ELECTROPHORETIC PATTERNS OF THE GENERAL PROTEINS OF FOUR SPECIES BELONGING TO THE FAMILY CARANGIDAE

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To My Parents & Binu

DECLARATION

I hereby declare that this thesis entitled **"Electrophoretic patterns of the general proteins of four species belonging to the family carangidae"** is based on my research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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CERTIFICATE

Certified that the dissertation entitled "**Electrophoretic patterns of the general proteins of four species belonging to the family carangidae**" *is a bonafide record of work done by* Ms. Bindhu Verghese *under our guidance at the Central Marine Fisheries Research Institute during the tenure of her M.F.Sc (Mariculture) programme of 1996-98 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.*

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सारांश

पेशी के प्रोटीन कई उपयोगी सूचनाएं प्रदान करते हैं और इस वजह से मछलियों के जातीय, वंशीय, पारिवारिक और वर्गिकीय स्तर के वर्गीकरण में कभी कभी इन्हें उपयुक्त किया जाता है । वर्तमान अध्ययन में होरिज़ोन्टल स्लाब पोलियाक्रिल अमाइड जेल इलक्ट्रोफोरसिस (जेल सांद्रता 7.5 ५) तरीके से कैरंजिडों की चार जातियों जैसे डेकाप्टीरस रसेली, डी. माक्रोसोमा, सेलार कुमनोफ्तालमस और मेगालास्पिस कोर्डिला में पेशी मयोजन की उपस्थिति की रिपोर्ट की जाती है । डी. रसेली, डी. माक्रोसोमा, एस. कुमनोफ्तालमस और एम. कोर्डिला के प्रोटीन खंडों और अनुमानित लोसी की संख्या क्रमशः 9, 7, 6 और 5 देखा गया । एस. कुमनोफ्तालमस में मूल्य का रेंज 21 से 84, एम. कोर्डिला में 2 से 72, डी. माक्रोसोमा में 15 से 89 और डी. रसेली में 18 से 96 था । प्रोटीन खंडों की भिन्नता, गतिशीलता और अभिरंजन तीव्रता से जातीय विशेषता व्यक्त हो जाती है । वर्तमान अध्ययन में उपयुक्त कैरंजिडों की सीमित नमूनों में से तीनों में अंतराजातीय बहुरूपता व्यक्त हो गई जो इस प्रकार है कि डी. रसेली में 3.5 ५, डी. माक्रोसोमा में 13.5 ५ और एस. कुमनोफ्तालमस में 6.6 ५ इस अध्ययन से यह भी व्यक्त हो जाता है कि कैरंजिडों के प्रोटीन खंडों को वर्गिकीय संदिग्धता पर होने वाले विवादों के निवारण केलिए सहायक जातीय - विशेष के आनुवंशिक सूचकों के रूप में भी उपयुक्त किया जा सकता है ।

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ABSTRACT

Muscle proteins usually provide useful information and therefore are frequently used as valuable diagnostic characters in the classification of fishes at species, generic, familial and higher taxonomic levels. The muscle myogen of four species of carangids. viz, *Decapterus russelli, D. macrosoma, Selar crumenophthalmus* and *Megalaspis cordyla* using horizontal slab polyacrylamide gel electrophoresis (Gel conc. 7.5%) is reported in the present communication. The number of protein fractions and the presumptive loci in muscle of *D. russelli, D. macrosoma, S. crumenophthalmus and M. cordyla* were found to be 9,7,6 and 5 respectively. The R_f value ranged from 21 to 84 for *S. crumenophthalmus*, 2 to 72 for *M. cordyla,* 15 to 89 for *D. macrosoma* and 18 to 96 for *D. russelli.* The difference in number of fractions, their mobility pattern and staining intensity indicated species-specificity. With the limited number of samples used in the present work, there has been an indication of intraspecific polymorphism among three species of carangids, being 3.5% in *D. russelli,* 13.5% in *D. macrosoma* and 6.6% in *S. crumenophthalmus.* In *M. cordyla* no polymorphism was observed. This work also revealed that protein fractions in carangids can be used as species - specific markers, which could be helpful in resolving disputes in the event of any taxonomic ambiguity.

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1. Introduction

Aquatic ecosystems and resources are of critical importance to human population from the time immemorial. Fishes probability surpass all vertebrate groups in their variety of social structure and kinds of life histories. Fish was not only significant as a portion of protein available for consumption but also as a good candidate for various research aspects such as taxonomy, biology, fishery, aquaculture and even genetics.

Species forms the taxonomic unit of fishery resource, conventional species identification in fishes was done based on the number of scales, scutes, fin rays, lateral line, calcified structures, life colour, body propotion etc. But this type of morphological identification may be deceiving as the morphological characters are susceptible for ecological changes (Menon, 1989). Species as a general fact cannot remain as a constant entity for long periods as it is susceptible to changes due to evolution which may lead to speciation. This type of speci ation may be due to reproductive isolation which transforms the population into different races and subspecies which ultimately result in evolution of new species.

The use of electrophoresis to detect allelic and non allelic forms of proteins had lead to a revolution in the population and evolutionary genetics of man, animals, plants and even in fishes (Kirpichnikov, 1981), by

differentiating heterozygotes and homozygotes. It is an efficient tool for identification of new species and for assigning right phylogenetic position of the organism. Study of electrophoretically separated protein fractions may directly give a good idea of the physiological, ecological and biological fractions of the organism. It may also prove useful as a tool in breeding programmes for identifying specific enzyme.

Proteins / Enzymes are used as genetic markers which play a significant role in fishery biology and management. The attributes to genetic markers are

* They occur naturally, so useful equally for identifying wild and hatchery stocks.

* They are inherited in Mendelian fashion which transmits the markers from the parents of one generation to offspring of the next.

* They are usually expressed throughout the life cycle of an individual; thus juveniles and adults are equally identifiable.

* Allelic frequencies at protein coding loci tend to be fairly constant over time (generations) which reduces the need to revise the standards for characterising a population

* Imposing the mark does not require the handling of individual fish, and so no trauma results from mark application and retention.

* The mark can be read (and the genotype determined) at a reasonable effort and cost.

It has been established long before that proteins are direct products of gene action. (Crick 1963; Nirenberg *et al.,* 1963; Ochoa, 1963). As gene controlled proteins form the structural basic source of genetic information at various levels of species organisation.

Proteins of different molecular sizes are present in different tissues of an organism. For the comparative genetic studies of different proteins, complex mixtures have to be isolated and visibly recorded by electrophoretic techniques. The principle of electrophoresis is that a charged ion or group of ions in a suitable medium of electric field migrate towards one of the electrode of opposite charge. Polyacrylamide is recently used for electrophoresis to get fine resolution, which can be obtained by controlling the pore size and shape of protein molecules as well as charge.

The advantage of protein electrophoresis primarily relate to its speed and relatively low cost compared to the DNA techniques; data on hundreds of individuals at several loci can be assessed with in a few days or weeks.

Equipment demands are modest and personnel can be trained quickly to interpret gel pattern.

Electrophoresis of proteins has been widely applied for direct study of genetic variation in fish population and identification of genetic stocks of commercially important fishery resources. The variation in the primary structure of protein, leading to surface change can be detected by electrophoresis and this technique was found to be useful in studying problems involving taxonomic ranks, from relationship between orders to relationship between species. Polyacrylamide gel electrophoresis (PAGE) is found to be more advantageous than other existing electrophoretic techniques as it gives a clear resolution of separated protein. Due to the existence of morphological plasticity among individuals, conventional morphological characters are often found to be deceiving in exact detection of a species. This can be overcome by using electrophoresis.

Carangids form the major fishery along the Southern states of India (Bal and Rao., 1984). In 1996, 1,47000 t of carangids were landed which formed about 6.1% of total marine fish landings. Of the 65 species of carangids found in the western Indian ocean about 35 species are found in the Indian waters. (FAO, 1984). It has been seen that the morphological identification of some species belong to carangidae is difficult. Taxonomic

ambiguity was found to exist between species of *Decapterus macrosoma* and *D. macarellus, D. kurroides and D. kurroides akaadsi, D. russelli* and *D. macrosoma, D. lajang* and *D. macrosoma* and *Caranx kalla* and *Caranx para* (FAO, 1984). So it becomes necessary to find out a species-specific marker for each fish to study their specific variation and determined their population size.

The present work was done using four species of carangids (*Decapterus russelli, D. macrosoma, Selar crumenophthalmus* and *Megalaspis cordyla*) to determine the amount of variation within and between the species to reveal their genetic relationship and to characterise species-specific markers.

2. Review of Literature

In the early history of fisheries research the fishes were identified solely based on morphological character (Schmidt, 1917; Heinke, 1988) like number of scales, finrays, vertebrae, body proportions, life colour, osteological features etc. Later, little more advanced outlook for identification came, which was based on the number of chromosomes and the protein patterns (Menon, 1989). The significance of biochemical genetic studies can be understood by its diverse application in fish genetic study concerning species separation, stock identification and hybridisation (Wishard *et al.*, 1980). Most of the earlier studies were based on morphological characters to assess genetic variability within a species, but to assess genetic variation based on morphological characters is very difficult and tedious (Alm, 1949; Swardson, 1979).

