KARYOMORPHOLOGY OF *LATES CALCARIFER*

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Shri. SUDHEESH, P. S.
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C E R T I F I C A T E

This is to certify that this Dissertation is a bonafide record of the work carried out by Mr. **SUDHEESH P.S.** under my supervision and that no part thereof has been presented before for any other degree.

Dr. GEORGE JOHN,
SCIENTIST S-2
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE,
COCHIN - 682 031.

Countersigned by:

Dr. P.S.B.R. JAMES,
DIRECTOR,
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE,
COCHIN - 682 031.

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PREFACE

Genetic research, particularly its applied aspects related to aquaculture organisms, is lagging far behind that of crop plants and farm animals. Todate, most effort in fish culture has been directed towards improved diets, health management and water quality management which deal with the environment in which the fish live. Basic genetic and breeding concepts deal with the animals itself and research in this area improve the biological potential of the fish.

Fish genetics is a virgin emerging field promising the production of cheap high quality fish protein. The chromosomal manipulation techniques of induced polyploidy, gynogenesis and androgenesis are likely to have significant applications in aquaculture. Other successful achievements include fast growing pond reared freshwater trouts and marine salmonids, the development of hybrid fishes, and recently the production of transgenic fishes using recombinant-DNA technology. An understanding of the genetic makeup and variability of aquaculture species is a prerequisite for these techniques and successful longterm viable aquaculture.

Study of karyotype of fishes is of much value in fish breeding. Karyotypic differences among species or taxa may be used to determine

phenetic similarities and phylogenetic relationships. In addition to understanding the systematic position of species, detecting gross genetic variation, cytogenetic studies would be an aid in experimental hybridization.

Though seabass (Lates calcarifer) culture is progressing tremendously in the Indo-Pacific region, the domestication of this species is still far away from the aquaculturists. The present study was aimed at the development of a suitable methodology for the chromosome preparations of Lates calcarifer. The chromosomes of L. calcarifer have been studied for finding the diploid number and also to examine the karyotype. Two populations from Cochin and Tuticorin were studied separately and karyotype prepared for both. The karyotypes of the two populations were compared. The chromosome morphology, the total length, relative length and arm ratios of the two populations were analysed and compared. This work is a priliminary step into the population genetics studies of L. calcarifer.

I express my deep sense of gratitude to my supervising teacher, Dr. George John, Scientist S-2, Central Marine Fisheries Research Institute, for his invaluable guidance and encouragement. I also express my thanks to the Director, Central Marine Fisheries Research Institute, Cochin for the facilities provided during the tenure of study. Thanks are also due to Dr. A. Noble, Scientist in charge of the PGPM for his help and

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INTRODUCTION

Cytogenetics is the study of chromosome morphology and the behaviour of chromosomes during meiosis and mitosis. Every organism is characterized by its own specific karyotype both in number and morphology. Fishes have been the subject of an increasing number of cytogenetic investigations in the areas of systematics, mutagenesis and aquaculture. Cytogenetic studies of fishes include those concerned with hybrid studies, polyploidy, chromosome morphology and behaviour, karyotype analysis, chromosome banding, sex chromosomes and supernumary chromosomes.

As cytogenetic studies of fishes are difficult compared to mammals and other groups, not much progress has been made in this field. Among approximately 20,000 - 23,000 living species of fishes, the chromosome number is known in only about 650 - 700 species and complete karyotyping has been made in about 500 species (Gold 1979). Early fish cytologists were handicapped by numerous technical difficulties resulting in several reports of chromosome number and morphology now considered incorrect (Chiarelli and Capanna, 1973; Denton, 1973; Ohno, 1974). Relatively recent developments of techniques have led to the current expansion of studies into the chromosomal basis of successful crossing and selection in economically important and cultivable fishes.

The development of cytogenetic studies by current air drying method combined with colchicine treatment has made accurate delineation

of chromosomes of somatic cells easier. Kosswig (1973) has reviewed the place of fish in genetic research. Fish cytogenetics has been reviewed in detail by Denton (1973), Gold (1979) and Blaxhall(1975).

The various techniques employed for the preparation of chromosome spreads of fishes include colchicine injections and squashes of the testes or haematopoietic tissues (Roberts, 1964; Ohno et al., 1965), corneal and conjunctival epithelium (Sick et al., 1962; Drewry, 1964), gill epithelium (Kligerman and Bloom, 1977; Mc Phail and Jones, 1966; Chen and Ebling, 1968), embryological material (Simon, 1963; Simon and Dollar, 1963; Swarup, 1959), sectioning of testes (Nogusa, 1960), scale epithelium (Denton and Howell, 1969) dry mount smears of gill epithelium and blastodisc smears (Stewart and Levin, 1968). Growth of various tissues in vitro (Roberts, 1964, 1966 and 1967; Chen, 1970), blood leukocytes in culture (Heckman and Brubaker, 1970; Ojima et al. (1970). Preparation of fish chromosomes by in vitro colchicine treatment was developed by Ida et al. (1978).

Advanced methods like the flow cytometry to determine cellular DNA content for screening polyploids and the various chromosome banding techniques, originally employed in mammals have been found useful in fishes.

Fishes are excellent examples of chromosomal polymorphism. Intrapopulation chromosomal polymorphism mainly due to Robertsonian translocation has been reported in Spicara, Mystus, white sea herring and African tooth carps (Kirpichnikov, 1981). He has also suggested that chromosomal variations between different populations of Aphanius, Lepomis, Lobitis and Salmo were related to intrapopulation or intra-individual variations of chromosome number.

Intraindividual variations of chromosome numbers were observed in rainbow trout (Ohno et al.,1965) and was found to be due to centric fusion (Junxiu, 1983). The variations in number was due to nondisjunction of chromosomes in the Atlantic Salmon (Barshiene, 1980).

The occurrence of Robertsonian polymorphism in different individuals of Rainbow trout was described by Thorgaard (1976). Post (1973) has discussed some hypothetical aspects on the formation of the large chromosome by fusion, translocation or by the development of heterochromatic substances.

A great deal of work has been done on cytotaxonomy of fishes (Booke, 1968, 1974, 1975). LeGrande (1975) revealed the evolutionary relationships among Pleuronectiformes by karyological studies. Karyotype studies were used for the classification of the trouts by Miller (1972). Ojima et al. (1973) studied the karyotypes of Acheilognathine fishes and discussed the phylogenetic problems.

Cytological studies in 6 species of cyprinidae was done by Nygren et al.(1976). Cataudella et al. (1973) worked out the karyology of some Mediterranean teleosts of the families Scorpaenidae, Serranidae, Labridae and Blennidae. Karyomorphology of mullets was studied in detail by Le Grande & Fitzsimons (1976), Cataudella and Capanna (1973). The karyology of Tilapia has been studied extensively (Badr and El-Dib, 1977; Chen and Chen, 1983; Majumdar and McAndrew, 1986).

Cytogenetic studies in North American minnows was done by Gold et al. (1981). Robinson and Potter (1981) investigated the chromosomes of lampreys. The karyotypic studies of 29 Mediterranean teleost fishes was done by Cano et al. (1982). The karyology of Chondrichthyes (Rajiformes) was done by Donahue (1974).

The problem of sex determination in eels was studied with the use of karyological methods by Passakas and Kelkowski (1973). The presence of ZW type of sex chromosomes were karyologically confirmed in Pacific anguilloid fishes by Park and Kang (1979).

The occurrence of lampbrush chromosomes in teleosts was described for the first time by Baumeister (1973). By the use of scanning electron microscope, Webb (1974) had demonstrated the three dimensional surface structure of the chromosomes and clearly visualized the centromeres.

Karyological investigations regarding 3 species of the family Percidae (Perca fluviatilis, Acerina cernua and Lucioperca lucioperca) were made by Nygren et al. (1968). Mayr et al. (1987) studied the karyomorphology of European Percidae by fluorescence banding technique and nucleolar organizer regions (NOR's) were located.

C-banding (constitutive heterochromatin) method in fish chromosomes was applied by Zenzes and Vioculescu (1975) followed by others (Ojima and Kurishita, 1980; Park and Grimm, 1981; Passakas, 1978). Ojima and Ueda (1982) identified ZW Chromosomes of conger eel by C-banding technique.

In India, the study of fish cytogenetics was started in 1960 on testes material using old techniques (Sharma et al., 1960). Fruitful karyotypic analysis was possible by the development of a kidney technique (Manna and Prasad, 1968). Natarajan and Subrahmanyam (1968) reported on the chromosomes of Tilapia mossambica. Subrahmanyam (1969) claimed that injection of 0.1% calcium chloride solution into estuarine mudskipper (Boleophthalmus boddaeri) enhanced mitotic divisions in gill epithelia, kidney and gonads. Subrahmanyam (1970) investigated the use of rotenone, a fish toxicant as a mitostatic agent useful for chromosome work.

Subrahmanyam and Natarajan (1970) noted the diploid complement in Therapon puta and T. jarbua to be 48 in both sexes and studied

the cytotaxonomy of the two species. Subrahmanyam and Ramamoorthi (1971) reported the diploid number in the estuarine worm eel Moringua linearis as 50.

