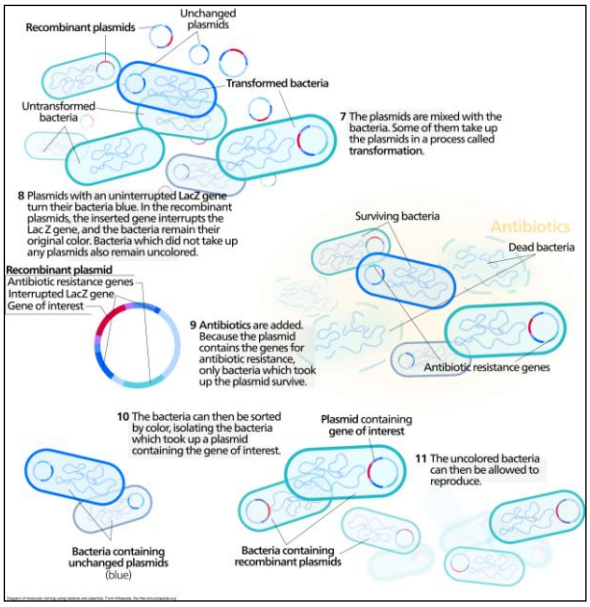
h) Transfer the supernatant, without disturbing the white precipitate to a new 1.5-ml tube.

i) Add 2.5-3 volume of cold ethanol to each tube and mix by inverting the tubes 5-10 times.

j) Spin down plasmid DNA precipitate at 10krpm for 10 min.

k) Discard the supernatant and remove the remaining liquid as much as possible by leaving the tube in upside down position on a paper towel for few minutes.





l) Keep the tubes in a tube holder and air dry for 5-10 min/ keep tubes at 37 °C heat blocker (DNA precipitate will turn to opaq white when it dry).

m) Resuspend the pellet in 50 µl TE and dissolve completely by repeatedly pipetting the solution up and down/gently tapping the tube.

(We can use RNAase to remove the RNA present in the plasmid DNA isolated either by add 1-5 μl (1 mg ml) RNAase to the digestion solution or add RNAase directly to the resuspension solution with a final concentration of 1mg ml.)

NB: Wear gloves while handling the reagents and Make sure that the vials and tips are DNAase and RNAase free

SUGGESTED READINGS

Casali, N., 2003. Escherichia coli host strains. In *E. coli Plasmid Vectors* (pp. 27-48). Humana Press.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids es 7(6): 1513-1523.

Next Generation Sequencing (NGS) - practical aspects

Wilson Sebastian
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Next generation sequencing is a term used to describe high throughput sequencing methodologies, including Roche 454 sequencing, Illumina sequencing, Ion torrent sequencing, SOLiD sequencing, Pack bio sequencing and Nanopore sequencing. In recent years the NGS technologies have been evolved rapidly to a phase that allow us to sequence DNA or RNA much more easily, quickly and cheaply than the sanger sequencing method. It has revolutionized the molecular biology, genomics and biomedical research drastically. With using NGS sequencing one entire human genome can be sequenced and assembled within a day, in contrast to sanger sequencing technology which took more than a decade to sequence first human draft genome. Generally NGS sequencing platforms carry out massively parallel/deep sequencing of base pairs in a DNA or RNA samples and produce an output with millions of short sequence or reads. It supports a range of biological research like chromosome counting and mapping, detection of chromosome and epigenetic changes, development of molecular markers for conservation and disease diagnosis, gene expression profiling, metabolic profiling, cancer diagnosis, personalized medicines and other molecular analysis.

NGS can be used to sequence entire genomes, specific areas of interest in the genome, small number of individual genes and whole transcriptome in tissue/cells by different library preparation methods. During sequencing, each of the bases pairs with sample sequence multiple times and thus it provides high depth and accuracy to the data generated. Bioinformatics tools are used to reconstruct the chromosome/combine together the fragments. Thus the choice of NGS platform and bioinformatics analysis is the center of NGS experiments. NGS technologies enables quick and cheap generation of big sequencing data but it needs high analytical skill and advanced tools to take the full advantage of every short reads generated. Highly efficient and fast processing programs are necessary for handling large volume of data.

Basic work flow of NGS Technique

Extraction of DNA/RNA from samples

Library construction

NGS

Sequence analysis

Basic work flow/Key tasks in NGS DATA analysis

Raw Data handling/Raw data formatting

Quality analysis

Sequence assembly- reference based/denovo

Alignment file handling

Variant calling

SNPs, genotypes

Structural variation

Visualization

Raw sequence data format

Formats are designed to represent sequence data and other important information about sequence like quality of the reads

Common data formats used in NGS data handling are:

SOURSE DATA	FILE FORMATS
Raw reads data	FASTQ, FASTA,SRF, SSF, CFFASTA etc.
sequence alignment data	SAM, BAM
Genome annotations data	GTF, GFF, BED
Variations Data	VCF

FASTQ format

FASTQ format is a text-based format to arrange a biological/DNA sequence data and its quality scores. It has been used as the standard file format for high throughput sequencing methods, as it consumes less data storage space

Example

@title @SRR010930.8436795/1
Sequence
ACCCCAGGATCAACACTTCACATGCATTAGCAGAGAGAG
ATAAATCAA
+ optional_text +
Quality
=>=??A?<@B@A:?B?D;AC@@CAAAD<AAA:99?:@=?@
B@77C><4

Line 1. begins with a '@' character and is followed by a sequence identifier
Line 2. include the raw sequence letters
Line 3. begins with a '+' character and is optionally followed by the same sequence identifier
Line 4. indicates the Quality values/ Quality score Q (PHRED quality scores encoded as ASCII) for each bases in the sequence (Line 2) and must contain the same number of symbols as letters in the sequence

Encoding	!	"	#	$	%	&	'	(	)	*	+	,	-	.	/	0	1	2	3	4
Q score	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

Quality analysis of raw data

All sequencing platforms have errors. So the first step of analysis after getting the sequence data is to evaluate the quality of the raw read/data obtained, followed by correcting, trimming and removal of sequence/reads which is not meeting the required standard. It is necessary to check the quality of reads, adapter contaminations, homopolymer associated base call error and perform necessary corrections to avoid making wrong conclusions.

PHRED quality scores/Quality score Q

Encodes the probability of an erroneous call and it is encoded as ASCII printable characters (ASCII 33–126)

ASCII encoding example
Standard offset 33 but older Solexa/Illumina variants used 64

PHRED quality scores/ Quality score Q can be defined as a property which is logarithmically related to the base calling error property P.