Morphological differences between fishes of different origin are caused by environmental factors and large fraction of evidence for genetic differences is circumstantial (Ricker, 1972) Realising the role of environmental parameters on phenotypic expression in fishes (Ryman and Stahl, 1981), modern investigators started applying biochemical technic for identification of gene-controlled phenotypes such as proteins and enzymes of different tissues of fishes. Protein differences used for comparing species dates back to 1906, when Nuttal used immunological methods to compare

serum of human with that of other primates. Phenotypically different haemoglobins in human blood were reported by Pauling *et al* .(1949).

Considerable theory concerning the dynamics of Mendelian genes had developed by the early 1930s. A conceptual framework and drift in the creation, maintenance, and distribution of Mendelian genes in natural populations were established through the works of Fisher (1930); and Wright (1931). For over 30 years, very little empirical information was available to match the theoretical ground work. Genetic studies during 1950's were focused on the immunological procedures till the clarification of the structure of DNA molecule by Watson and Crick (1953), which led to the understanding of direct relationship between genes and proteins. Starch gel electrophoresis (Smithies, 1955), enhanced by application of histochemical staining method (Hunter and Market, 1957) added simplicity and sensibility of protein variation. In 1959, Raymond and Weintraub introduced polyacrylamide as an effective electrophoretic medium which showed high resolving power due to its adjustable pore size.

After the introduction of starch gel electrophoresis, genetic polymorphism was increasingly studied in fish (Sick, 1961; Ferguson, 1994). Protein polymorphism were then used for identifying hybrids (Tsuyuki and Roberts, 1965b), for defining intraspecific protein structure

(Tsuyuki *et al.*, 1968) and also for describing allelic frequency variation at protein coding loci among conspecific population (Hodgins *et al.*, 1969).

Genetic variation is the raw material in species populations, which enable them to adapt to changes in their environment. New genetic variation may arise in a population due to spontaneous mutation of a gene or by immigration from population of genetically different individuals. In several organisms including fish species, individuals possessing the most genetic variation have shown to have better survival rate or high relative growth. Interpretation of genetic variance was done by Utter *et al.*, (1974). Considerable work has been done on genetic variation in different fish species. Tsvetneko (1991) had made an analysis on the juveniles and mature specimen of *Liza auratus, Mugil cephalus, L. saliens* and *M.soiuy*. The result showed that the mullet was similar to majority of fishes in term of genetic variability. Lacson (1992) reported intraspecific variation among sample of six species of reef fishes (*Chromis cyanea, stegaster partitus, S. planitrons, S. lucostiatus, S. dorsopunicans, Thalassoma bifasciatum*) and found that genetic distance and indices of genetic subdivision were small in all the six study species. Matsuoko *et al.* (1995) studied genetic relationship and protein variation of two morphologically similar congeneric fishes of family osmeridae, (*Hypomisus nipponensis* and *H. pretiosus*) and found

that the genetic variation between the two was very low. Genetic divergence in Maldivian and Micronesian dames of Pomacentridae (*Stegastes nigricans, Chrysiptera biocellata, C.glauca, and C. lucopoma*) was studied by Lacson and Clark (1995) suggest that the Maldivian and Micronesian samples represent separate dames.

Genetic relationships in carangids was studied by Kijma *et al.,* (1986). Genetic divergence and systematic of sparid fishes was investigated by Tanguichi *et al.,* (1986). The amount of genetic divergence among seven cut throat (*Salmo clarki*) subspecies and rainbow trout (*Salmo gairdneri*) which helped in identifying genetically pure populations of these taxa was estimated by Leary *et al.* (1987).

Menezes *et al.,* (1988-90) studied interspecific divergence and intraspecific genetic divergence of sciaenids. Menezes (1990) reported genetic divergence in three carangids *(Decapterus ruselli, Selar crumenophthalmus, Selaroides leptolepis)* and found that the three carangids were clearly divided into two groups. Studies on *Mugil Cephalus, Liza subviridis* and *Valamugil cunnesius* was conducted by Menezes *et al.,* (1992). Genetic divergence and phylogenetic relationships among Pomfrets *(Parastromatus niger, Pampus argenteus, Pampus chinensis)* were studied by Menezes (1993). The results showed average genetic distance among

species which increased with level of taxonomic category which helped to divide the three species studied into two distinct groups.

The occurrence of polymorphic locus among the individuals of same species is called intra specific variation or polymorphism which occur at molecular level as protein or enzyme variability. The electrophoretic analysis of intra and inter specific organisms mostly showed species-specific patterns. Tsuyaki *et al.,* (1965a) have discussed the value of muscle myogen patterns in phylogenetic studies and intra specific protein variation as diagnostic character of stock analysis. Earlier the only method for the study of intraspecific variation and racial variation was morphometric and meristic characters. Now biochemical genetics have become a useful tool to supplement the same (Chakraborty, 1989). Nemipterids *(Nemipterus japonicus, N. mesoprion and N. delagoae)* showed species specificity for eye-lens proteins; but for muscle protein, the patterns were uniform for all the three species studied (Chakraborthy, 1989). Species-specific electrophoretic patterns were detected in *Oreochromis mossambicus* and *O. urolepsis hornorum* as well as in their F_1 hybrids (Urib - Alcocer *et al.,* 1989). A comparative study made by Dobrovolov (1994) on the non-enzymatic protein of five species of Gobio species *(G. gobio, G. albipinnatus, G. uronoscopus* and *G. kessleri)* revealed species specificity

for myogenic, esterase and malate-dehydrogenase. Species specificity in European barbels *(Barbus barbus, B.meridionaks* and 4 native barbels) of South East Asia was studied by Huriuax *et al.* (1992).

Menezes (1976) studied the eye-lens and serum proteins of *Sardinella fimbriata and S. longiceps.* She found that the eye-lens proteins of two species did not show marked differences in the number motility or staining intensity of protein fraction.. Studies on serum proteins revealed major difference between the proteins fractions mobility and the amount of protein in various fractions. Intra and interspecific relationship of marine catfish, studied by Suzuki and Phan (1990) revealed species specificity. Species-specific band patterns were showed by *Cynoglossus macrolepidotus, Pseudorhombus zebrias guagg and Asopia cornuta* (Apte and Rao, 1992). El-Deeb *et al.,* (1992) found that muscle myogen of different phenotype of red tilapia *(Oreochromis niloticus and O. aureus)* were not similar or species-specific.

In marine and fresh water fishes, the proteins which have been most extensively studied included haemoglobins (Sick, 1961), serum plasma protein (Nyman, 1965 a,b), muscle myogens (Tsuyuki and Roberts, 1965b; 1966; Tsuyuki *et al.,* 1965a) and organ protein (Nyman 1965 b). The major feature of the patterns of such bands on the gel medium has shown to

be species-specific. Species-specific protein in freshwater fishes and their suitability for a 'protein taxonomy', was studied by (Nyman, 1965a). Investigation on eye-lens proteins of three flat fishes*(Psettodues erumei, Brachiurus orientalis and Psuedorhombus arsius)* by Menezes (1979) indicated significant differences in the number of proteins fractions, their migratory distance and staining intensity, all the indicated characteristics of species. Whatt (1987) studied utility of species differences in tissue patterns for systematic and evolutionary analysis. Chatterjee (1989) did some works on cytotaxonomy and electrophoretic investigation of Indian air breathing fishes.

Molecular genetic approaches have proved invaluable, both in clarifying taxonomic problems and in enabling the identification of eggs and larvae. Genetic techniques are becoming increasingly used, both in the taxonomy and systematics. Taxonomical investigations have been greatly benefited by species-specific electrophoretic band patterns of protein. Today taxonomists are concerned with more of recognising and accounting variations within a species (Menon 1989). In recent years, the major biological tool applied in the taxonomy of fish is electrophoresis. Study on protein pattern have become a valuable tool in elucidation of taxonomic problems. In electrophoretic technique, closely related species share many

electrophoretic alleles, but also differ at some gene loci at which they are fixed for different alleles (Smith *et al.,* 1990).

Protein differences between species were used in Australian studies to identify fish fillets (Shaklee and Keenam, 1986), to identify teleost larvae (Smith and Crossland, 1977) and prawn post larvae (Larvey and Staples, 1990) to resolve taxonomic problems in teleosts (Smith *et al.,* 1979; Smith and Robertson, 1981) molluscs (Richardson *et al.,* 1982) prawns (Boulton and Knott, 1984) and to identify teleost hybrids (Shearer and Mulley, 1978). In addition, the use of electrophoretic techniques has revealed cryptic species in some fishes (Daly and Richardson, 1980). The arrow squid fishery around Newzealand was thought to be based on a single species (Robert, 1979) until an electrophoretic study reveals two species fixed for different alleles at enzyme locus (Smith *et al.,* 1981). Conversely, no electrophoretic differences had been found in 33 enzyme loci between two species of rock lobsters, *Jasus edwardsii* and *J. novaehollandias* as for Newzealand and Tasmania, respectively (Smith *et al.,* 1980).

Genetic divergence between species has been observed in many fishes using biochemical markers (Ayala, 1983), which is usually protein or enzyme. The search of genetic markers with true mendelian inheritance originated from the intuitive feeling that these markers would discriminate

genetically between populations of most fish species (Ridgway and Klontz, 1960). Electrophoretic technique is very useful in the development of enzyme and non-enzymatic proteins as genetic markers which help in stock identification. Genetic markers are superior to artificial markers and tags, as they are natural and can be found in all stages of animals (Kapila and Kapila, 1996). A number of biochemical genetic markers for stock identification and assessment have been identified for Tuna sp. (Fujino, 1966; Fujino and Kang, 1968b), *Tilapia zilli* (Cruz *et al.*, 1982), marine teleost (Smith *et al* ., 1990), corigonid (Slecttitova *et al.*, 1992) and in several fresh water fishes.