Prasad (1971) reported the occurrence of interpopulation chromosomal variation in Clarius. Chatterjee and Majhi (1973) showed both sexes of Mugil parsia to possess 2n = 48 chromosomes, all acrocentric and without distinguishable sex chromosomes. Rishi (1973) studied karyotypes of eighteen marine fishes. Chromosomes of Channa and Anabas have been studied in detail (Manna and Prasad ,1973a, b).

Manna and Prasad (1974) reported the intrapopulation chromosomal variation in Mystus vittatus. Natrajan and Subrahmanyam (1974) studied the chromosomes of 13 estuarine teleost species collected from Porto Novo waters. Both rotenone and colchicine were used as mitostatic agents. Khuda-Bukhsh and Manna (1974) studied chromosomes of seven marine teleost fishes. Prasad and Manna (1976) carried out chromosome studies on Tilapia mossambica. Rishi and Bala (1977) investigated the chromosomes of marine fishes.

A checklist of diploid number in different species of fishes was prepared by Manna and Khuda-Bukhsh (1977b). The morphometrical analysis of chromosome complements of a number of fresh water species was carried out by Manna and Prasad (1977), Manna and Khuda-Bukhsh (1977a, 1978).

Ravindran and Ravindran (1978) reported the cytological abnormalities caused due to water pollution from factory effluents. Chowdhury et al. (1979) analysed the karyotypes of six marine fishes. Patro and Prasad (1979) studied the chromosomes of six marine percoid fishes.

Mukherjee and Manna (1980) investigated the effect of malathion on chromosomes of Tilapia mossambica. Patro and Prasad (1981) studied the chromosomes in five species of flat fishes and reported the presence of sex chromosomes. Chowdhury et al. (1982) reported the chromosome morphology in five species of Tetradontiform fishes and the diploid number was found ranging from 40-48.

Manna and Som (1982) have stressed the importance of fishes as the best monitor for the effects of genotoxic agents in polluted water. The occurrence of sex chromosomes in Mystus gulio was reported by Das (1983) and indicated that chromosome number in different species of marine fishes unlike freshwater forms were less variable.

Thus Indian cytologists determined the number, morphology and behaviour of some 125 species of fishes belonging to 40 families of teleosts of both inland and marine waters (Manna, 1983). The progress of fish cytogenetic research in India has been comprehensively reviewed by Manna (1984).

Cytogenetic information on cultivable brackishwater and marine fishes of India is by and large insufficient and inexhaustive. Since Lates calcarifer is a highly priced food fish and an exciting sport fish, the karyomorphological studies would be an aid in the genetic improvement programmes of the fish. Khuda-Bukhsh(1979) has reported on the chromosomes of Lates calcarifer. For the present study specimens from two populations of Lates calcarifer from Cochin and Tuticorin were karyologically analysed and compared.

MATERIALS AND METHODS

Collection and maintenance of experimental animals:

Lates calcarifer (family : Centropomidae, order : Perciformes) is widely known in parts of the tropical Pacific and Indian Ocean regions. In India it is distributed mainly on the East coast. It is found in Chilka lake, Hooghly-Mahtlah estaury, Mahanadi estuary, Tuticorin bay and sparsely in Cochin backwaters. For the present study the specimens of the size 80-120 mm were collected from the backwaters of Puduvaippu (Cochin) in Kerala and Tuticorin in Tamil Nadu.

Maintenance in the laboratory:

The live animals were transported to the laboratory in oxygenated polythene bags and maintained in fibreglass tanks containing water of salinity 5-10 ppt. Since the animals are highly carnivorous and predaceous, live prawns were given as food. The fishes were acclimatized for about one week before the experiments were conducted.

Based on available literature a number of methods for chromosome studies were tried as under. Modifications were also included in order to suit the test species.

METHOD 1: LeGrande and Fitzsimons (1976)

The animals were well fed and maintained in tanks with aeration. All specimens were immatures of undetermined sex. Live fishes received

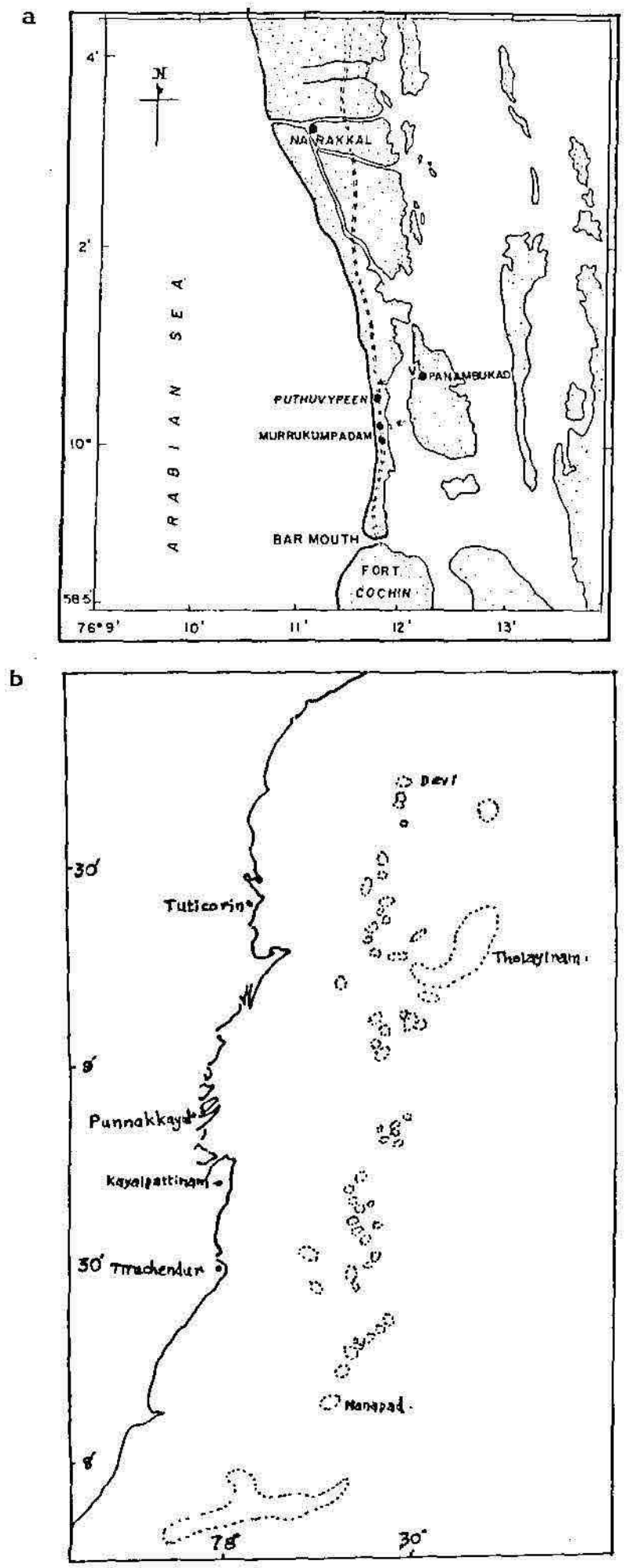

Fig. 1(a & b). Map showing the area of collection of specimens.

an intramuscular injection of about 0.01 ml. of 0.005% Colchicine (Sigma) per gram of body weight. The injected animals were allowed to reside in the tank with vigorous aeration. After 3 hrs the fishes were sacrificed by pithing and kidney and gill tissues dissected out. The tissue was minced in 2-3 ml of 1.0% sodium citrate solution at room temperature and allowed to stand for 30 minutes. After citrate treatment, the suspension was centrifuged 5-7 minutes at about 1500 rpm. The supernatant was decanted and the cell button fixed with absolute methanol:glacial acetic acid (3:1). After three washes in fixative, they were stored in the refrigerator till the spreads were made.

Before dropping the cell suspension on the slides, they were removed from the refrigerator and allowed to reach room temperature. Suspensions were dropped from a height of 15 cms on to the slides stored in chilled 50% alcohol and ignited. The slides were stained in a Giemsa working solution for 25 minutes. The working solution was prepared by adding 2 ml. of Giemsa stock solution to 98 ml of Sorenson's buffer (pH 6.8). After staining the slides were rinsed in distilled water and dried. The dried slides were stored in slide boxes for screening.

METHOD 2: Chourrout and Happe (1986).

The fishes kept in well aerated tank for 3½ hrs,after 0.005% colchicine (1 ml/100 gm body weight) had been injected into the dorsal muscle, were then sacrificed. The kidney and gills were dissected out and each tissue was transferred to 2 ml of 0.4% KCl solution for 30 minutes at room

temperature. The gill arch was removed from the base of the gill tissue. Both the tissues were cleared by removing blood and other impurities and cut into small pieces using scissors. The bigger particles were removed and the tissue suspension centrifuged at 1000 rpm. for about 7 minutes. The supernatant was decanted and the fixative (methanol : acetic acid (3:1)) added to the residue, resuspended and kept for 25 minutes at 4°C and then centrifuged again for 7 minutes at about 700 rpm. The supernatant was poured off and fresh fixative added. The fixed material was stored in the refrigerator. Before dropping the cell suspension on the slides, they were removed from the refrigerator and allowed to reach room temperature. Suspensions were dropped from a height of 15 cms on to the slides stored in chilled 50% alcohol and air dried. The slides were stained in a Giemea working solution for 25 minutes.