Quality score Q = –10 log 10 P
Error probability P = 10 – Q/10
A data with Q = 30 has error probability P = 10^{-3} = 1 in 1000

Low quality reads < 20% PHRED quality scores

Various tools have been available to perform the different stages of quality analysis

PROGRAM	Operating systemsupported	INPUT FILE SUPPORTED	OUT POT
FastQC	Linux, Mac, Windows	FASTQ,SAM,BAM	HTML
NGSQC	Linux	FASTA,FASTQ, QUAL FASTA	HTML
PRINSEQ	Linux, Mac, Windows, *Web interface*		FASTQ,FASTA,HTML
FASTX-Toolkit	Linux, Mac, *Web interface*	FASTQ,FASTA	FASTQ,FASTA
CLC Bio	Linux, Mac, Windows	FASTQ,FASTA, TXT etc.	FASTQ,FASTA,TXT, HTML etc.
ContEST	Linux, Mac, Windows	FASTA,VCS,BAM,	TXT

FastQC

FastQC is a simple too to quickly analyse the quality of a FASTQ file before they are used for downstream analysis.

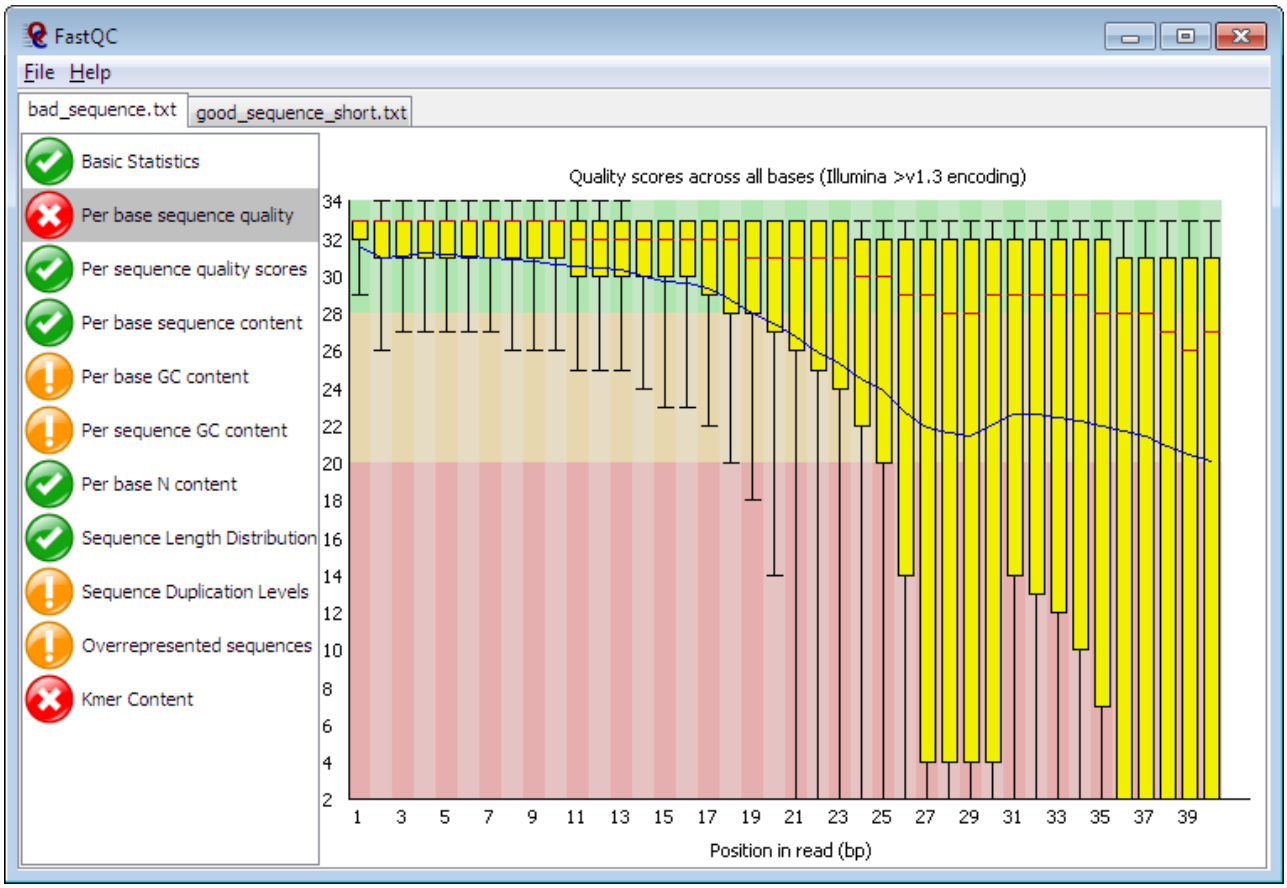
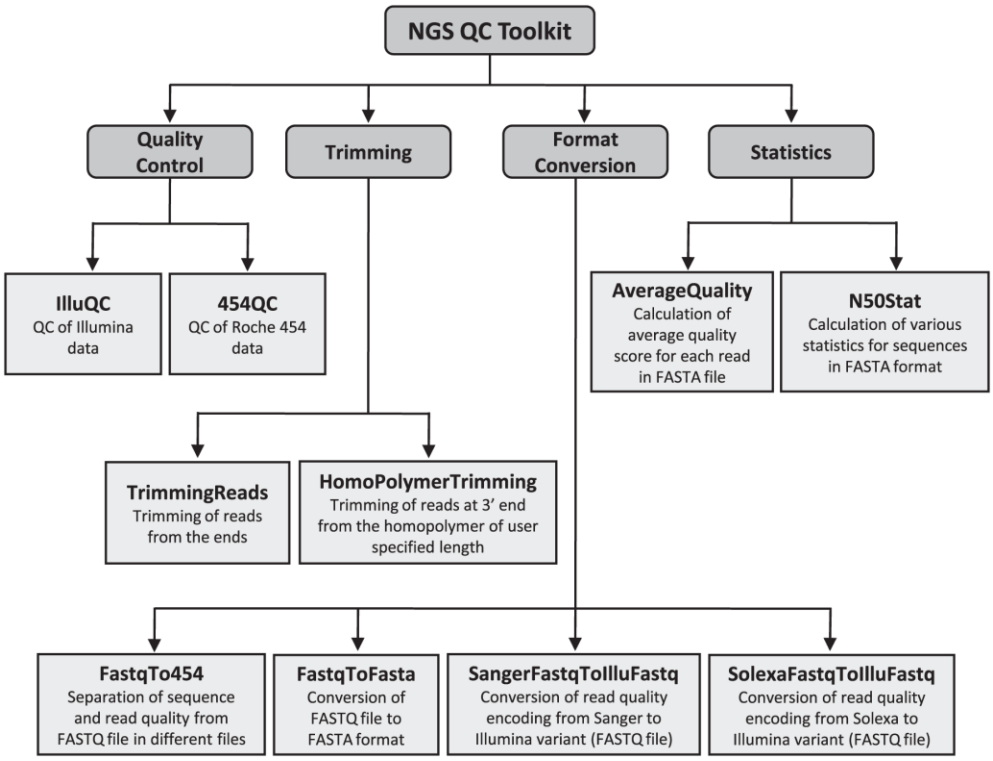