Genetic variants at loci, identifiable by biochemical procedure have proved valuable in identifying discrete breeding populations and in estimating contribution to stock mixture (Utter, 1991). Differences in the morphology and the life history pattern among specific fish populations lead to the expectation of genetic differentiation within many fish species and contributed to the basis of stock concept (Ryman & Sahl, 1981; Berst & Simon, 1981). An abundance of morphologically and ecologically distinct groups of Atlantic herring (*Clupea harengus* L.), resulted in attempt to classify divergent stock at different taxonomic levels (Parrish and Saville, 1965; Iles & Sinclair, 1982). Genetic variability studies in populations of

Atlantic mackeral, *Scomber Scombrus* L (Jamieson and Smith, 1987), Sardine, *Sardinops sagax melanostica* (Kenya *et al.,* 1989), red drum, *Scianops ocellatus* (Bohlmeyer *et al.*, 1991), Atlantic salmon, *Salmo salar* L. (Mc. Elligot *et al.*, 1991), American plaice, *Hippogiossoidae platissoides* (Stott *et al.,* 1992) and rainbow trout (Herhberger, 1992) were studied using polyacrylamide gel electrophoresis.

Biochemical genetic studies of Indian fishes are scanty and are of preliminary nature, which are mainly the attempts to find out interspecies or species specific protein differences. Some of the examples are flatfish (Kasinathan *et al* ,1992), marine fishes (Manohar and Velankar, 1973), Goboids (Natarajan et al., 1975), Bombay duck (Kurian, 1977), oil sardine (Rao and Dhulkhed, 1976), mackeral (Dhulkhed and Rao, 1976; Menezes, 1986; Menezes *et al.,* 1990), *Mugil cephalus* (Bhosle, 1977), Mullets (Rao, 1981), *Channa stewartii* and *Danio dangila* (Bhattacharya and Alfred, 1982), *Etroplus suratensis*, *Liza macrolepis* and *Mystus gulio* (Kamalakara Rao *et al.,* 1985), grass carp (Padhi and Khuda-bukhsh, 1989), and carangids (Menezes, 1990). Investigations were also done on shrimps and oysters (Thomas, 1981; Puthran Prathibha, 1984; Ponniah, 1988; Kasinathan and Natarajan, 1980-81). Stock identification studies were done in mullets by Reddy (1977) and Rao (1981), cichlids (Mahobia, 1987),

penaeid prawns (Philip Samuel, 1987) and *Mugil cephalus* (Vijayakumar, 1992). Basic information on the techniques involved in fish genetics has been emphasised in C.M.F.R.I. special publication (Bye and Ponniah, 1983). Jhingran (1984); Das and Jhingran (1989) emphasised on the importance of fish genetic resources, its conservation and management in India.

The efficiency of biochemical genetics to ratify taxonomic status of fish species and identifying polymorphism is well recognised. The above review of literature points to the large volume of work being carried out in the world over in fish genetics using protein electrophoresis, since 1950's. The present literature review also reveals scarcity of works in Indian context. It in important to understand that fishery management should go hand in hand with biochemical genetics. The lack of such collaboration reflects the confusion and debates over the taxonomic status of several fish groups.

3. Materials and Methods

3.1. MATERIALS

3.1.1. Collection of sample.

The samples *(Decapterus russelli, D. macrosoma,. Selar crumenophthalmus* and *Megalaspis cordyla)* (Plate 1,2,3 &4) and for the study were collected from trawl and gillnet landings at Cochin fisheries harbour. Majority of the carangid catch were obtained from the trawlers operating at 25 km from the shore and 100-120m depth *(09^0 57'N, $76^0$14'E).* The fishes were collected soon after landings and were transported in crushed ice. Initially muscle, liver and eye-lens were dissected out from the samples for analysis. However, subsequently only muscle was used because it was giving more number of bands. Extreme care was taken to avoid red muscle while preparing tissue samples. Approximately, 1g of tissue was taken by weighing in an electronic balance and after that the tissue was stored in aluminium foil at —20^0C prior to homogenisation.

Plate 1 Decapterus russelli

Plate 2 Decapterus macrosoma

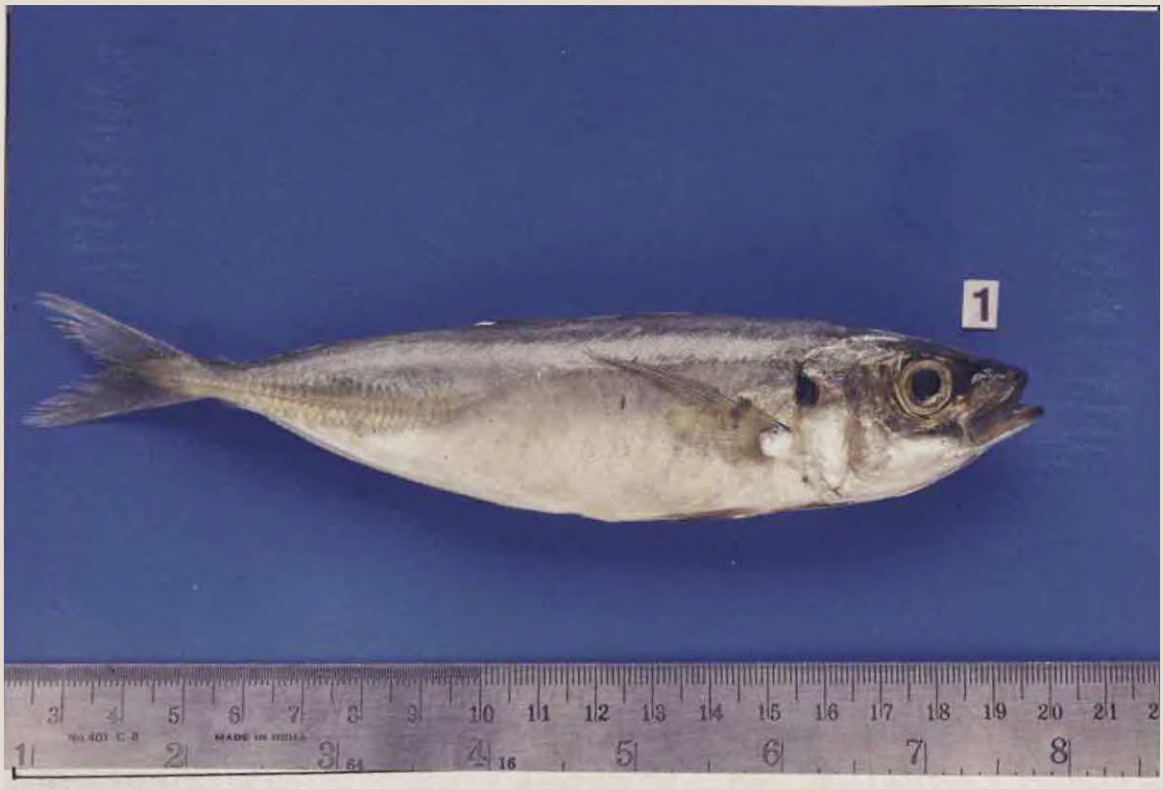

Plate 3 Selar crumenophthalmus

Plate 4 Megalaspis cordyla

3.1.2. Sample extraction

Protein extraction was done using double distilled water and 0.2M sucrose medium at different ratios of the sample, and homogenising media, (W/V) (1:1, 1:2 and 2:1)and 2:1 was found to be ideal. Homogenisation was done by using a manual glass homogeniser as well as a mechanical homogeniser (Remi). The homogenised sample was then transferred to eppendorf tubes and centrifuged at 4^0C and 10K. Duration of centrifugation was varied at 15 min, 30min and 1h. for standardisation. The supernatant obtained was then transferred to another eppendorf and kept at —20^0C for later use for electrophoresis.

3.2. METHODS

3.2.1. POLYACRYLAMIDE GEL ELECTROPHORESIS.

Horizontal slab polyacrylamide gel is the most recent material used for electrophoretic analysis of the samples. The principle of electrophoresis is that, the charged ions or groups will migrate towards one of the electrodes when placed in an electric field. Since proteins have different net charges and different molecular size, they will migrate at different rates within an electric field. The rate of electrophoretic migration depends on the net

charge differences, pH and the size and shape of the molecules. The rate of migration differ in different ions and their differences is used to separate components of a protein mixture.

3.2.2. REAGENTS

3.2.2.1. Gel buffer

Two types of gel buffers were used, one was the separating gel buffer and the other stacking gel buffer.

3.2.2.1.a. Separating gel buffer.

Tris buffer of pH 8.9 and 1.8 M was used as separating buffer, which was prepared by mixing 10.9g Tris buffer (Tris hydroxy methyl) Amino methane with 125µl TEMED. Separating gel buffer mixture was then dissolved in 50 ml double distilled water and the pH of the solution was adjusted to 8.9 by using 1N HCl as required. The prepared solution was then kept at 20^0C.

3.2.2.1.b. Stacking Gel Buffer

Tris buffer of 0.5M and pH 6.8 was used as stacking gel buffer which was prepared by using Tris buffer (Tris-Hydroxy Methyl) Amino methane = 3.02g

Tris buffer was made upto 50ml using double distilled water and pH of the solution was adjusted to 6.8 using Con. HCl and the solution was stored in bottles and kept at 4^0 C.