METHOD 3: Denton and Howell (1969). Modified.

About 5 cm sized animal was allowed to swim in well aerated colchicine solution (0.01%) in a beaker for 4½ hrs. The animal was sacrificed and the gills dissected out. They were given hypotonic treatment with 0.3% KCl for 20 minutes. The cell suspension made was centrifuged at 1000 rpm. for about 5-7 minutes. The supernatant was discarded, fresh fixative was added and the material kept in the refrigerator. Slides were prepared as in the method (2).

METHOD 4: Reddy and John (1986)..

The conditioned fishes were injected intramuscularly with 0.005% colchicine (1 ml/100 gm body weight) and kept in well-aerated tank for 3 hrs. The specimens were sacrificed by pithing and the kidney dissected out. After clearing the blood vessels, it was then transferred to 1% sodium citrate solution and cut into small pieces with scissors. It was kept at room temperature for 30 minutes and then transferred to a glass tissue homogenizer and gently agitated. After removing the large tissue particles, the cell suspension was centrifuged for 5 minutes at 1500 rpm. The supernatant liquid was decanted. About 4 ml of fixative was poured to the material and allowed to stand for 20 minutes. The material was again centrifuged before giving the final change of fixative and kept under refrigeration overnight. The slides were prepared as in method (2).

METHOD 5: (Squash method) Mc Phail and Jones (1966) Modified.

The fish was given a 0.005% colchicine (1 ml/100 gm body weight) injection into the anterior dorsal musculature and allowed to reside in a well aerated tank for 2 hrs. The animal was sacrificed and the posterior gill arch removed. It was hypotonized in 0.4% KCl at room temperature for 30 min and stained in2% Giemsa's stain for 20 minutes. The stained arch was shaken lightly on a clean slide until a light slurry of cells was deposited on the slide. Large pieces of tissues were removed. The slurry was immediately covered with a clean cover glass and squashed manually using a number 5 or 6 rubber stopper.

METHOD 6: Stewart and Levin (1968)

The fishes were given intramuscular injection of 0.001% colchicine (1 ml/100 gm body weight of the fish) and kept in well-aerated tank for 3 hrs. The fish was sacrificed and the fourth branchial arch dissected out. It was then transferred to 0.1 M KCN solution for 30 seconds and then hypotonized in double distilled water for 5 minutes. The tissue was then applied to a clean slide and dispersed with pressure from a clean scalpel blade and the smear of monolayered cells allowed to air dry. The slides were stained for 1 hour in 5% Giemsa, rinsed in distilled water and air dried.

METHOD 7 : Kligerman and Bloom (1977)

The fishes were allowed to reside in a well-aerated tank after an intramuscular injection of 0.001% colchicine (1 ml/100 gm body weight of the fish). After 2½ hrs the fishes were sacrificed by pithing and the kidney and gills dissected out. The individual tissues were transferred to 10 times their volume of 1% sodium citrate or 0.4% KCl hypotonic solution for 30 minutes. The blood vessels, mucous and other impurities were removed. The tissues were then fixed in methanol : glacial acetic acid (3 : 1) by slowly adding the fixative drop by drop. The fixative was poured off and fresh fixative added. The tissues were kept in a refrigerator. After about 1 hr. the fixative was again changed. For preparing slides, a few pieces of the tissue were removed from the fixative and touched to a piece of filter paper to remove excess fixative. The tissue was placed in an embryo cup and 5-8 drops of 50% acetic acid was added to it. The

tissue was minced gently for about 1 minute to form a cell suspension. This was dropped onto clean, grease-free slides heated to between 40 and 50°C, using a Pasteur pipette. The suspension was dropped from a height of about 8-15 cm and immediately after dropping it was withdrawn back into the pipette leaving a ring of cells approximately 1 cm diam. on the slide. Two or three rings were made on one slide. The slides were air dried and stained in 2% Giemsa's stain (2 ml of Giemsa's stain in 98 ml of Sorenson's buffer at pH 6.8) for 25-30 minutes. The summary of different treatment schedules are given in tables 1 and 2.

In all the above methods the slides could be observed with or without mounting. Mounting was done in DPX. About 14 specimens from Cochin and 10 specimens from Tuticorin were used for preparing metaphase chromosome spreads.

Karyotype preparation:

Metaphase plates of well spread chromosomes with distinct morphology were used for karyotyping. Since the prints meant for karyotyping should be as large as possible without loss of definition, prints with good magnifications were used for the study. The individual chromosomes were cut out from a photographic print with good contrast. Sharp scissors were used for cutting the chromosomes. The cut out chromosomes were placed in a petridish to prevent their loss. The homologous pairs were arranged and sticked to a hard white paper according to the morphology and total length. Terminology for centrometric positions followed the criteria established by Levan et al. (1964). If the centromere is median the chromosome

is said to be metacentric. If the centromere is submedian, its type is submetacentric. If the centromere is terminal, the type is acrocentric or telecentric and if the centromere is subterminal, the chromosome is subtelocentric. Sometimes chromosomes with terminal and subterminal centromeres are described as acrocentric. Levan et al., (1964) suggested that the chromosomes with these centromeric types may be designated as m., sm, st and t chromosomes, respectively. Each category of chromosome type was given definite numerical values based on arm ratios (length of the long arm divided by the length of the short arm) (L/S). Table 3 gives these values relative to the centromeric positions and chromosome types.

Well spread metaphase plates without overlapping chromosomes were photographed on KB21 EFKE Black and White Film 135-36, using an Olympus Diaplan microscope with 100X objective. Total length, relative length (100X chromosome length/total diploid length) and arm ratio were calculated for each chromosome in a spread. The NF value was calculated by giving points for each chromosome pair as NF of metacentric and submetacentric=2 and that of acrocentric=1.

Table : 1. Summary of different treatment schedules

Generalised schedule	Method 1 : LeGrande and Fitzsimons (1976)	Method 2 : Chourrout and Happe (1986)	Method 3 : Denton and Howell (1969)	Method 4: Reddy and John (1986)
Colchicine administration	1 ml (0.005%) Soln/ 100 gm body wt. exposure 3 hrs.	0.005% solution(1 ml/ 100 gm body weight), exposure 3½ hrs.	Animal kept in 0.01% solution 4 hrs.	0.005% (1 ml/100 gm body weight) solution for 3 hrs.
Tissue	Kidney and gill.	Kidney and gill.	Gill.	Kidney.
Hypotonic treatment	1% sodium citrate (30 min.).	0.4% KCl (30 min.).	0.3% KCl (20 min.).	1% Sodium citrate (30 min.).
Cell suspension	In hypotonic solution.	In hypotonic solution.	In hypotonic solution.	In hypotonic solution.
Fixative	Ethanol : acetic acid (3 : 1)	Ethanol : acetic acid (3 : 1)	Ethanol : acetic acid (3 : 1)	Ethanol : acetic acid (3 : 1)
Slide preparation	Suspension dropped on wet chilled slides, ignited/air dried.	Suspension dropped on wet chilled slides/warmed air dried.	Suspension dropped on wet chilled slides/ warmed, air dried.	Suspension dropped on wet chilled slides/ warmed, air dried.
Staining	2% Giemsa pH 6.8 20 minutes	2% Giemsa pH 6.8 30 minutes	2% Giemsa pH 6.8 30 minutes	2% Giemsa pH 6.8 20 minutes

2, 1 Continued

Table : 2. Summary of different treatment schedules

Generalised schedule	Method 5 : Mc Phailand Jones (1966) Modified	Method 6 : Stewart and Levin (1968) Modified	Method 7: Kligerman and Bloom (1977)
Colchicine administration	0.005% (1 ml/100 gm body weight). Solution for 2 hrs.	0.001% (1 ml/100 gm body weight), solution for 3 hrs.	0.001% (1 ml/100 gm body weight) solution for $2\frac{1}{2}$ hrs.
Tissue	Posterior gill arch.	Fourth branchial arch.	Kidney and gill.
Hypotonic treatment	0.4% KCl (30 minutes)	Treated with 0.1 M KCN for 30 seconds and then in double distilled water for 5 minutes	1% sodium citrate or 0.4% KCl (30 minutes).
Cell suspension	Squash method	Smear preparation	Fixed in methanol : acetic acid (3:1) and cell suspension in 50% acetic acid, ring of cells deposited on warm, clean, dry slides.
Fixative			
Slide preparation			
Staining	2% Giemsa pH 6.8 20 minutes	5% Giemsa pH 6.8 for 1 hour	2% Giemsa pH 6.8 25 minutes

Table : 3. Nomenclature for designating chromosome type

Centromeric position	Chromosone type	Arm ratio (L/S)	Anaphase configuration
Medial	Metacentric	1.00 - 1.70	V - shaped
Submedian	Sub-metacentric	1.71 - 3.00	J - shaped
Subterminal	Sub-telocentric	3.01 - 7.00	Rod - shaped
Terminal	Acrocentric or Telocentric	7.01	Rod - shaped


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R E S U L T S

The results are presented as three aspects.

a) Methodology,

b) Diploid number
   i) Specimens from *Cochin*
   ii) Specimens from Tuticorin

c) Karyotype
   i) Specimens from *Cochin*
   ii) Specimens from Tuticorin.

a) Methodology:

Various methods were tried based on the investigations by earlier workers on the preparation of fish chromosomes. The results of the various methods are summarized below.