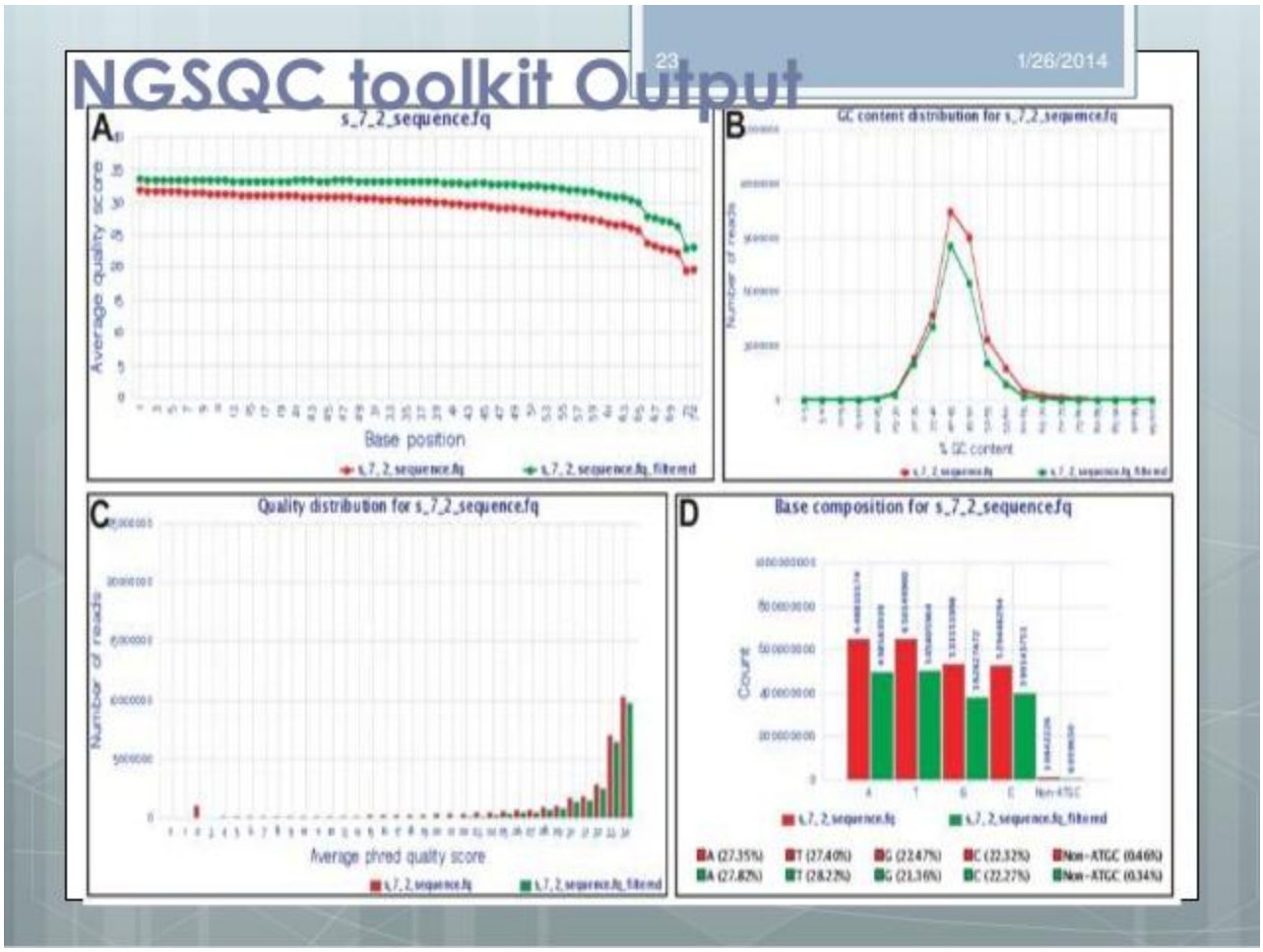
Figure 2. Example of FastQC out put

NGSQC



https://doi.org/10.1371/journal.pone.0030619.g001

Figure 3. Flow chart showing various tools included in NGS QC Toolkit.



QC analysis of sequence data is extremely important for success of full downstream analysis and avoiding wrong conclusions, especially for data assembly and single nucleotide polymorphism (SNP) detection.

Programs used for Align/assemble to a reference genome or sequence

BFAST
Bowtie
BWA
ELAND
Exonerate
GenomeMapper
GMAP-
Gnump-
MAQ
MOSAIK
MrFAST and MrsFAST
MUMmer
Novocraft-
PASS
RMAP
SeqMap
SHRiMP
Slider
SOAP
SSAHA
SOCS
SWIFT
SXOligoSearch
Vmatch
Zoom

Programs used for RNA-Seq Analysis

1) de-novo based
Velvet-Oases
Soapdenovo-Trans - Alternative splicing, differential expression level
Trinity - Reconstruction of transcriptome from RNA-seq data
Trans-AByss - Estimate gene expression level, identify potential polyadenylation sites and candidate gene-fusion events
2) Reference based
Program
Scripture
cufflinks

Variant annotation tools

ANNOVAR
AnnTools
NGS-SNP
Seattleseq
snpEff
SVA
Variant

Visualization Tools

EnsembleGenome Browser
UCSC genome Browser
VEGA genome
Browser
Artemis
IGV
Savant
Circos
Tablet

Bacterial genomic DNA isolation-practical guidelines

T.G. Sumithra, Anusree V. Nair, P.V. Amala
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Extraction and purification of nucleic acids (DNA and RNA) is one of the most crucial procedures in molecular biology and represents the primary step for many downstream applications such as PCR, cloning, sequencing, genotyping, development of diagnostic kits, vaccines, *etc*. Bacteria contain two main types of **DNA namely,chromosomal** DNA and plasmid DNA. Chromosomal DNA contains most of theessential genes of the organism for basic survival in ordinary surroundings. Therefore, it is longer and higher in molecular weight than plasmid DNA. Plasmids are small circular, double stranded extrachromosomal DNA molecules that can replicate independently. Earlier plasmid DNA was thought to be unique to bacteria. It is now obvious that replicative forms of DNA structurally and experimentally analogous to bacterial plasmids are common in archaea and eukaryotic organisms such as fungi and higher plants. Genomic DNA refers to the entire DNA contained within a cell including chromosomal and plasmid DNA. If the organism carries no plasmid, then genomic DNA equals chromosomal DNA.