3.2.2.2. Tank buffer

Electrode buffer or tank buffer was prepared from Tris glycine at pH 8.3. Tank buffer was made by dissolving 36.04g of Tris glycine in 2.4 l of double distilled water. The pH was adjusted to 8.3 by adding 2 M Tris buffer stock..

3.2.2.3. 30% Acrylamide-Bis-acrylamide stock.

Following was the composition of the stock solution.

Acrylamide	= 29.1g
Bis- acrylamide	= 0.9g
Double distilled water	= 100ml

The solution was filtered using whatman No.1 filter paper and was stored in amber coloured screw capped bottles at 4^0C.

3.2.2.4. Ammonium persulphate

Ammonium persulphate (10%) stock was always prepared afresh by dissolving 0.1g of Ammonium persulphate in 1ml double distilled water.

3.2.2.5. Loading buffer

Composition of the loading buffer was as follows:

Glycerol	= 2ml
Double distilled water	= 7ml
Bromophenol dye	= 1ml

The loading buffer was kept in small vials at 4^0c. .

3.2.3. STANDARDISATION OF METHODOLOGY:

Standardisation of gel concentration was done with three different gel concentration such as 7%, 7.5% and 8% (Table 1) and there was no resolution of bands in 7% gel. Though with 7.5% and 8% gels resolved bands, better results from the former. Finally, Standardisation of the tissue

Table. 1 Gel composition used in electrophoretic analysis of carangids

Gel composites	7.0%		7.5%		8%	
	Separating gel (50ml)	Stacking gel (25ml)	Separating gel (50ml)	Stacking gel 25(ml)	Separating gel (50ml)	Stacking gel (25ml)
30% Acrylamide bis-acrylamide	11.67	2.91	12.5	3.125	13.33	6.67
Tris buffer (pH 8.9)	6.25	-	6.25	-	6.25	-
Tris buffer (pH 6.8)	-	6.25	-	6.25	-	6.25
Double distilled water	7.08	15.85	6.25	15.62	5.62	12.08
10% APS	+400*	100*	+400*	100*	+400*	100*
TEMED	-	25*	-	25*	-	25*

+ 0. 16% of APS in 25ml double distilled water.

* Micro litres.

for electrophoresis was done. Eye-lens, liver and muscle were taken for the analysis and electrophoretic pattern of muscle protein showed better result.

3.2.3.1. Casting of 7.5% gel

The mounting glass plates were first cleaned and wiped dry and then the two plates were fixed properly using clamps. To obtain 7.5% gel, first separating gel was made by mixing 12.5ml from the 30% acrylamide-bisacrylamide stock and 6.25ml double distilled water in a beaker. From the 10% APS stock solution, 400µl were taken and made upto 25ml. This APS solution was mixed with the acrylamide mixture in a beaker and poured in between the glass plates immediately. A layer of butanol was spread over it and the gel was allowed to set for some time. A layer of water was formed in between the gel and the butanol layer which gave an indication that polymerisation had taken place and the gel got set. Then the butanol and water layer were discarded and rinsed thoroughly with distilled water. Moisture was removed from the gel, using a filter paper. After that, the stacking solution was added on top of the separating gel. The stacking solution contained 2.91g arcylamide-bis-arcylamide, 6.25ml stacking gel buffer, 15.3 ml double distilled water, 100µl APS and 25µl TEMED. The gel was then allowed to set and was kept for 20min at 4^0C.

3.2.3.2. Sample application and Electrophoresis

After keeping the gel at 4^0C for 20 min, it was taken out and the glass plates with moulds for wells were lifted slowly from the gel. The gel along with the lower plane glass plate was then kept for a prerun for 15-20 minutes at 75mA current in a horizontal gel electrophoretic unit. Prior to that, the power pack was switched on and the cooler was set at 10^0C. While the prerun was going on, the sample was prepared with 30µl of loading buffer and 60µl of the sample supernatant. As soon as the prerun was completed, the 60µl of that sample was loaded into each well. The current flow of the powerpack was then regulated at 65mA. The run took about 2-2 1/2 h. Once the marker dye reached the anodic end, it gave an indication that the run was over and care was taken while running, so as to prevent run out of the marker . The gel was then taken out and the stacking gel part was cut using a gel cutter and was removed . The separating gel was then transferred to a base for staining.

3.2.3.3. Staining procedure for protein (Huriaux and Focant, 1977)

The separating gel was stained in coomassie brilliant blue (0.25%). Coomassie brilliant blue of 0.25% was prepared by adding 1.25g of

Coomassive brilliant blue, 230ml of double distilled water, 230ml of methanol and 40ml glacial acetic acid.

The stain was poured on to the gel and kept to take stain for 1 1/2-2 h

3.2.3.4. Destaining

After 1 1/2-2 h, the gel kept in staining solution was transferred to the destaining solution which was prepared by mixing 150ml methanol, 70ml acetic acid and 780ml double distilled water . The gel was then kept in destainer to remove the excess stain.

3.2.4. ANALYSIS OF THE GEL

The band patterns were observed in a transilluminator over visible light and zymogram of each gel was recorded carefully on a graph sheet for species/genera wise comparison and for scoring intraspecifc variation; samples were always run in the same gel and under identical conditions, exactly similar in all the respects. In the zymogram, bands were serially numbered, with the fastest band among all the species getting the first number and the slowest, the last. The most commonly occurring protein profile of each species was considered as the base picture of that species

and any variation to this pattern was separately recorded to study intraspecific polymorphism, if any.

The relative frequency of each fraction was estimated using the following formula.

$$\text{Relative frequency } (R_f\,\%) = \frac{\text{Distance travelled by the fraction}}{\text{Total distance travelled by the marker dye}} \times 100$$

4. Results

4.1. GENERAL PROTEIN PATTERNS OF CARANGIDS

Comparative picture of the sarcoplasmic protein profile of all the four species is presented in Fig 1. The number of protein fractions of the skeletal muscle of carangids under study ranged from 5 to 9. Maximum number of bands (9) were found in *Decapterus russelli* (Presumptive loci = 9) and minimum number was in *Megalaspis cordyla* (Presumptive loci =5) . *Selar crumenophthalmus* exhibited 6 bands (Presumptive loci = 6), while *Decapterus macrosoma* showed 7 bands (Presumptive (loci = 7).

The relative frequency (R_f)value of protein fraction in percentage for muscle is given in Table II. The R_f value ranged from 2 to 96 with different species. In *S. crumenophthalmus,* R_f value range was from 21 to 84; in *M. cordyla,* from 2 to 72; in *D. macrosoma,* from 15 to 89 and in *D. russelli,* it ranged from 18 to 96. The staining was most intense for the fraction 5,6,8,10 and 11 for *D. russelli*; 10 for *D. macrosoma*; 6,9 and 11 for *M. cordyla* and 6,8,10 and 11 for *S. crumenophthalmus.*

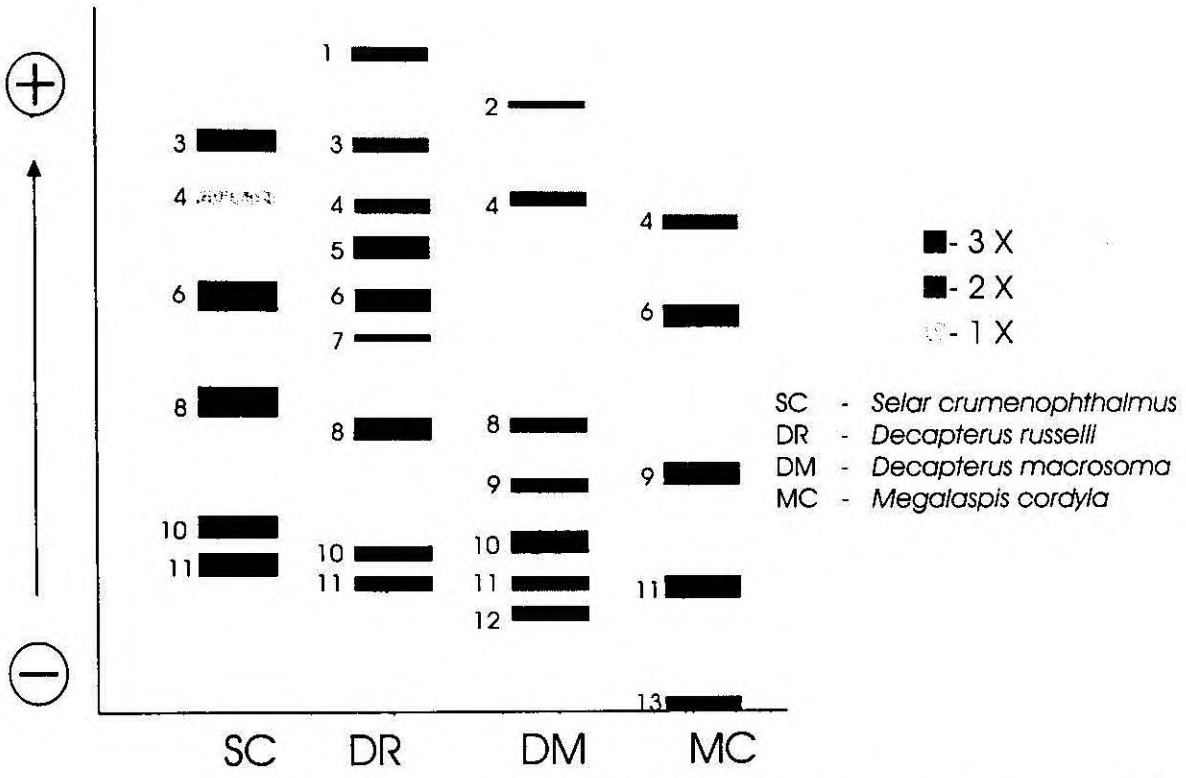

Fig.1. Zymogram of general protein in muscle tissues of carangids showing interspecific/inter generic difference

4.2. INTER-SPECIFIC COMPARISONS

4.2.1. *Decapterus russelli* with *Decapterus macrosoma*

Clear cut Interspecific variations were observed in the protein profile of *D. russelli and D. macrosoma* (Fig.1,Plate 5). In *D. macrosoma,* total number of bands were 7, while 9 bands were observed in *D. russelli.* The protein banding pattern of the two species showed much variation especially, with regard to the bands in the middle region. In *D. russelli,* the middle region is occupied by intensely staining bands 5,6 and 7; while in *D. macrosoma,* the entire region appeared without any bands. Of all the bands, only bands 4,8, 10 and 11 alone showed similar R_f values in both the species. (Table II).