Method 1: Le Grande and Fitzsimons (1976).

This method was found unsuitable because the chromosomes were highly contracted and clumped. *Sodium citrate* was effective in inducing cell swelling but the metaphase plates were incomplete and unfit for cytological evaluation. Few slides provided countable metaphase spreads, but the chromosome morphology was unsuitable for Karyotyping.

Method 2: Chourrout and Happe (1986):

The swelling of cells was less and chromosome spreads were rare. The chromosomes could not be counted because of clumping and shrinkage of chromosomes. The cell debris was accumulated over the chromosomes.

Method 3: Denton and Howell (1969).

Chromosomes were very small and unevaluable. The metaphase chromosomes were highly contracted and found unsuitable for screening.

Method 4: Reddy and John (1986).

Metaphase spreads were found but the plates were incomplete and the chromosomes with distorted morphology. The cell debris formed, interfered with chromosomes. The method appeared unsuitable.

Method 5: Mc Phail and Jones (1966).

The chromosomes were highly contracted and present as dark clumped bodies with severely distorted morphology. This method did not yield suitable metaphase plates for cytological evaluation.

Method 6: Stewart and Levin (1968).

Suitable metaphase plates were not available with this method. The method appeared unsuitable.

Method 7: Kligerman and Bloom (1977).

Excellent results were obtained by this method. Low concentrations of colchicine were tried and found very useful to maintain the morphology of chromosomes intact. The chromosomes were larger compared to the results from other methods. The centromeric position could be easily located. The 0.8% sodium citrate hypotomic treatment was found suitable to get optimum swelling of the cells. The hypotonic treatment at temperatures around 4°C was found to give better results. This method provided sufficient number of metaphase plates per slide, comparable to in vitro methods and thus enabled Karyotyping. Some variations tried are summarized in Table 4.

Both gill and kidney tissues gave fairly good results. In kidney tissue preparations, the connective tissues had to be removed to prevent them from mixing with the chromosomes. Cell swelling was more in kidney tissue preparations. However, gill tissue was found more suitable than kidney tissues.

Small fishes of 8-15 cm size gave more dividing cells while in large specimens metaphase spreads were fewer.

b) Diploid numbers:

Chromosome spreads were examined from 14 animals collected from Cochin backwaters and 10 animals from Tuticorin. The frequency

Table 4: Treatment and results of method 7 (Kligerman and Bloom - 1977).

	Tissue	Colchicine solution	Colchicine exposure	Hypotonic medium, soidum citrate solution	Hypotonic treatment	Fixative	Acetic acid suspension	Results
1.	Gill and kidney	0.005%	2.5 hours	1%	15 minutes at room temperature	Methanol-acetic acid (3.1)	40%	No chromosome spreads
2.	Gill and kidney	0.005%	2.5 hours	1%	15 minutes at 4°C temperature	Methanol-acetic acid (3.1)	40%	No chromosome spreads
3.	Gill and kidney	0.001%	2.5 hours	0.8%	30 minutes at room temperature	Methanol-acetic acid (3:1)	50%	Chromosome spreads seen
4.	Gill and kidney	0.001%	2.5 hours	0.8%	30 minutes at 40°C temperature	Methanol acetic acid (3:1)	50%	Very good chromosome spreads seen

of diploid numbers of specimens from Cochin and Tuticorin studied separately. The frequency of diploid numbers of specimens from Cochin and Tuticorin are shown in Tables 6 and 8 respectively. The animal wise distribution of diploid numbers are given in Tables 5 and 7 for Cochin and Tuticorin specimens respectively.

In both the populations the modal diploid number was found to be 48. It was observed in the maximum number of 123 metaphases counted from all of the 14 animals collected from Cochin. The counts were negatively skewed. No other modes were observed. The remaining diploid numbers observed seem to fall into the pattern of a normal distribution.

The modal number of 48 was observed in 128 metaphases counted from all of the 10 specimens from Tuticorin. As in the specimens from Cochin the counts were negatively skewed. Counts above 48 was observed in Tuticorin specimens. The high diploid counts are ascribed to the mixing of adjacent metaphase plates during dropping. Counts below 48 presumably represented chromosome loss during slide preparation. Metaphase plates showing diploid number of 48 are shown in plates 2 and 3 for Cochin and Tuticorin specimens respectively.

c) Karyotype:

The karyotype of specimens from Cochin and Tuticorin are shown in plates 4 and 5 respectively. The karyotype consists of 19 pairs of acro-

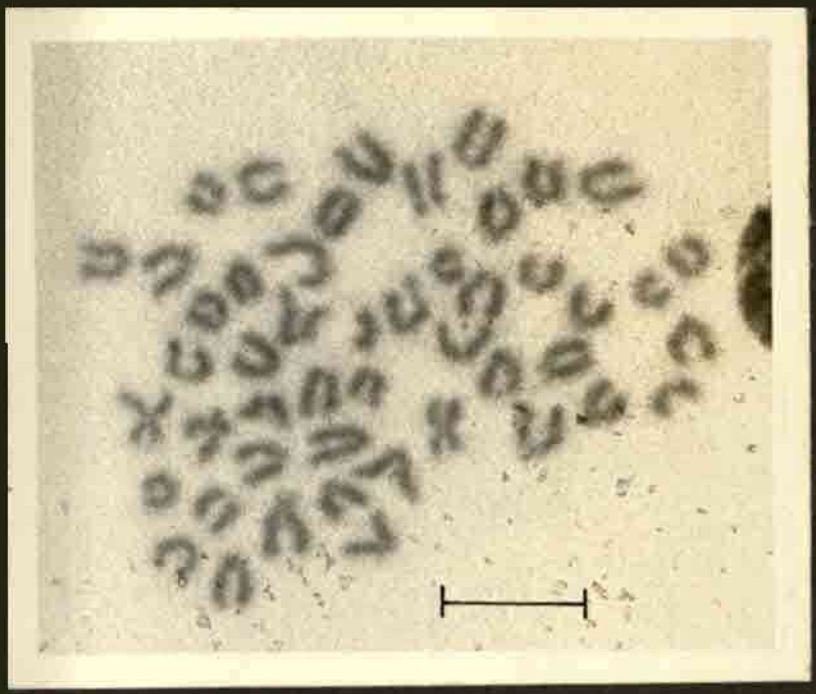


PLATE - 2 Somatic chromosome complement of L. calcarifer (Cochin)
Bar = 10 μ.

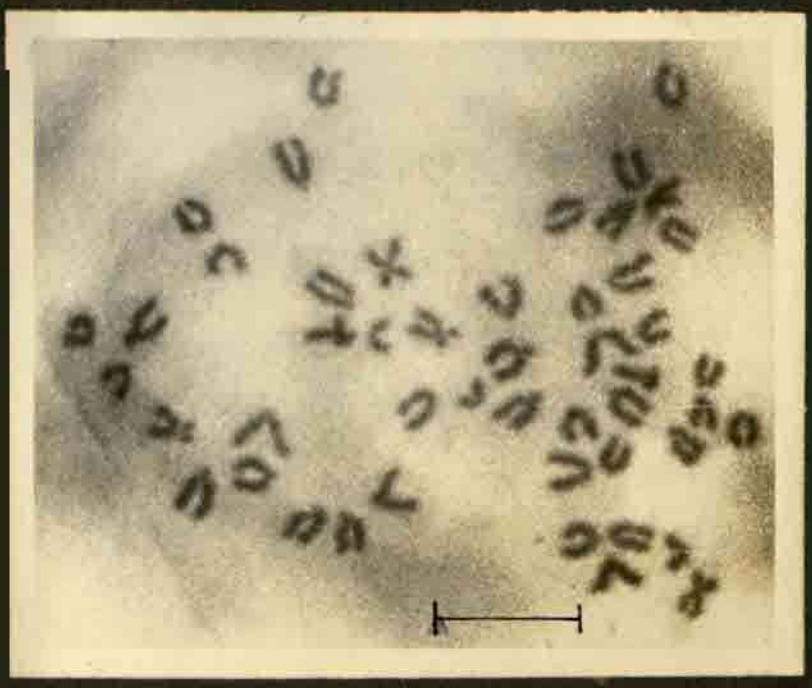


PLATE - 3. Somatic chromosome complement of L. calcarifer Tuticorin

Table 5: Animalwise distribution of diploid numbers of specimens from Cochin.

Test animals	Diploid Numbers																						
	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1.	1	1									1					1				1	1		6
2.	1				1													1		1	2	1	8
3.				1					1								1			3	2	1	6
4.													1				2	1				1	9
5.															1					1			
6.											1			1		1	1		1				3
7.																	1		1		1	1	10
8.																1	1	1			1	1	14
9.														1	2								5
10.																							14
11.																					1	2	10
12.																						1	6
13.		1		1													2				2		18
14.							1									2				1	3		14

Table 6: Frequency of Diploid numbers, metaphases counted from 14 animals from Cochin.