The most significant goal during the extraction of nucleic acids is the achievement of the highest purity DNA/RNA which is necessary to ensure the successful downstream applications. If we are targeting chromosomal DNA, it is important to avoid plasmid DNA and RNA from final product. Similarly, selective removal of chromosomal DNA and RNA is important during extraction of plasmid DNA. Many commercial kits designed for selective isolation of genomic/ chromosomal/ plasmid/ RNA are available in the market. They are efficient and easy to use, even if often expensive. Conversely, the abundance of commercial kits has resulted in the lack of basic knowledge of the principle and processes involved in each step. The deficiency of this essential knowledge in students and early stage researchers can hinder the ability to modify a technique as required and troubleshoot if necessary. Hence, it is more valuable to follow the conventional protocols for students and early stage researchers.

EXTRACTION OF BACTERIAL GENOMIC DNA-PRINCIPLE

Genomic DNA isolation is less demanding than selective extraction of chromosomal or plasmid DNA as it needs only to separate total DNA from RNA, protein and lipid. However, for whole genome sequencing purposes, it is necessary to collect the intact genomic DNA in the longest fragments. Therefore, methods to

extract high-molecular-weight genomic DNA using magnetic separation for sequencing purposes has been developed and commercial kits using the same principle are available now. In general, there are three basic steps in genomic DNA isolation from bacteria.

1. Cell disruption/ Lysis of bacterial cell to release nucleic acid

In general, the most common method for disrupting bacterial cell for attaining genomic DNA is through enzymatic digestion and detergent lysis in a buffered medium containing EDTA (Ethylene Diamine Tetraacetic Acid), a metal chelating agent. The alkaline pH (8-9) of the buffer reduces electrostatic interactions between basic proteins and DNA, thus assists in inhibiting nuclease activities and denaturing other cellular proteins. EDTA binds divalent cations (Mg2+, Ca2+, Mn2+) so that stability of cell wall and cell membrane will be reduced making more susceptible to lysis step. EDTA also inhibits the activity of nucleases which have a requirement for divalent metal cations.

The enzymatic lysis can be achieved by using lysozyme and protease K. Lysozyme, a member of muramidase class breaks β-1,4-glycosidic linkage between N-acetyl muramic Acid-N-acetyl glucosamine repeating unit of peptidoglycan layer in the cell walls of most bacteria. Lysozyme is highly effective for disrupting cells of a wide range of bacterial species especially, when used in combination with EDTA. However, prokaryotes as Archae are resistant to lysozyme activity due to the lack of peptidoglycan in their cell walls. Additionally, some bacterial species are less susceptible to lysozyme due to the presence of capsule or slime which protect the peptidoglycan. Proteinase K, a serine protease is another enzyme which cleaves the peptide crosslinking interbridges of peptidoglycan layers of cell walls. As Proteinase K is insensitive to the action of EDTA and lysozyme, it can be used in combination with these reagents. However, the different combinations of aminoacids in the peptide interbridges of cell walls of different bacterial species make them more or less susceptible to Proteinase K lysis. Additional bacterial cell-disrupting enzymes like other muramidases, mutanolysin and lysostaphin, Subtilisins and chromopeptidase have been reported for applying in bacterial genomic DNA extraction (Moore et al., 2004).

Detergents allow the effective and relatively gentle disruption of bacterial cells by strongly binding to proteins, causing irreversible denaturation. Detergents are particularly effective when bacterial cell walls are damaged through metal chelating agents, lysozyme and Proteinase K prior to their addition in cell suspension. Sodium dodecyl sulfate (SDS) is the most commonly used detergent in bacterial genomic DNA extraction. SDS binds effectively to cellular proteins and lipoproteins denature them and promote the dissociation from nucleic acids. Further, SDS inhibits action of nucleases and does not interact with hydrophilic nucleic acids. N-lauroylsarcosine

(Sarcosyl), another detergent is reported to be more effective at denaturing cellular polysaccharide and can be used for the disruption of bacteria having copious amounts of capsule. Cetyltrimethyl ammonium bromide (CTAB), a cationic detergent, has also been proven suitable for genomic DNA extraction from bacteria by denaturing and precipitating cell wall lipopolysaccharides and proteins.

2. Nucleic acid extraction

The nucleic acid extraction from the disrupted bacterial cells involves the application of organic solvents like phenol and chloroform. These interact with hydrophobic components of protein and lipoprotein and cause their denaturation. Afterwards, the precipitate of denatured cellular material that remains within the organic phase is separated by centrifugation. Phenol is effective for denaturing protein, while chloroform is effective for polysaccharide materials. Therefore, extraction of DNA using mixtures of phenol/chloroform are more efficient than either alone. Isoamyl alcohol is also added along with Phenol: Chloroform in order to reduce foaming and stabilize the interphase between the aqueous and organic phase. High purity phenol saturated and equilibrated with buffer (pH 8) should be used for DNA extraction.

3. Recovery of DNA

The recovery of DNA is usually achieved by using absolute ethanol or isopropanol, which cause reversible denaturation and precipitation of nucleic acids. This is followed by centrifugation step. As DNA precipitates poorly in salt free solutions, precipitations have to be done in the presence of a monovalent cation (at least, 0.1 M concentration). Therefore, precipitation is done by adding 0.1 suspension volume of 3 M sodium acetate (having pH 5.2) and double/triple suspension volumes (after addition of salt) of absolute ethanol/ 0.5–1.0 volumes of isopropanol. For suspensions with low DNA concentration, a higher ratio of absolute ethanol/isopropanol to suspension volume will assist in DNA precipitation. Instead of sodium acetate, 0.5 volumes of 7.5 M ammonium acetate (pH 8) can also be used. If ammonium acetate is used, small nucleic acid fragments (~150 nucleotides and smaller), will not be precipitated (may be beneficial in some cases). Usually DNA precipitations with absolute ethanol is carried out at extreme low temperature (-200C or −70 ◦C), data of Zeugin and Hartley (1985) recommend that precipitations at extreme low temperatures has no significant improvement over precipitations carried out in ice water (0◦C) and in reality, may be counterproductive. Further, even though majority of DNA in concentrated suspensions is recovered within 5 min by centrifugation (12,000–15,000 × g), the recovery of DNA from dilute suspensions may require centrifugations for 30 min.