4.2. 2. *Selar crumenophthalmus* with *Decapterus russelli*

In *S. crumenophthalmus*, the protein fractions corresponding to bands 1,5 and 7 of *D. russelli* were absent (fig. 1,Plate 6), this was the only striking difference between the two genera. The five out of 6 bands of *S. crumenophthalmus* showed similar staining intensity with five out of 9 bands of *D. russelli.* In *S. crumenophthalmus* and *D. russelli,* the R_f values

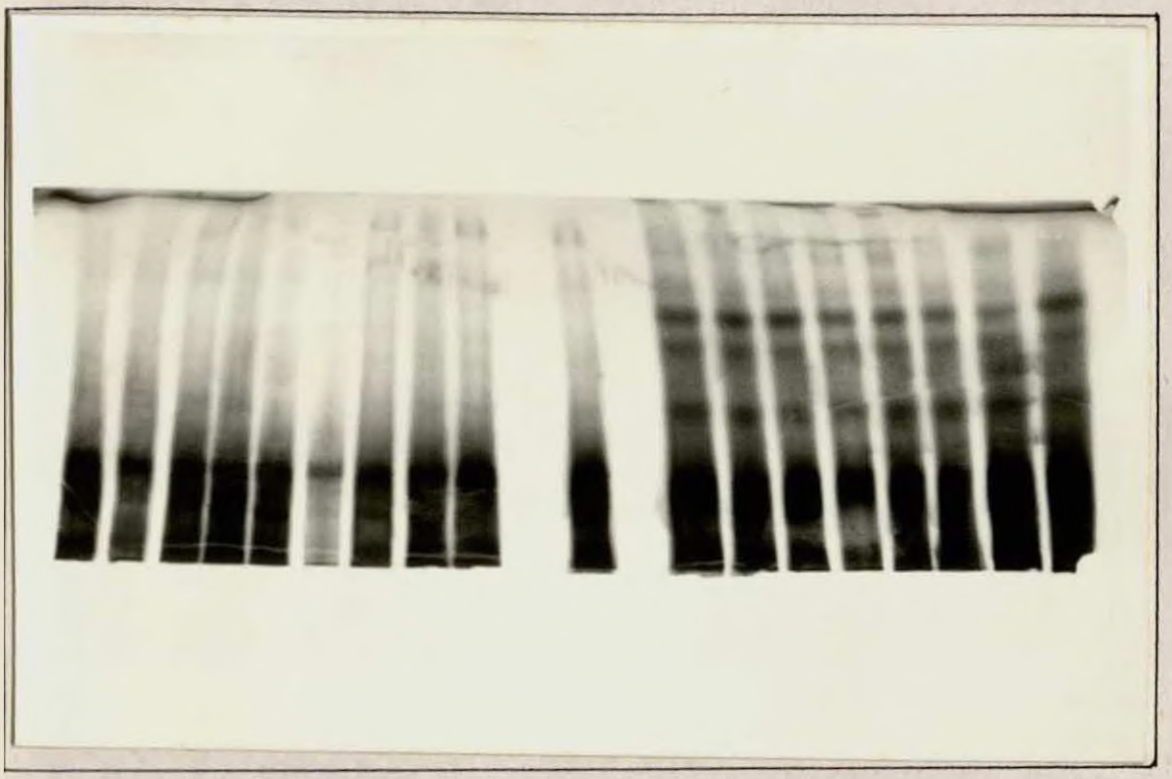

Plate 5 Sarcoplasmic protein patterns observed in *D. russelli*

(2-11, *D. macrosoma*; 13-20 *D. russelli*)

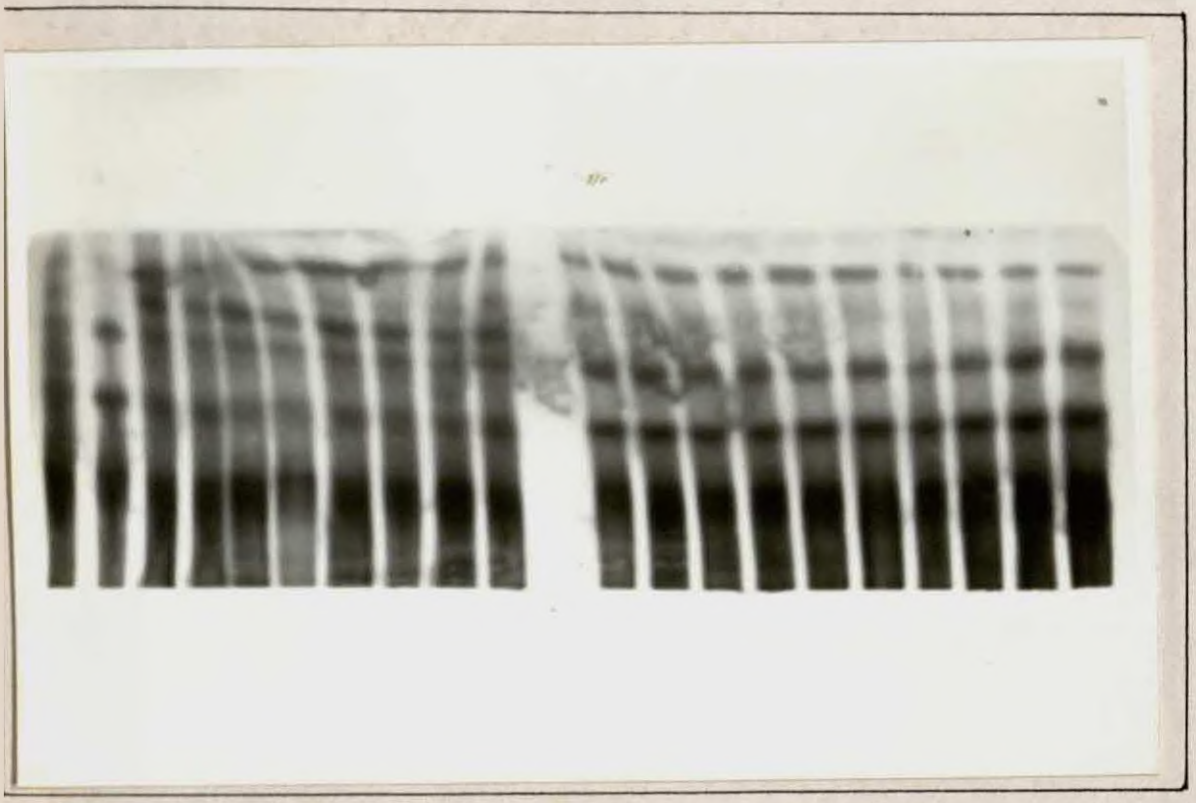

Plate 6 Sarcoplasmic protein patterns observed in *D. russelli* and *S. crumenophthalmus*

(2-10, *D. russelli*; 12-20 *S. crumenophthalmus*

were similar for bands 3,4,6 and 11 (Table II). Overall, both the genera appeared to exhibit similar protein band patterns.

4.2. 3. *Selar crumenophthalmus* with *Decapterus macrosoma*

The banding pattern and staining intensity differed widely between the two species (Fig 1). Among these two species, only one fraction (band number 10) showed same staining intensity, and only four bands, viz. 4,8,10 and 11 showed common R_f value (Table II).

4.2. 4. *Megalaspis cordyla* with other species.

The protein profile of *M. cordyla* was distinct with a prominent additional band at cathodal end (band number 13) which was not found in any of the other three species compared (Fig 1,Plate 7). Totally, there were five protein fractions. Two fractions, the fastest (band number 4) and the slowest (band number 11) showed same R_f value and staining intensity with other 3 species. Bands 4,6 and 11 of *M. cordyla* showed similar R_f value (Table II) and staining pattern of corresponding bands of *S. crumenophthalmus*. The intensely staining band 9 of this species showed similar R_f value with a less intense staining fraction of *D. macrosoma*.

Plate 7 Sarcoplasmic protein patterns observed in *M. Cordyla*, *D. macrosoma*, *D. russelli* and *S. crumenophthalmus*.

(1-5, *M. codyla*; 7-10, *D. macrosoma*; 12-15, *D. russelli*; 17-21, *S. crumenophthalmus*)

4.2. 5. Comparison of all the four samples.