Diploid numbers	Frequency
26	2
27	2
28	-
29	2
30	1
31	-
32	1
33	-
34	1
35	-
36	2
37	-
38	1
39	2
40	3
41	5
42	8
43	3
44	2
45	7
46	13
47	8
48	123
Total number of metaphases 186.	

Fig. 2. Frequency of diploid numbers of Cochin specimens

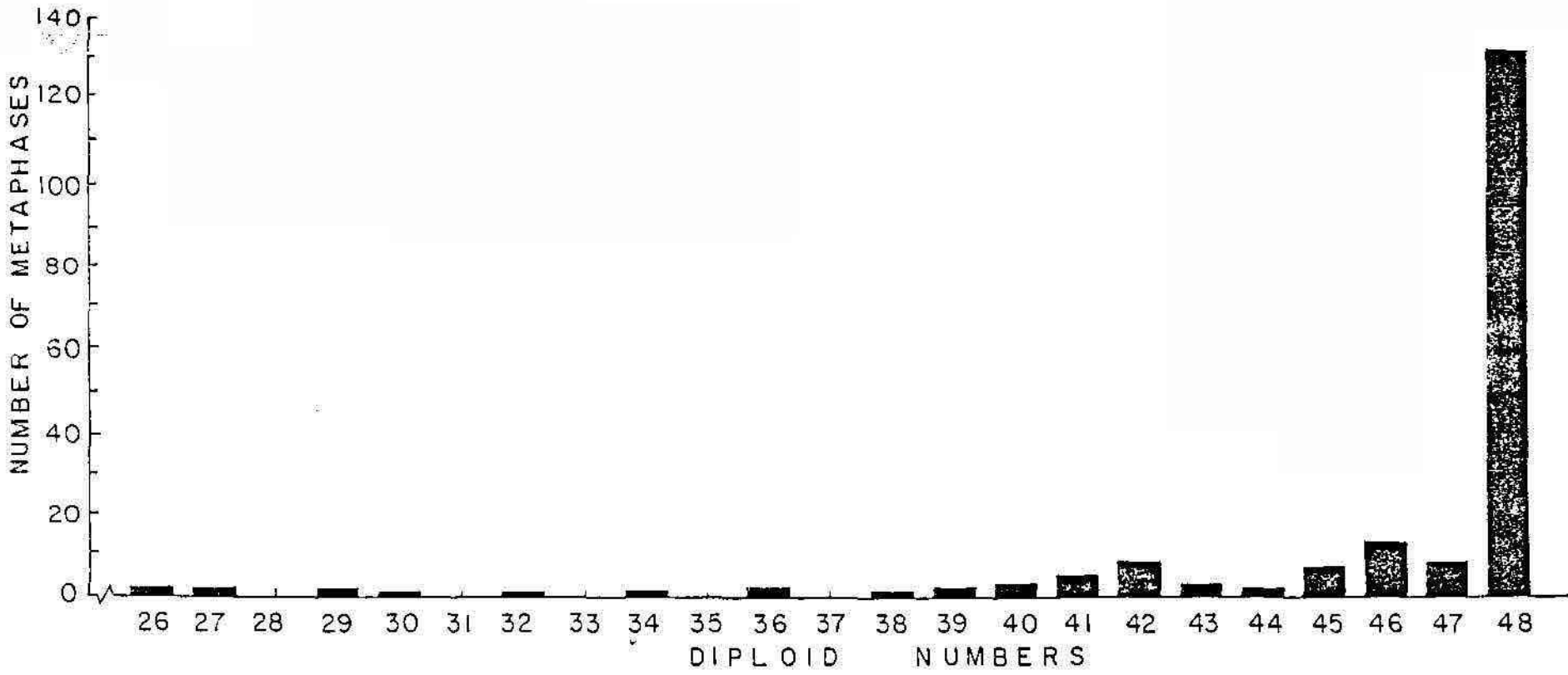


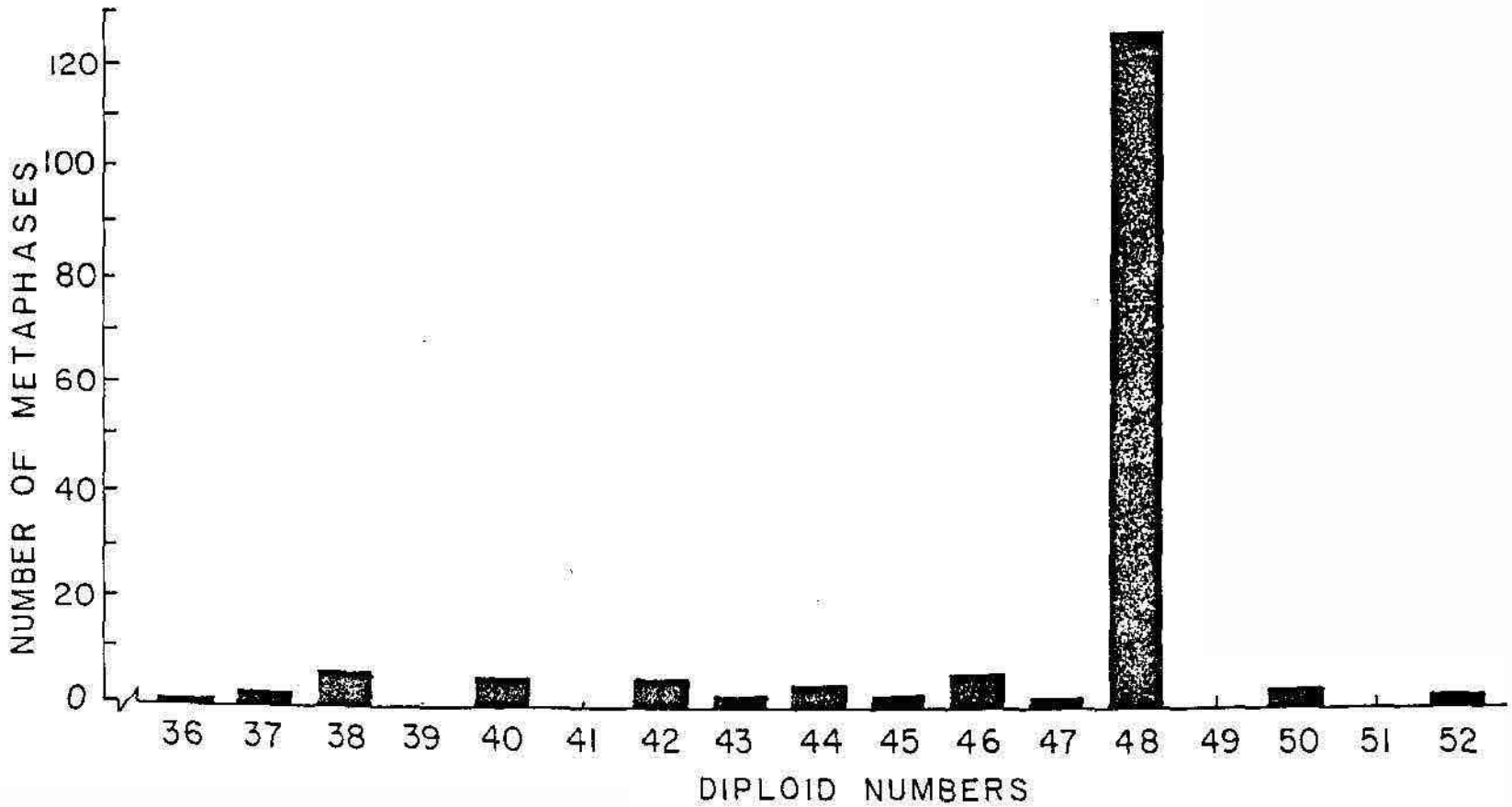
Table 7: Animalwise distribution of diploid numbers of specimens from Tuticorin.

Test animals	Diploid numbers																
	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
1.	1				1		1		1				7		1		
2.			2								2		12				
3.			1					1		1		1	8				
4.		1			1				1		1		14				1
5.					1			1					6		2		
6.			2				1						26				
7.		1			2				1				9				
8.							1				2		18				
9.			1							1			13				
10.							2		1		1		15				1

Table 8: Frequency of diploid numbers; metaphases counted from 10 animals from Tuticorin

Diploid numbers	Frequency
36	1
37	2
38	6
39	-
40	5
41	-
42	5
43	2
44	4
45	2
46	6
47	1
48	128
49	-
50	3
51	-
52	2
Total number of metaphases 167.	

Fig. 3. Frequency of diploid numbers of Tuticorin specimens



centric chromosomes. 4 pairs of submetacentric Chromosomes and 1 pair of metacentric chromosomes. All chromosomes were homomorphic pairs. Sex chromosomes were morphologically unidentifiable in the two populations. Morphometric analysis of chromosomes were done on four metaphases each from Cochin and Tuticorin specimens. The total length and the relative length of the 24 pairs of chromosomes and the arm ratios estimated for metacentric and submetacentric chromosomes of L. calcarifer from Cochin are given in Table 9 and that of Tuticorin specimens are given in Table 10. The total length of the chromosomes ranged between 3.7504 /um and 1.7875 /um in the specimens from Cochin. The average chromosome length was estimated to be 2.9284 /um. The NF value (Fundamental number of arms) was found to be 55.