MATERIALS REQUIRED

Microcentrifuge, water bath, 1.5/2 ml centrifuge tubes, Shaking incubator, Micropipettes, Gloves

LB broth, Tris base (Molecular weight 121.4), EDTA, Glucose, lysozyme, Proteinase K, CTAB (cetyl trimethylammonium bromide), Sodium chloride, Phenol-Chloroform-Isoamyl alcohol (25: 24: 1), Absolute ethanol, RNAase, 70% Ethanol, SDS, Sodium acetate

Reagents-Composition

1. TEG buffer(pH 8.0):25 mM Tris,10 mM EDTA, 150 mM GlucoseAdjust pH to 8.0 with 2 N HCl

2. 1X TE Buffer (pH 8.0): 10 mM Tris, 1 mM EDTA

PROCEDURE

1. Inoculate one well isolated colony of bacteria into 5 ml of LB broth and incubate overnight in a shaking incubator at optimum temperature.

2. Harvest the cells by centrifugation at 10,000 rpm for 15 min.

3. Re-suspend the pellet in 567 µl of TEG buffer + lysozyme (5 mg/ml of TEG buffer) mixture and vortex. Add 30 µl of 10% SDS and 3 µl of Proteinase-K (20 mg/ml), mix and incubate in water bath at 60°C for 1 h.

4. Add 100 µl of 5M NaCl and 80 µl of CTAB (10% CTAB in 0.7 M NaCl). Mix and incubate in water bath at 65°C for 15 min.

5. Add equal amount of Phenol-Chloroform- Isoamyl alcohol (25: 24: 1), mix gently by inverting the tubes and centrifuge at 10,000 rpm for 15 min at 4°C.

6. 6. Collect the aqueous phase in another micro-centrifuge tube without disturbing the interphase and lower phase.

7. 7. Repeat the process (Step 6) once again and collect the supernatant in a fresh tube

8.

9. 8. To the aqueous phase, add 1/10th volume of 3M sodium acetate and 2.5 times ice cold absolute ethanol and incubate at -200C overnight.
Or
9. Add 1/10th volume of 3 M sodium acetate and equal volume of isopropanol and incubate at room temperature for 10-15 min.

9. 10. After incubation, centrifuge for 15 min at 12,000 rpm.

11. Discard the supernatant and add 500 µl of 70% ethanol. Centrifuge again at 10,000 rpm for 15 min at 4°C. Discard the supernatant.

12. Air dry the tubes and re-suspend DNA in 30 µl of DNA dissolving buffer (TE/elution buffer).

13. Add RNase solution [2 µl of RNase A (10 mg/ml) to 20 µl of DNA] and mix the tubes 2-5 times and incubate at 37°C for 2-4 h to digest RNA. Cool the sample to room temperature and then store at -20°C.

SUGGESTED READINGS

Moore E, Arnscheidt A, Kruger A, Strompl C. and Mau M. 2004. Simplified protocolsfor the preparation of genomic DNA from bacterial cultures. Molecular Microbial Ecology Manual, Second Edition, Kluwer academic publishers, Netherlands, 1.01: 318.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular Cloning: A LaboratoryManual. Cold Spring Harbor Laboratory, Press, Nova York.

Genomic DNA Extraction from Fungi-Practical Aspects

P.V. Amala, T.G. Sumithra, Anusree V. Nair
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

Increasing incidences of fungal infections in both humans and animals have catalyzed the new research and activities in mycology. Additionally, as fungi are recognized as superior microbes than bacteria in terms of metabolic versatility and efficacy, research for better exploration of beneficial fungi are also expanding in recent decades. Extraction of pure DNA is crucial in many of these mycological research as it is a prerequisite for downstream applications such as precise identification and characterization, gene isolation, southern blotting, construction of genomic DNA libraries, genome sequencing, microarrays *etc*.

Principle

Generally, two basic steps are involved in fungal genomic DNA isolation; initially, disruption of cell for DNA extraction, followed by purification and precipitation of genomic DNA from cell lysate. The major challenge for genomic DNA extraction from fungi is the difficulty in lysing the rigid cell walls having high polysaccharide content (mannan, β-glucans and chitin) (Fredricks et al. 2005). Fungal nucleases and rigid cell wall (Chaffin et al.,1998) necessitate additional lysis steps during extraction of DNA from fungi (Alaey et al., 2005).

Cell disruption

In this step the fungal cell and nucleus are broken up to release the DNA. Various methods are applied for lysing fungal cell walls such as, grinding of mycelia using glass rods, liquid nitrogen (Lee et al. 1988, Wu et al. 2001), magnetic beads (Faggi et al. 2005), microwave exposure (Goodwin and Lee 1993), dry ice (Griffin et al. 2002),benzyl chloride (Xue et al. 2006), enzyme digestion (Wilson 1990; Li et al. 2002), or bead-vortexing/SDS or CTAB (Cetyl trimethyl ammonium bromide) lysis (Sambrook and Russel 2001; Doyle and Doyle 1987) *etc*. A combination of above methods is also proved to be useful (Zhang et al. 2008).

Usually, cell disruption is done by mechanical disruption using tissue homogenizer followed by lysis using detergents and enzymes in an extraction buffer. The extraction buffer contains Tris (hydroxyl methyl amino methane) which helps to bind with lipopolysaccharides helping to permealize the membrane and to maintain a constant pH in the solution. The chelating agent EDTA (Ethylene

diamine tetra acetic acid) present in extraction buffer inhibits cellular DNAase activity by chelating with magnesium ions which are essential for enzymatic activity. The detergent, CTAB, a positively charge strong cationic surfactant that solubilizes membranes, degenerate proteins and facilitates the separation of polysaccharides from cell wall. SDS, another detergent will remove negative ions from proteins and lipids that frames membranes.

Nucleic acid purification and precipitation

Phenol-chloroform extraction technique is used to purify nucleic acids from the contaminants. Phenol is a nonpolar compound with high density than water. When phenol is added to cell homogenate, water and phenols remains separated. As nucleic acids are polar, they do not dissolve in phenol. Consequently, following centrifugation two "phases" will be formed, top aqueous phase with nucleic acid and water, and lower organic phase with denatured cell components. Phenol is often used in combination with chloroform as phenol is effective for denaturing protein, while chloroform is effective for polysaccharide materials. Isoamyl alcohol is also added along with Phenol: Chloroform for better separation of aqueous and organic phase. High purity phenol saturated and equilibrated with Tris (pH 8) should be used for DNA extraction.