The comparative picture of the four species provided clear cut species - specific banding patterns (fig.1,Plate 7). In *D. russelli,* band number 5 was specific to that species which was not found in any of the other species studied. For *D. macrosoma,* band 12 was specific and in *M. cordyla,* band 13 was specific. Band 8 was more prominently stained in *S. crumenophthalmus*. Close similarity of protein fractions was seen between *S. crumenophthalmus* and *D. russelli,* with regard to the position and staining reaction of protein fractions, rather than pattern found in the individuals of the same genus, *D. macrosoma* and *D. russelli.* Three out of five bands of *M. cordyla* (fraction 4,6 and 11) were also found to be closely similar with the same fractions of *S. crumenophthalmus.*

4.3. INTRA-SPECIFIC COMPARISON

4.3. 1. *Decapterus russelli*

The variation in the number of bands was comparatively very less within the species (Fig 2,Plate 8). Only one out of thirty samples exhibited an additional band between first and third bands, with staining intensity 2x

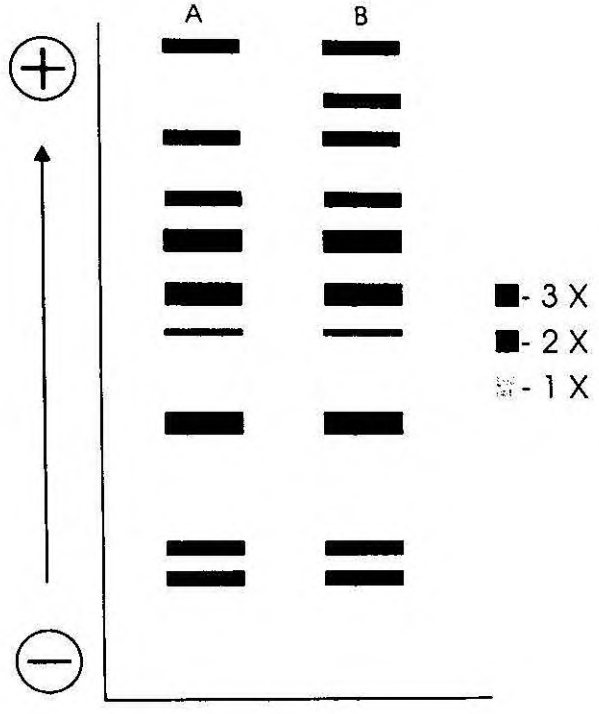

Fig.3. Zymogram showing intraspecific polymorphism in sarcoplasmic protein pattern of *Decapterus russelli*
A=96.5%
B=3.5%

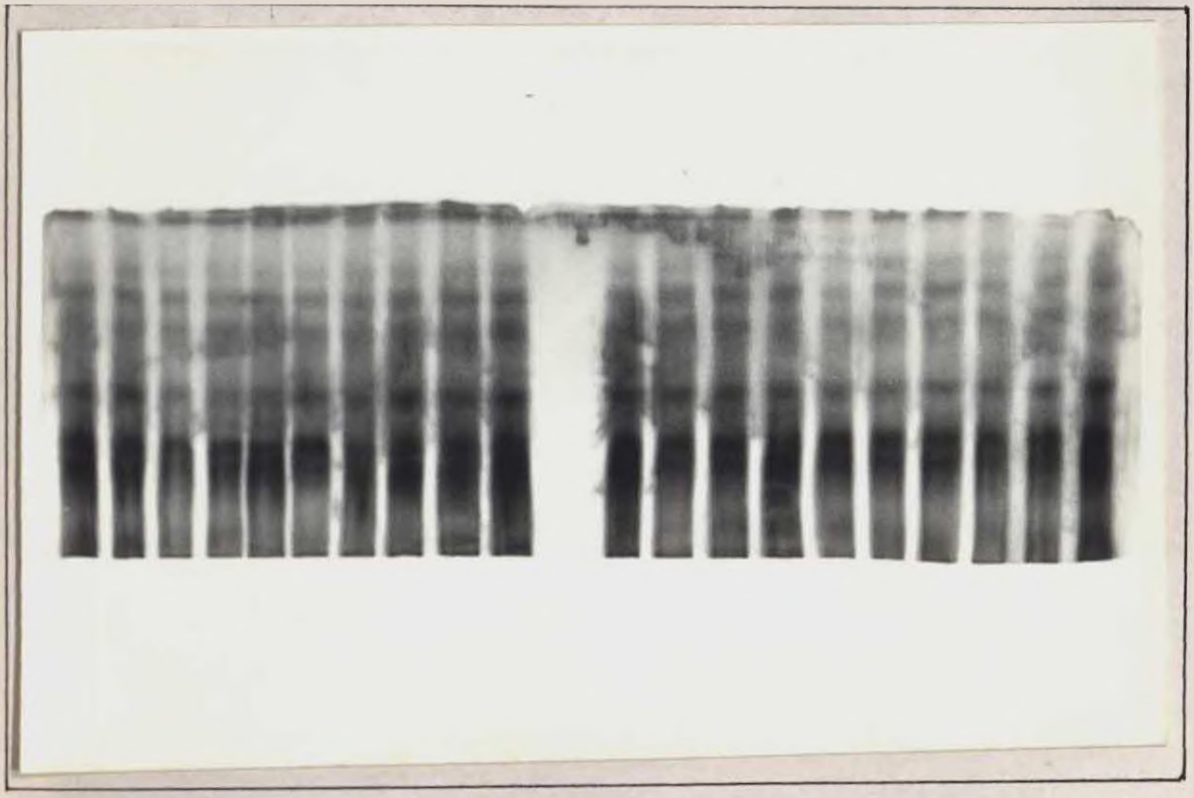

Plate 8 Sarcoplasmic protein patterns, observed in *D. russelli*.

and R_f value 87 (Table II). The indicative polymorphism in *D. russelli* was found to be 3.5 %.

4.3. 2. *Decapterus macrosoma*

In the thirty samples of this species studied, all of them except four showed similar protein fraction (fig.3,Plate 9). Out of the four, three exhibited a double banding pattern of intensity 2x and R_f values of 54 and 58 was found between bands 4 and 8 in the middle zone(Table II). The fourth specimen exhibited a single band in the place of double band which showed an intensity of 2x and shared the same R_f value 58 of the faster band of the former variant. In *D.macrosoma* the indicative polymorphism was 13.5%.

4.3. 3. *Selar crumenophthalmus*

All the samples showed similar banding patterns except in two individuals (fig.4,Plate 10), in which there was an additional protein fraction towards cathodal end with intensity 2x and R_f values 17 (Table II). The indicative polymorphism was found to be 6.6%.

4.3. 4. *Megalaspis cordyla*

All the thirty samples showed similar banding patterns(fig.8).

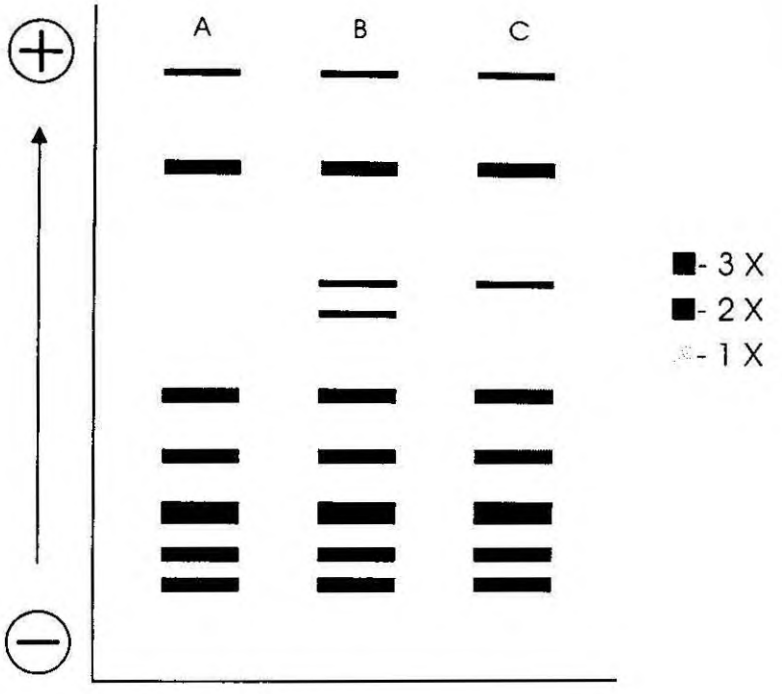

Fig.2. Zymogram showing intraspecific polymorphism in sarcoplasmic protein pattern of *Decapterus macrosoma*