In the specimens collected from Tuticorin the total length of the chromosomes ranged between 3.7319 /um and 1.9471 /um. The average chromosome length was estimated to be 2.988 /um. As in the case of specimens from Cochin, the NF value was found to be 55. An idiogram of L. calcarifer is shown in Fig.6.

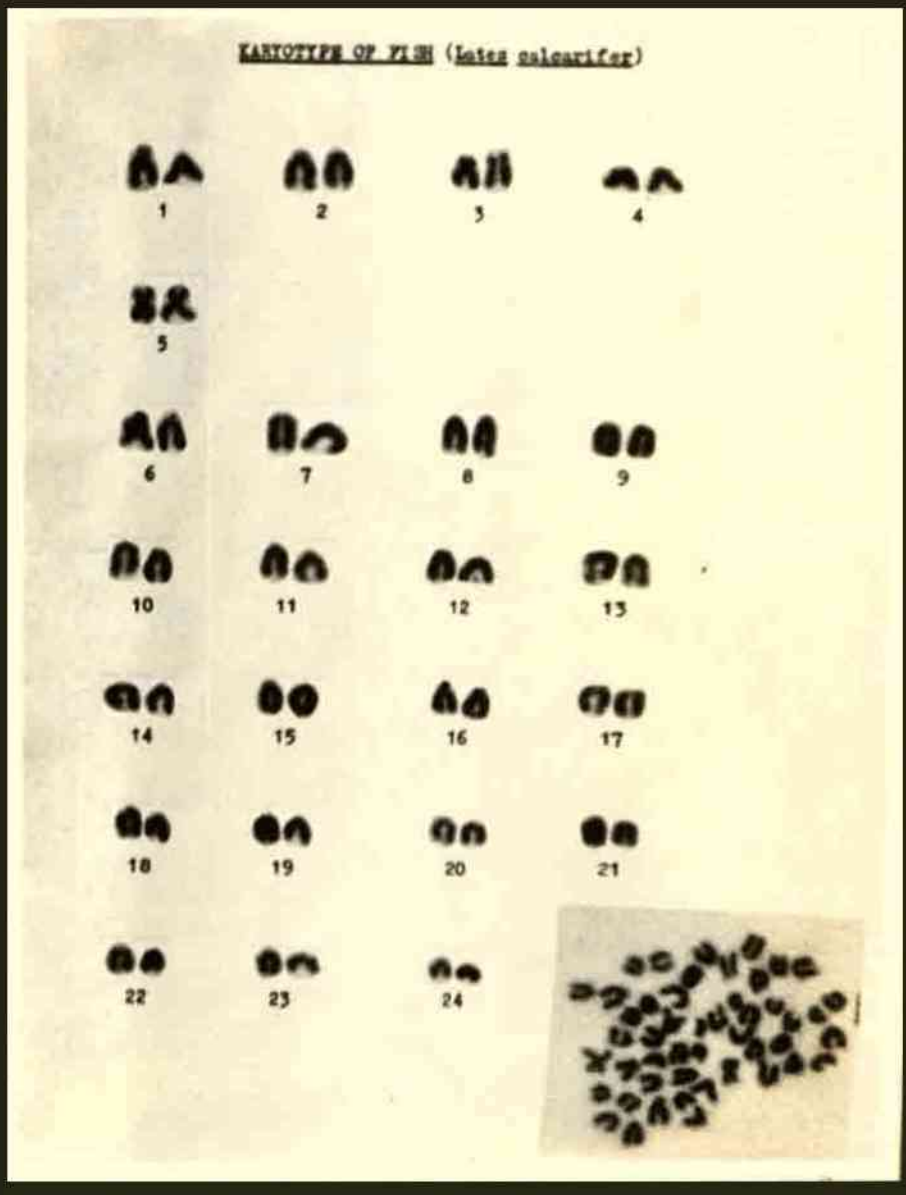


PLATE - 4. Karyotype of L. calcarifer (Cochin).

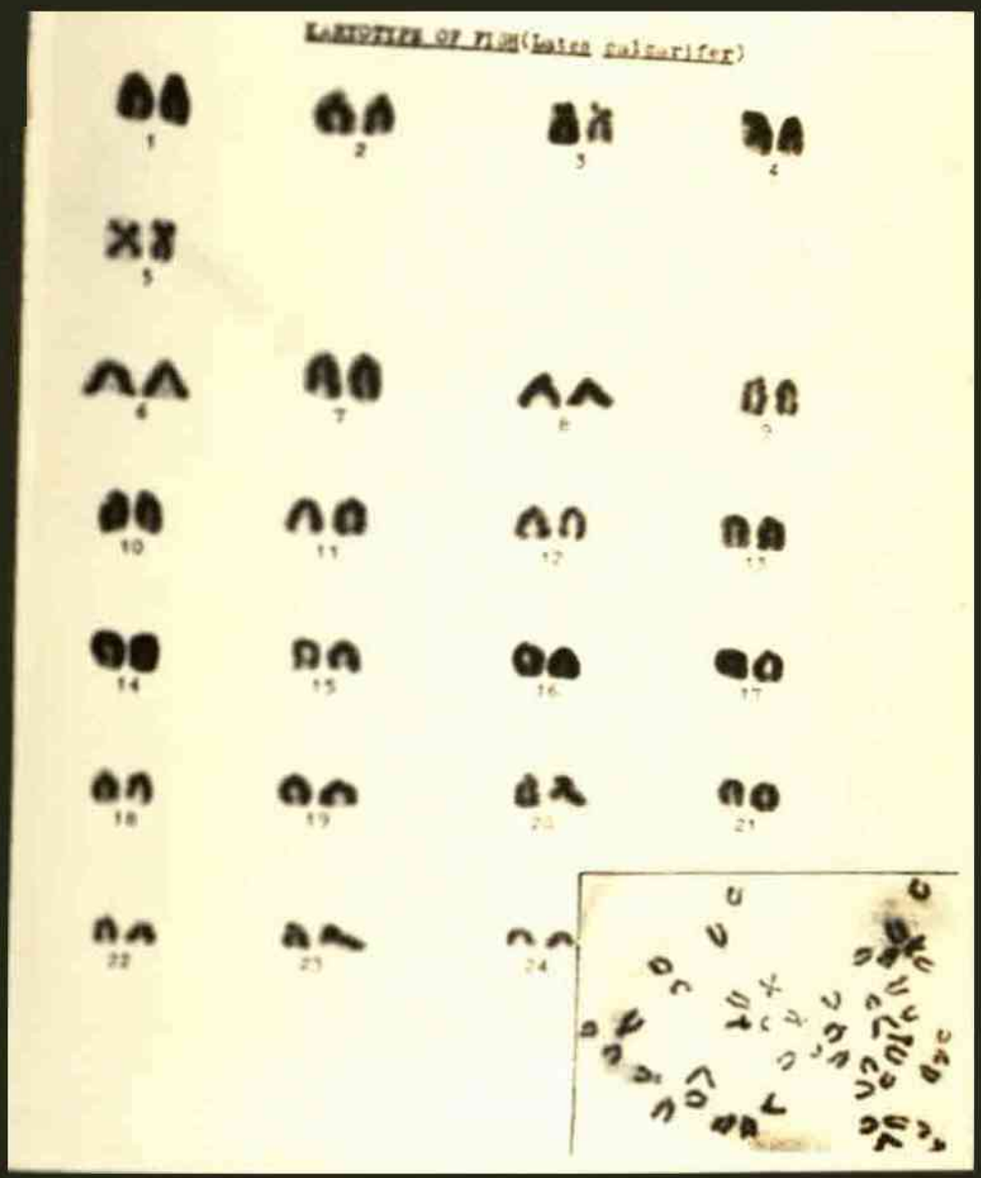


PLATE - 5. Karyotype of L. calcarifer (Tuticorin).

Table 9: Total chromosome lengths, relative lengths, and arm ratios for L. calcarifer collected from Cochin.