Ethanol precipitation is commonly used for precipitation of nucleic acids. This requires diluting the nucleic acid with a monovalent salt such as sodium acetate (pH 5.2; most commonly used for most of the routine precipitations), lithium chloride (0.8 M final concentration; does not efficiently precipitate DNA, protein or carbohydrates and loss of tRNAs, 5S RNAs, snRNAs, and other RNAs < 250-300 nt are reported), sodium chloride (0.8 M final concentration; use if sample contains SDS) and ammonium acetate (2-2.5 M; do not use if downstream reactions include phosphorylation step). The basic principle in this step is that, salt and ethanol forces the precipitation of nucleic acid that can be pelleted out by centrifugation. Remnants of salt and alcohol are removed by next step *i.e.* washing with 70% alcohol.

MATERIALS REQUIRED

Microcentrifuge, water bath, 1.5/2 ml centrifuge tubes, shaking incubator, micropipettes, gloves, mortar and pestle, sterile tips

Glucose peptone broth/Potato dextrose broth/ Sabouraud dextrose broth, Tris base, EDTA, proteinase K, CTAB (cetyl trimethyl ammonium bromide), sodium chloride, Phenol-Chloroform- Isoamyl alcohol (25: 24: 1), absolute ethanol, RNAase, 70% ethanol, SDS, sodium acetate

Reagents-Composition

1. Extraction buffer (pH 8.0): 25 mM Tris, 10 mM EDTA, 3% SDS, 150 mM glucose.

2. 1X TE buffer (pH 8.0): 10 mM Tris, 1 mM EDTA

PROTOCOL

1. Inoculate fungal culture in any of the mentioned media and incubate at optimum temperature for five to seven days

2. Centrifuge about 8 ml of the culture at 10,000 rpm for 15 min to harvest the cells

3. Re-suspend the pellet in 2810 µl of extraction buffer and homogenize the mixture by grinding with sterile mortar and pestle.
4. Aliquot 700 µl of the mixture into 2 ml centrifuge tubes and add 5 µl of

   Proteinase-K (20 mg/ml), mix and incubate in water bath at 60°C for 1 h.

5. Add 120 µl of 5M NaCl and 100 µl of CTAB (10% CTAB in 0.7 M NaCl). Mix and incubate in water bath at 65°C for 15 min.
6. Add equal amount of Phenol- Chloroform-Isoamyl alcohol (25: 24: 1), mix gently by inverting the tubes and centrifuge at 10,000 rpm for 15 min at 4°C.

7. Transfer the upper aqueous layer into fresh micro-centrifuge tube without disturbing the interphase and organic phase.

8. Repeat the process (step 7 and 8) once again and collect the supernatant in a fresh tube

9. To the supernatant, add 1/10th volume of 3M sodium acetate and equal volume of isopropanol and incubate at room temperature for 10-15 min.

   Or add 1/10th volume of 3 M sodium acetate and 2.5 times ice cold absolute ethanol and incubate at -200C overnight.

10. After incubation, centrifuge for 15 min at 10,000 rpm.

11. Discard the supernatant and DNA pellet is washed with 1 ml of 70% ethanol. Centrifuge again at 10,000 rpm for 15 min and discard the supernatant.

12. Add RNase solution [2 µl of RNase A (10 mg/ml) to 20 µl of DNA] and mix the tubes 2-5 times and incubate at 37°C for 2-4 h to digest RNA. Cool the sample to room temperature and then store at -20°C for further use.

SUGGESTED READINGS

Sambrook J and Russel DW. 2001. Rapid isolation of yeast DNA. In: SambrookJ,RussellDW (Eds) Molecular cloning: A laboratory manual, 2nd Edn, Cold Spring Harbor Laboratory, New York, pp 631–632.

Motkova P and Vytrasova J. 2011. Comparison of methods for isolating fungal DNA.*Czech J. Food Sci.29: S76–S85.*

Microalgal genomic DNA isolation - practical guidelines

K.V. Jaseera, P. Kaladharan
Fishery Environment Management Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

The very first step in any of the molecular techniques is to isolate DNA with good purity, efficiency, yield and intensity. Isolation of DNA from algae is quite difficult. The complex algal cell wall impedes DNA isolation, and they often possess mucilaginous polysaccharides, polyphenolic compounds, diverse pigments and other secondary metabolites which also interfere with efficient extraction procedure. Many of these compounds co-purify with the nucleic acids during extraction procedures, and often interfere with downstream processing also. Several protocols are currently used for DNA extraction from algae, and usually employ a variety of physical disruption methods of cell lysis, including liquid homogenization, sonication, and grinding in liquid nitrogen. Apart from nuclear DNA algae also contain mitochondrial and plastid DNA.CsCl (Cesium chloride) density gradient is commonly used technique to separate nuclear DNA from others.

Genomic DNA extraction protocol

The first experimental steps are always the same in every protocol and aim at separating the algal cells from the medium and is achieved by centrifugation and washing with buffer or ddH2O. The step is repeated to completely remove of growth medium. The cell pellet is re-suspended in proper buffer.

1. Cell lysis: Several methods are developed to disrupt cells to aid release of DNAfrom intact cells which include simple mechanical shearing of cells (Mortar and pestle with liquid nitrogen/ glass beads, or the usage of sonicator) enzymatic digestion and detergent lysis. Most protocol employ combinations of cell disruption protocol to get pure DNA in good quantity and quality. EDTA in the buffer act as cation chelating agents and binds divalent cations such as calcium and magnesium and there by inhibit nuclease activity. These ions also help to maintain the integrity of the cell membrane, removal of them with EDTA destabilizes the membrane. Tris act as main buffering component and maintain the pH of the buffer at a stable point, usually 8.0. This alkalinepH reduces electrostatic interactions between basic proteins and anionic DNA, thusassists in inhibiting nuclease activities and denaturing other cellular proteins. Moreover, Tris also interacts with the LPS (lipopolysaccharide) in the membrane causing membrane destabilisation. Upon cell destruction, DNA and contents spill into the buffer. The

cellular soup and fragmented RNA and proteins influences the pH of the solution. Tris buffer the soup and maintain the pH at a steady point and protect the DNA from pH change. Enzyme lysis involves usage of proteinase K, main proteolytic enzyme with pH-optimum in the range between pH 7.5–12.0.Proteinase K cleaves peptide bond in proteins adjacent the carboxyl group of hydrophobic amino acid residues (aliphatic and aromatic).