A=86.5%
B=10%
C=3.5%

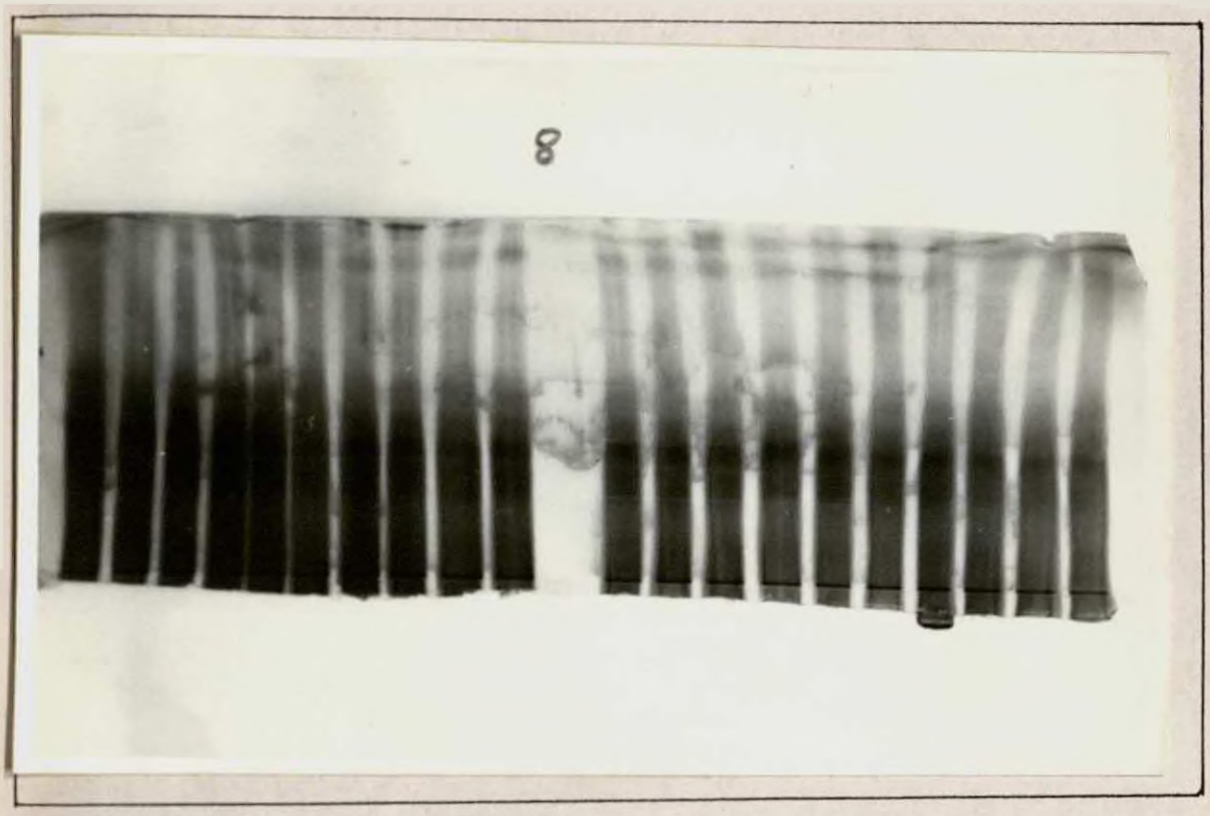

Plate 9 Sarcoplasmic protein patterns observed in D. macrosoma.

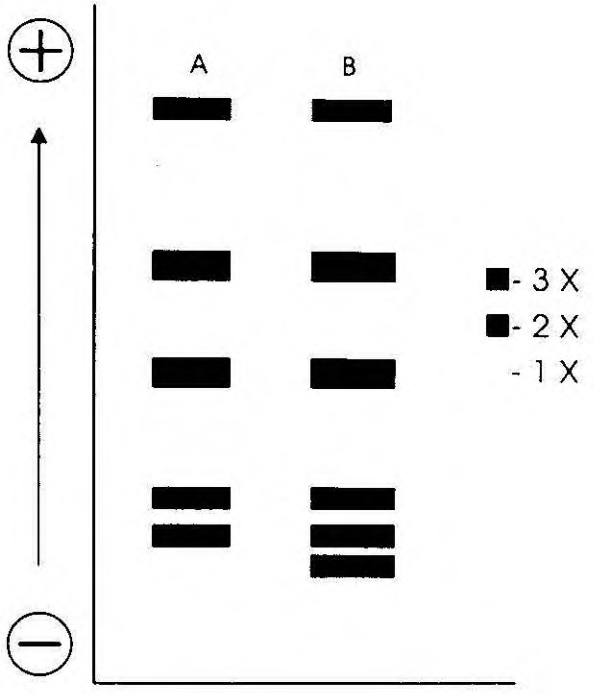

Fig.4. Zymogram showing intraspecific polymorphism in sarcoplasmic protein pattern of *Selar crumenophthalmus*

A=93.4%
B=6.6%

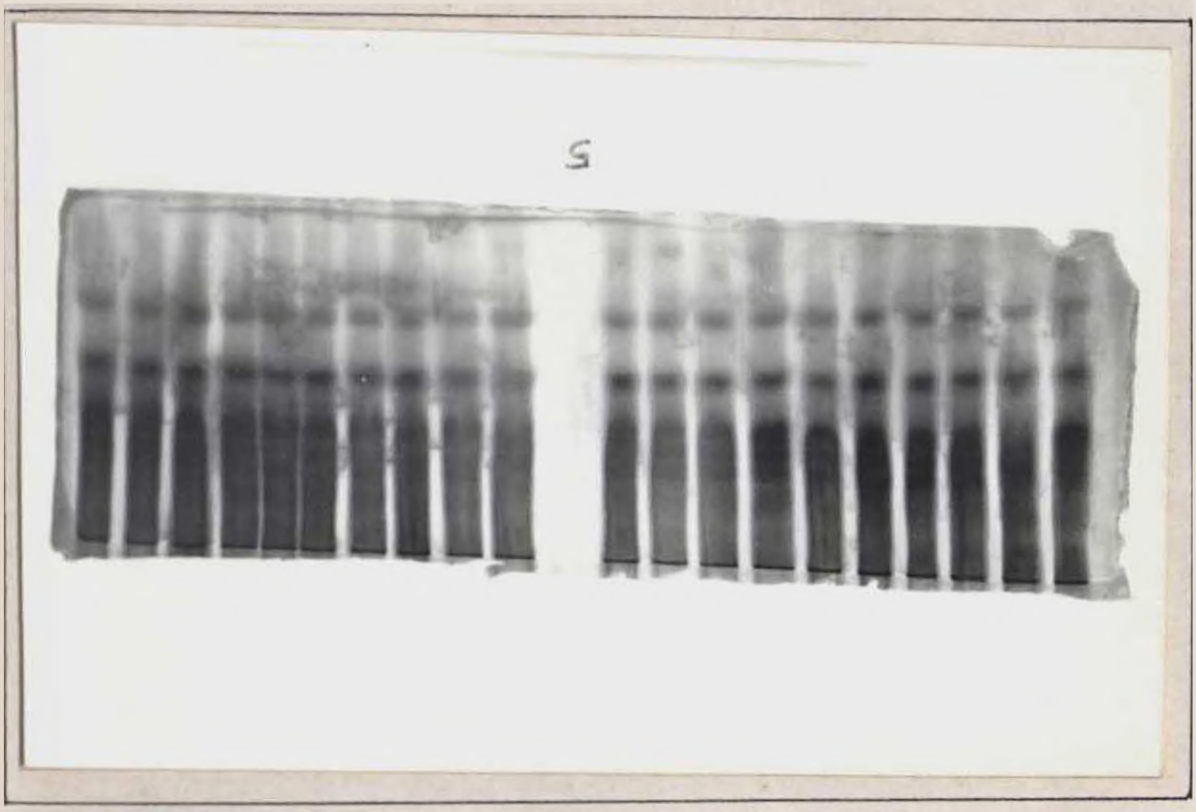

Plate 10 Sarcoplasmic protein patterns observed in S. crumenophthalmus.

Table II. R_f values of the four species of carangids

Fraction	R_f Values			
	Decapterus russelli	*Decapterus macrosoma*	*Selar crumenophthalmus*	*Megalaspis cordyla*
1	96	-	-	-
2	-	89	-	-
3	83	-	84	-
4	74	75	75	72
5	68	-	-	-
6	61	-	61	58
7	55	-	-	-
8	42	42	45	-
9	-	33	-	35
10	23	25	26	-
11	18	18	21	18
12	-	15	-	-
13	-	-	-	2

+

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5. Discussion

Molecular genetic approaches began to be used in fisheries in early 1950's with the of introduction of 'Zone electrophoresis' by Smithies (1955) and 'Zymogram' method byHunter and Market (1957). This added sensitivity and simplicity to the study of Protein Variation which enabled research workers to reveal various unknown treasures of knowledge about molecular facts and significance of protein study in numerous vertebrate and invertebrate organisms. These discoveries opened a new world of genetics 'the biochemical genetics' which proved that electrophoresis is a powerful tool for genetic studies.

Recent researches in biochemical genetics enabled technology perfection and formulation of procedure, but adopting it blindly may not give satisfactory result as it is known that the gel concentration, buffer composition, pH, amount of current applied, incubation period,species selected etc. can affect electrophoresis and the result. So experiments have to be done to standardise the procedure. The degree of resolution depends on the reliability and method adopted.

In the present short term electrophoretic study on some fishes of the family Carangidae, the general protein profile was examined using horizontal gel electrophoretic unit. Modifications were made with respect

to gel concentration, tissue ratio, homogenising media and time of homogenisation etc. from the standard methodology. According to Smith(1968) the upper range of gel concentration can go up to 30% generally electrophoretic studies in fishes is carried out at 7.5% gel (Herzberg and Pasteur, 1975; Khan and Gadru, 1988). In the present work with carangids too, 7.5% was found working best among the two concentrations, viz. 7%, 7.5% and 8% tried.