*Chromosome pair no.	Total length (μm) ($\bar{x}$ ± S.D.)	Relative length (%) ($\bar{x}$ ± S.D.)	Arm ratio ($\bar{x}$ ± S.D.)
1.	3.7504 ± 0.2739	2.6681 ± 0.01947	1.8293 ± 0.08422
2.	3.5839 ± 0.0251	2.5496 ± 0.01785	1.9385 ± 0.04037
3.	3.3718 ± 0.03108	2.3987 ± 0.02210	2.5851 ± 0.03226
4.	3.2599 ± 0.02279	2.3191 ± 0.01622	2.04295± 0.0821
5.	3.2792 ± 0.03262	2.3328 ± 0.02323	1.009978±0.069638
6.	3.2954 ± 0.03671	2.3327 ± 0.02265	-
7.	3.2790 ± 0.01949	2.3444 ± 0.00309	-
8.	3.2537 ± 0.0354	2.3170 ± 0.03073	-
9.	3.2413 ± 0.0019	2.3059 ± 0.001109	-
10.	3.0604 ± 0.07654	2.1772 ± 0.05447	-
11.	3.0194 ± 0.0576	2.1480 ± 0.04097	-
12.	2.9912 ± 0.0390	2.1279 ± 0.03028	-
13.	2.9723 ± 0.0086	2.1145 ± 0.006375	-
14.	2.8508 ± 0.1179	2.0281 ± 0.08503	-
15.	2.7893 ± 0.1394	1.9943 ± 0.09481	-
16.	2.7707 ± 0.0873	1.9710 ± 0.06215	-
17.	2.7274 ± 0.1243	1.9403 ± 0.04686	-
18.	2.6361 ± 0.0625	1.8754 ± 0.02770	-
19.	2.5938 ± 0.07639	1.8452 ± 0.05121	-
20.	2.5783 ± 0.0738	1.8360 ± 0.05769	-
21.	2.5037 ± 0.0709	1.7811 ± 0.05043	-
22.	2.4311 ± 0.0339	1.7295 ± 0.02411	-
23.	2.2563 ± 0.0423	1.6051 ± 0.03010	-
24.	1.7875 ± 0.493	1.2716 ± 0.03512	-

*Chromosome pair no.1 to 4 submetacentrics, 5 metacentric and 6 to 24 acrocentrics. Total complement length = 140.5632 μm.

Fig.4. Histogram of chromosome lengths of L. calcarifer (Cochin).

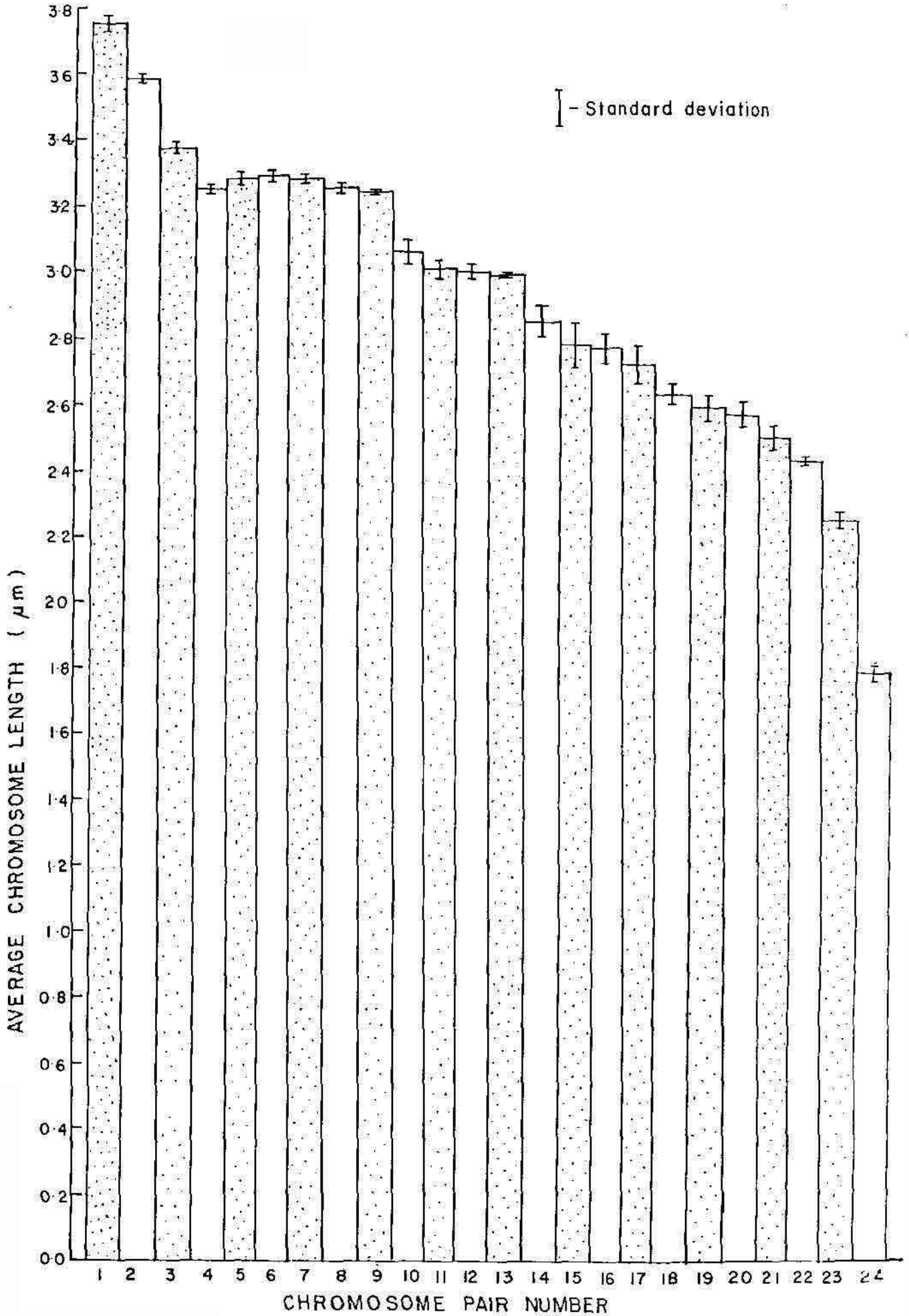
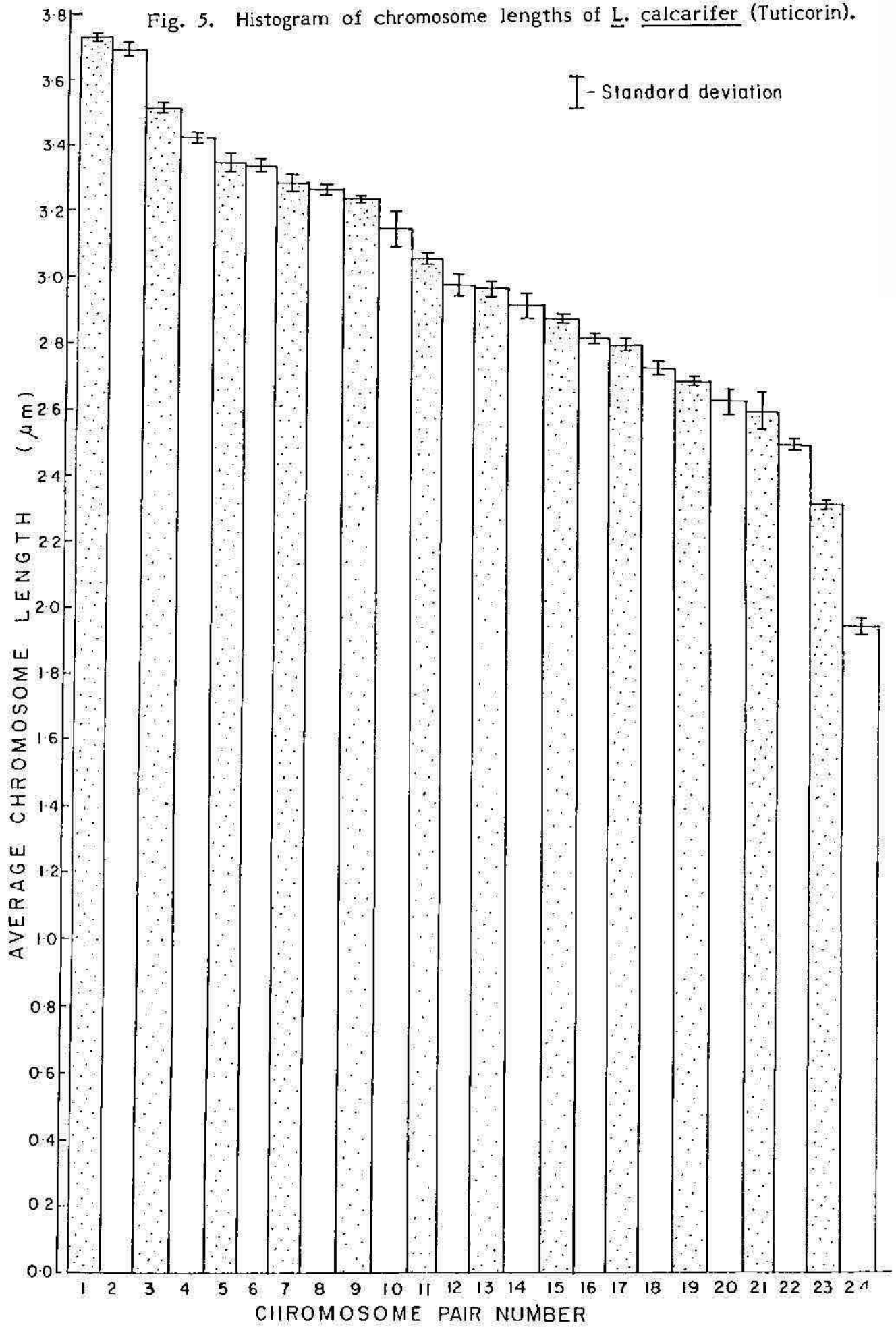


Table 10: Total chromosome lengths, relative lengths, and arm ratios for L. calcarifer collected from Tuticorin.