Proteinase K works better in the presence of a detergent (SDS, sarkosyl, triton X 100, etc.) or a chaotropic salt (guanidinium isothiocyanate or hydrochloride) and at elevated temperatures. Proteinase K is resistant to denaturation by heat and detergents. It is thermostable and can function in the range from 25˚C to 65˚C. It is often used at higher temperatures (50-65˚C) because most nucleases that would chew up your DNA are denatured/inactivated at these temperatures. All these conditions help to denature proteins, exposing many more hydrophobic amino acid residues buried in the hydrophobic core of the protein.

SDS (Sodium dodecyl sulphate) is an anionic detergent that solubilise protein and lipids from the membranes and thereby cause damage to protein structure and lose of membrane integrity and finally cell lysis. It also denatures nuclear envelope and histone proteins associated with DNA. CTAB (Cetyl Trimethyl Ammonium Bromide), a cationic detergent, is used to facilitate the separation of polysaccharides during purification. In some cases, additives, such as poly vinyl pyrrolidone are also used which aid in removing polyphenols. The purification of DNA using CTAB exploits that polysaccharides and DNA have different solubility in CTAB depending on the concentration of sodium chloride. At high salt concentrations, polysaccharides are insoluble, while at lower concentrations DNA is insoluble. So by adjusting salt concentration, both polysaccharides and DNA can be differentially precipitated. Nowadays 100µl of cocktail of enzymes containing lyticase, cellulase, pectolyse and pectinase are also used to facilitate cell disruption.

2. Nucleic acid extraction: Phenol and chloroform extraction method is thetraditionally used method to extract nucleic acid from disrupted cells. It is a liquid-liquid extraction technique used to separate DNA from proteins and lipids. Phenol is effective for denaturing protein, while chloroform act on polysaccharide materials. These two interact with hydrophobic components of protein and lipoprotein and cause their denaturation.Since phenol: chloroform mixture is immiscible with water mixing and centrifugation yield two distinct phases. The proteins and hydrophobic lipids will partition into the lower organic phase (phenol: chloroform) while the nucleic acids remain in the upper aqueous phase. The upper aqueous phase is pipetted off with care by avoiding pipetting any of the organic phase or material at the interface. This procedure is often repeated 2-3 times to increase the purity of the DNA. Isoamyl alcohol is employed as anti-foaming agent and

stabilizes interphase (coagulated proteins) between the aqueous (DNA) and organic phase (Lipids). It also benefits the precipitation of proteins and carbohydrates.

3. Precipitation and isolation of DNA: Precipitation of nucleic acid is achieved

by the addition of salt and ethanol to the aqueous solution, which forces the precipitation of nucleic acids out of solution. After precipitation, centrifugation allows the nucleic acids to separate from the rest of the solution. The pellet was washed in cold 70% ethanol and then removes the ethanol by centrifugation. The nucleic acid pellet was allowed to dry and then re-suspended in buffer. Water is a polar molecule and has a partial negative charge near the oxygen atom and partial positive charges near the hydrogen atoms due to unshared electrons. Because of these charges, polar molecules, like DNA or RNA, can interact electrostatically with the water molecules, allowing them to easily dissolve in water and can therefore be described as hydrophilic Nucleic acids are hydrophilic due to negatively charged phosphate (PO3-) groups along the sugar phosphate backbone.

The salt neutralizes the charges on the sugar phosphate backbone. A commonly used salt is sodium acetate. In solution, sodium acetate dissociates into Na+ and [CH3COO]-. The negative charge on the PO3- groups on the nucleic acids was neutralized by the positively charged sodium ions making the DNA molecule much less hydrophilic, and therefore less soluble in water.

MATERIALS REQUIRED

Micro-centrifuge, water bath, 1.5/2 ml centrifuge tubes, Shaking incubator, Micropipettes, Gloves

F/2 media, Tris base (molecular weight 121.4), EDTA, Glucose, Lysozyme, Proteinase K, CTAB, Sodium chloride, Phenol-Chloroform- Isoamyl alcohol (25: 24: 1), Absolute ethanol, RNAase, 70% Ethanol, SDS, Sodium acetate

Reagents-Composition

1. **TEG buffer**(pH 8.0):25 mM Tris,10 mM EDTA, 150 mM Glucose

2. **1X TE Buffer** (pH 8.0): 10 mM Tris, 1 mM EDTA

PROTOCOL

1. 1. Centrifuge culture at exponential growth phase and wash pellet in DDW or TE buffer

2. 2. Add 450 µl TEG buffer with lysozyme (5 mg/ml) and vortex with glass beads in 1.5 ml tube. Add 50 µl SDS (10%)

3. 3. Incubate for 15 min in ice, 10 min at room temperature (RT), then at 60°C for 10 min.

4. 4. Add 5-10µl of Proteinase K (20mg/ml). Mix and incubate at 37 °C for overnight or 60 °C for 3 hours

5. After lysis add 100 µl of 5M NaCl and 80 µl of CTAB (10% CTAB in 0.7M NaCl) mix and incubate in water bath for 10-15 min

6. Add equal volume of Phenol-chloroform-isoamyl alcohol (25:24:1) mix gently by inverting the tube and incubate at RT for10 min. Centrifuge at 4 °C for 10 min 10000 rpm

7. Save aqueous phase in a fresh tube and repeat above step

8. Save aqueous phase and add equal volume of Chloroform: Isoamyl alcohol

   (24:1). Mix well by inverting the tube and centrifuge.

9. Save aqueous phase. Add 1/10th volume 3M sodium acetate and approximately 400ul isopropanol/ethanol. Notice precipitation of DNA in thread form

10. Centrifuge and decant supernatant. Wash the pellet in 70% ethanol and allow drying.

11. Dissolve DNA in TE buffer (30-50 µl)

12. Add RNase solution [2 µl of RNase A (10 mg/ml) to 20 µl of DNA] and mix 2-5 times and incubate at 37°C for 2-4 h to digest RNA.

13. Cool the sample to room temperature and then store at -20°C for future use.

SUGGESTED READINGS

Wu XQ, Zarka A, Boussiba S (2000) A simplified protocol for preparing DNA from filamentous cyanobacteria. Plant Mol Biol Rep 18: 385-392.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Press, Nova York.