The samples for electrophoresis was usually homogenised either in sucrose, Tris buffer or double distilled water. A comparative study using 0.2M sucrose and double distilled water was done and found that double distilled water was ideal for muscle tissues of carangids as the extract in double distilled water produced sharp bands with less trailing. Puthran (1984) also found that double distilled water was giving better resolution compared to Tris buffer and sucrose medium in *P. monodon.*

The electrophoretic methods have been used successfully for fishes by various workers (Utter *et al.,* 1974; Kornfield and Koehn, 1975) Muscle proteins usually provide very valuable information. So are frequently used as a valuable diagnostic characters in the classification of fishes at species, generic, familial and higher taxonomic levels. The value of muscle myogen in phylogenetic studies and inter and intraspecific protein variation was

discussed by Tsuyuki *et al.* (1965a). Earlier the only method available for the study of intraspecific and racial variation was morphometric and meristic characters. However at present biochemical genetics has become a useful tool to supplement the same. Genetic inheritance of muscle protein pattern was also reported by interspecific hybridisation experiments (Tsuyuki and Roberts, 1965b).

Protein differences between species is specific for individuals representing a group. This could elucidate taxonomic problems in the case of disputed species (Smith *et al.,* 1979; Smith and Robertson, 1981). In electrophoretic technique, closely related species share many electrophoretic alleles but also differ at some gene loci at which they are fixed for different alleles (Smith *et al.,* 1990). Species-Specific nature of myogen patterns have been demonstrated in many fishes (Tsuyuki *et al.,* 1963, 1965, 1968; Herzberg *et al.*, 1975; Mackie and Jone, 1977).

If two different species have same number of electrophoretic fraction, further close comparison of relative mobility of one or more bands could reveal well defined species - specific differences (Puthran 1984). Thus in electrophoretic studies, species-specificity of a protein should mean primarily the electrophoretic mobility differences of one or more bands. Present study with four representatives of the family carangidae showed

species-specific banding pattern. In all the four species compared, the band number was different so they can be easily identified as species- specific bands. In the study, the banding pattern and mobility observed in each species was also specific *D. russelli, M.cordyla, S. crumenophthalmus,* and *D. macrosoma* exhibited 9,5,6 and 7 number of bands respectively. The R_f value of protein fraction also showed species-specificity which ranged from 21 to 84 in *S. crumenophthalmus*, 25 to 72 in *M.cordyla*, 18 to 96 in *D. russelli* and in *D. macrosoma* from 15 to 89. Similar work was done by Menezes (1976) in the eye lens and serum proteiins of *Sardinella longiceps* and *S. fiimbriata* and found that two species revealed major differences in protein fractions, mobility and amount of protein fractions with species specificity. Menezes (1979) conducted another similar study on flatfishes *Psettodus erume*i, *Brachiurus orientalis* and *Pseudorhombus arisus* and obtaned characteristic protein patterns of species. Herberg *et al.* (1975) and Mackie *et al.* (1977) studied on water soluble muscle proteins of different species of fish and found it to be species-specific. Menezes (1984) and Chakraborty (1989) found species-specific banding pattern in flying fishes and sciaenids respectively.

Chatterjee (1989) studied on primary structure of proteins in Indian air-breathing fishes and found that variation in primary structure of proteins

can be used in studying problems involving different taxonomic ranks. Taxonomical investigations have been greatly benefited by species-specific electrophoretic banding patterns of protein. (Menon, 1989). In the present study with four species, the electrophoretic banding patterns confirms their status as separate species which have undergone speciation long back indicating that the four species are wide apart in their origin.

The individuals of one species may differ from another if they have distant origin. It is not necessary that morphologically identical species should have similar protein banding, this characteristic of the protein banding is used to resolve problems of taxonomical ambiguity and other related disputes (Ponnaiah, 1988). In a comparative study of *D. russelli* and *D. macrosoma* it was found that even though they belong to same genus, *D. macrosoma* lacks the entire middle zone present in *D. russelli.* Of the nine bands in *D. russelli* and seven bands of *D.macrosoma* only band 4,8,10 and 11 alone showed similar R_f values in both species. This result shows that the two species were wide apart from the biochemical point of view eventhough morphologically they are almost identical. *D.russelli* and *S. crumenophthalmus* has similar banding patterns, the only striking difference being the presence of an additional band in the former (R_f value 68) compared *to S. crumenophthalmus.* The R_f values of both species

showed similarity at bands 3,4,6 and 11. These indicate a close relationship between the two species, despite the fact that they belong to two genera. Further studies have to be undertaken using isozymes, mt DNA, nuclear DNA etc. to arrive at a final conclusion on their genetic similarity. *M. cordyla* was found to have closer banding pattern to *S. crumenophthalmus* with similar R_f value for bands 4,6 and 11. Which leads to a possibility that *S.crumenophthalmus*, *D. russelli* and *M. cordyla* are closely related or they have common origin; however to confirm this, further work in required using molecular genetic tools. Matsuoka *et al.* (1995) in a similar work on genetic relationship of coregonid fishes, *Hypomesus nipponensis* and *H. pretiosus japonicus* proved despite their morphological similarity they formed separate species.

Genetic divergence between species can be used as genetic markers (Ayala, 1983). The genetic markers are usually proteins or enzymes. Ridgway and Klontz (1960) found that genetic markers could be used to genetically discriminate between populations of most fishes. Band 5 was prominent and intensily stained in *D. russelli,* siince this band was present only in this species this could be considered as species diagnostic protein marker. Similarly, band 12 for *D. macrosoma,* 13 for *M. cordyla* and 8 for *S. crumenophthalmus* were found to be species specific. Kapila and Kapila

(1996) found that genetic markers are superior to tags. Genetic markers was identified using isozymes in *Tilapia zilli* (Cruz *et al.*, 1982) Tuna (Fujino, 1966) and in several other fishes. These genetic markers will help to identify stock hybridisation and in various other analytical studies like stock assessment.

The first report of intraspecific muscle protein variation in marine fish species is that of *Anoplomoma fimbria* (Tsuyuki *et al.*, 1965a). Intraspecific variation is the raw material for population studies. The occurrence of polymorphic locus among individuals of same species which occur at molecular level is a valuable tool to determine heterozysosity which determine the potential of a population. In-depth study on Intraspecific polymorphism was done by Slechtova *et al.* (1992) in European white fish and peled fishes of family coregonidea. Which showed polymorphism in locus 13. Sacroplasmic protein of Nemepterids also exhibited polymorphism (Santos *et al.*, 1993). In the muscle and liver tissue of *Schilbe intermedius* 28-30% polymorphism was seen (Engel brecht *et al.*, 1994).

Present study on *D.russelli* showed comparatively low variation of about 3.5% with one of the protein fractions. The polymorphic band was in between the Ist and 3rd band with staining intensity 2x and R_f value 87.

In *D. macrosoma* the polymorphism observed was 13.5%. The samples of this species showed two types of variations two individuals with double band (10%) and one with single band (3.5%) No band(s) with similar R_f value(s) and intensity was observed in remaining samples (86.5%) of *D. macrosoma* indicating that it can be a non-genetic variation. To reach a proper conclusion, an in-depth study is needed using more number of samples from the same population of the species. In *S. crumenophthalmus* approximatly 6.6% polymorphism was observed.

The present work leads to a conclusion that the four species under study are valid species and exhibited well detectable species-specific protein banding patterns which can be used as diagnostic genetic markers. Much confusion exists in taxonomic identification of certain carangids, such as *D.russelli and D.macrosoma, D. kurroides and D. kurroides akaadsi, Caranx kalla* and *C.para, D. lajang and D. macrosoma, Carangoides cili* and *Citula diversa* based on morphological characters (FAO, 1984). The present study revealed that the sarcoplasmic protein profiles of carangids can be used as a valuable tool in resolving such

taxonomic ambiguity. It can also be applied to study the occurrence of natural hybrids, if any along the coasts. *D. russelli*, *S. crumenophthalmus* and *M. cordyla* are closely related to each other with respect to banding patterns. On the other hand *D. macrosoma* and *D. russelli* of the same genus showed entirely different banding pattern indicating that they could be distantly related.

6. Summary

- The present study deals with the electrophoretic profile of protein in four marine teleosts of the family carangidae (*Decapterus russelli, D.macrosoma, Selar crumenophthalmus* and *Megalaspis cordyla*) from Cochin.

- Electrophoretic methodology was standardised for separation of sarcoplasmic proteins for all the four species and found that 7.5% gel concentration was ideal. Ratio of the sample to homogenising media was also standardised and 2:1 (w/v) was found to be ideal. Double distilled water gave better resolution than 0.2M sucrose.

- The total number of electrophoretic fractions differed from species to species. In *D. russelli*, *D. macrosoma*, *M. cordyla* and *S. crumenophthalmus*, the number of bands observed were 9,7,5 and 6 respectively.

- The R_f values ranged from 21 to 84 in *S. crumenophthalmus*, 2 to 72 in *M.codyla*, 15 to 89 in *D. macrosoma* and 18 to 96 in *D. russelli*.

- Each species provided clear cut species specific banding patterns. In *D. russelli* band 5 was specific, whereas bands 12, 13 and 8 were specific for *D. macrosoma*, *M. cordyla S. crumenophthalmus,* respectively.

- Intraspecific polymorphism was indicated in three of the species studied. In *D. russelli,* polymorphic bands were seen in 3.5% of samples, in *D. macrosoma* in 13.5% samples and in *S. crumenophthalmus* 6.6% samples. No polymorphic bands were observed in *M. cordyla.*

- The present study gives a base-line picture of the sarcoplasmic protein profile of four species of carangids available along Indian coast. The electropherograms of all the four species studied were species-specific, hence can be used as diagnostic genetic markers for identification of these species. The protein patterns obtained can also be correlated with the same species or of other species of the family carangidae, to resolve disputes in the event of any taxonomic ambiguity.

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