PCR amplification of *16s rRNA*gene- universal marker for bacterial identification

Anusree V. Nair, T.G. Sumithra, P.V. Amala
Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi

INTRODUCTION

16S rDNA sequencing has a key role in the discovery of novel bacteria and in the preciseidentification of bacterial isolates, from last a few decades. The complete *16S rRNA* gene sequencing and finding bacterial identity using NCBI-BLAST program has become a routine practice in molecular systematics for bacterial identification. This is based on the fact that the *16S rRNA* gene region is highly conserved between different species of bacteria and archaea. Analysis of *16S rRNA* gene sequence further helps to better identify less defined (produce variable reactions in phenotypic identification scheme), biochemically inert, hardly isolated, phenotypically uncharacterized and slow-growing bacteria. Other genes like *23S rRNA,* 16S-23S rRNA internal transcribed sequences (ITS), *rpoB* (encodes β subunit of RNA polymerase), *groEL* (encodes heat-shock protein, HSP),*gyrB* (encodes β subunit of DNA gyrase) and *recA* are also found in almost all bacteria and therefore, have been used for bacterial identification by means of conserved sequences as universal primers in PCR. Still, *16SrRNA* gene is the most common target for bacterial identification and the number of sequence depositions into databanks such as GenBank(http://www.ncbi.nlm.nih.govt/genbank) and RIDOM(http://www.ridom rdna.de)

The *16S rRNA* gene sequence of bacteria is about 1,550 bp long and consist of nine hypervariable regions that are separated by nine highly conserved regions. These 9 “hypervariable regions” exhibit substantial sequence diversity between different species of bacteria, thus can be exploited for species identification (Van de Peer et al., 1996). Nine hypervariable regions covered nucleotides 69-99, 137-242, 433-497, 576-682, 822-879, 986-1043, 1117-1173, 1243-1294 and 1435-1465 for V1 through V9 respectively, in which the numbering is based on *E. coli* (Brosius et al., 1978). The highly conserved regions flanking the hypervariable regions enable PCR amplification of target sequences using universal primers (Baker et al., 2003; Lu et al., 2000; McCabe et al., 1999; Munson et al., 2004). Various universal 16S PCR primers are thus designed to target the conserved regions which helps to amplify the *16S rRNA* gene in an extensive array of diverse microorganisms. *16S rRNA* gene sequencing is now widely used to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. It functions as a quick and

less time consuming alternative to conventional phenotypic methods of bacterial identification.

***16S rRNA* gene- the most common housekeeping genetic marker for bacteria??**

Present in almost all bacteria

Size of *16S rRNA* gene (~1,500 bp) is adequate for bioinformatics works

Function of the *16S rRNA* gene has not been changed by evolution

16S rRNA gene sequence depends on a significant inter-species differenceand a small intra-species difference which aided in bacterial identification

Precautions

The assay should be done only on pure cultures of bacteria. Molecular identification of bacteria directly from specimens using universal primers is compromised as other contaminating bacteria introduced during sample collection, specimen processing and PCR setup, will also produce amplicon in PCR, contributing to a mixed DNA sequence that cannot be interpreted. Therefore, identification of bacteria directly from specimens are allowed only in those specimens which are from normally sterile sites like blood, CSF, aspirates from joints *etc*. In these specimens also, the assay may be attempted when organisms are visible by microscopy or when there is histological evidence of a bacterial infection. When using the assay on such specimens, the results must be interpreted together with supplementary medical and laboratory evidences.

MATERIALS REQUIRED

Thermal Cycler
Gel electrophoresis unit
Sequencer
Nuclease free water
10X *Taq* buffer
Taq polymerase (1.5 U/µL)
10 mM dNTP mix
Primers
Template DNA

Primer details

Primer name	Sequences (5'-3')	Position	Product size
8F	5'AGAGTTTGATCCTGGCTCAG-3'	8-27	~1500bp
1492R	5'-ACGGCTACCTTGTTACGACTT- 3'	1492-1513	

Composition of reagents

10X *Taq* buffer (Sigma): 100 mM Tris (pH 8.3 at 25˚C), 500 mM KCl, 15 mM MgCl2,

0.01% gelatin

10 mM dNTP mix: 2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dCTP, 2.5 mM dTTP

PROCEDURE

To the sterile 0.2-ml microfuge tube, add the reagents in the following order

Reagents	**Volume**
Autoclaved nuclease free water	19.75 µl
10 X *Taq* buffer	2.5 µl
Forward Primer (10 picomol)	0.5 µl
Reverse Primer(10 picomol)	0.5 µl
dNTPs	0.5 µl
Taq polymerase	0.25 µl
DNA Template (50 ng)	1 µl
Total volume	**25 µl**

Mix the contents properly and spin down.

Keep the reaction mix in the thermal cycler.

Create the PCR program as given below and save.

Steps	**Temperature**	**Time**
Initial denaturation	94˚C	5 min
Denaturation	94˚C	30 sec
Annealing	58˚C	30 sec
Extension	72˚C	1 min
Final extension	72˚C	10 min
Hold	94˚C	∞
Number of cycles		35

After completing the reaction, 2-5 µl of amplified product is withdrawn and analyzed by agarose gel electrophoresis (1.5%) to check the presence of band.

Marker should be loaded to check the amplified product is of correct size

Purify the amplicon using PCR purification/ gel extraction kit

Send the amplified product for sequencing along with the primers

After receiving the sequencing results, compare our sequence (s) to the known sequences NCBI database using NCBI-BLAST

Cut-off value of sequence similarity for the species and genus level identification is 99 and 97% respectively (Janda and Abbott, 2007).

SUGGESTED READINGS

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173(2): 697-703.

Chakravorty S, Helb D, Burday M, Connell N, Alland, D. 2007. A detailed analysis of *16S ribosomal RNA* gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods*, 69(2): 330-339.

James G. 2010. Universal bacterial identification by PCR and DNA Sequencing of *16S rRNA* Gene. In: Schuller M, Sloots T, James G, Halliday C, Carter I. (eds) PCRfor Clinical Microbiology. Springer, Dordrecht.

Indian Council of Agricultural Research
(Ministry of Agriculture and Farmers Welfare)
Central Marine Fisheries Research Institute -ICAR CMFRI
PB No:1603, Ernakulam North P.O,Kochi-682018, INDIA
Phone: +91 484 2394357, 2394867 Fax +91 4842394909 E-mail: contact@cmfri.org.in
www.cmfri.org.in

सी एम एफ आर आइ
CMFRI

भाकृअनुप
ICAR

हर कदम, हर डगर
किसानों का हमसफर
भारतीय कृषि अनुसंधान परिषद

Agrisearch with a human touch