*Chromosome pair no.	Total length (μm) ($\bar{x}$ ± S.D.)	Relative length (%) ($\bar{x}$ ± S.D.)	Arm ratio ($\bar{x}$ ± S.D.)
1.	3.7319 ± 0.02149	2.6013 ± 0.01825	1.7648 ± 0.0621
2.	3.6951 ± 0,03742	2.5757 ± 0.01816	2.2674 ± 0.0279
3.	3.5127 ± 0.01984	2.4486 ± 0.01719	2.6197 ± 0.0525
4.	3.4249 ± 0.02631	2.3876 ± 0.0231	2.1836 ± 0.0723
5.	3.3426 ± 0.0491	2.3300 ± 0.01278	1.0290 ± 0.0213
6.	3.3319 ± 0.03726	2.3225 ± 0.0026	
7.	3.2825 ± 0.05216	2.2881 ± 0.01243	-
8.	3.2645 ± 0.03729	2.2755 ± 0.03448	-
9.	3.2312 ± 0.01219	2.2523 ± 0.0468	-
10.	3.1482 ± 0.1049	2.1945 ± 0.0672	-
11.	3.0567 ± 0.0286	2.1307 ± 0.0239	-
12.	2.9729 ± 0.06249	2.0723 ± 0.0282	-
13.	2.9614 ± 0.0437	2.0643 ± 0.03742	-
14.	2.9173 ± 0.07243	2.0335 ± 0.01249	-
15.	2.8761 ± 0.02149	2.0048 ± 0.04214	-
16.	2.8124 ± 0.02671	1.9604 ± 0.0521	-
17.	2.7921 ± 0.03124	1.9463 ± 0.0395	-
18.	2.7219 ± 0.04149	1.8973 ± 0.03319	-
19.	2.6814 ± 0.02681	1.8691 ± 0.06312	-
20.	2.6294 ± 0.07216	1.8328 ± 0.04292	-
21.	2.5914 ± 0.1129	1.8064 ± 0.0297	-
22.	2.4921 ± 0.03218	1.7371 ± 0.02614	-
23.	2.3124 ± 0.02267	1.6119 ± 0.05721	-
24.	1.9471 ± 0.05718	1.3572 ± 0.04821	-

*Chromosome pair no. 1 to 4 are submetacentrics, 5 metacentric and 6 to 24 acrocentrics. Total complement length = 143.46 μm.

Fig. 5. Histogram of chromosome lengths of L. calcarifer (Tuticorin).



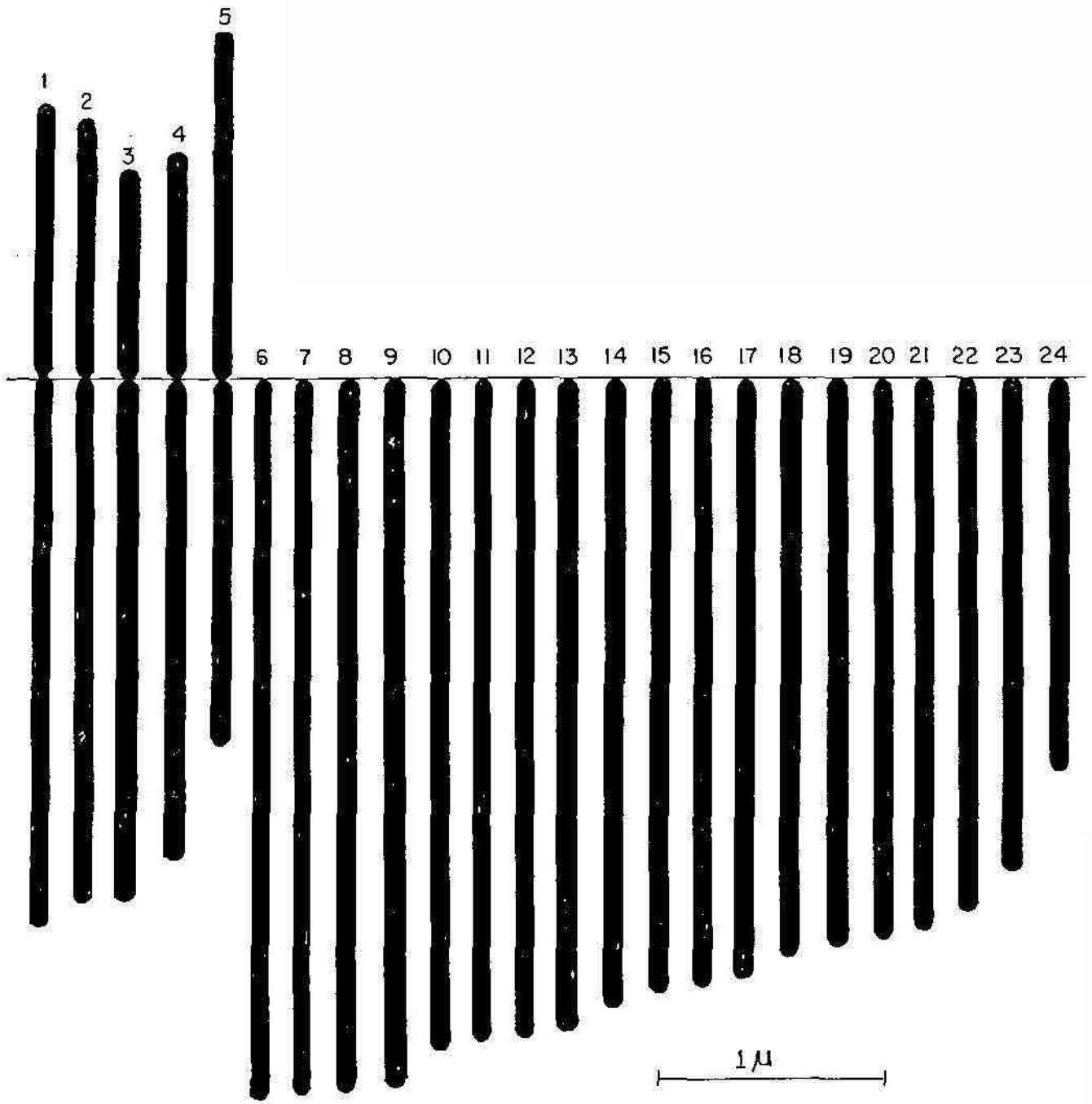


Fig. 6. An idiogram of L. calcarifer; chromosome pairs 1-4 are submetacentrics, 5 metacentrics and 6-24 acrocentrics.

DISCUSSION

The discussion is presented as a) Evaluation of methods, b)Diploid numbers, c) Karyotype analysis.

a) Evaluation of methods:

Though a number of methods have been evolved for the chromosome preparation of fishes, each species requires specific standardized methods for obtaining well spread chromosomes with clear morphology. For the present study different methods were tried to achieve a suitable methodology for the particular species. The in vitro studies are best suited for chromosome studies but is usually impossible as it requires a lot of sophisticated laboratory facilities.

Methods 3 and 5 were generally found unsuitable. Metaphase plates were virtually absent from these two methods. Method of Mc Phail and Jones (1966) gave no metaphase spreads due to the damage of chromosomes during manual squashing. The sodium citrate hypotonic treatment of LeGrande and Fitzsimons (1976) was helpful for inducing cell swelling. Method 2 and 4 yielded only incomplete metaphase spreads at a very low frequenty and hence found unsuitable for screening. The incomplete metaphase spreads are possibly due to the loss of chromosomes during centrifuging.

The method of Kligerman and Bloom (1977) gave excellent results with well spread chromosomes. The treatment of low concentration of 0.001% colchicine was helpful to get well-spread non-contracted chromosomes. Modification of LeGrande and Fitzsimons (1976) method's hypotonic treatment of 1% sodium citrate to 0.8% gave excellent swelling of cells. Preparations made from kidney tissues were interfered by the debris derived from the connective tissues. Gill preparations were totally free from such debris.

b) Diploid number:

In fishes both haploid and diploid sets contain chromosome numbers characteristic for the species (Denton, 1973). Out of about 1400 species of fishes the diploid numbers ranged between 16 in Sphaerichthyes osphromonoides (family : Belontidae) (Calton and Denton, 1974) and 239 in Acipenser naccari (family : Acipenseridae) (Ojima, 1981) with 48 as the peak in 450 species belonging to 22 orders. The next lower peak of 2 n = 46 was found in 225 species belonging to Atheriniformes, Beloniformes, Bericiformes, Cypriniformes, Cyprinodontiformes, Gadiformes, Osteoglossiformes, Perciformes, Salmoniformes, Scorpaeniformes, Siluriformes and Tetradontiformes. The diploid number of 50 have been shown in 140 species belonging to widely distributed orders (Manna, 1984).

The primitive teleost Karyotype is thought to have consisted of 46-48 chromosomes (Neyyar 1966) and was most likely 48 acrocentric

chromosomes (Ohno et al., 1968; Ohno, 1970; Fitzsimons, 1972; Le Grande 1975; Denton, 1973; Nogusa, 1960). The diploid number of L. calcarifer is determined to be 48 chromosomes. This finding is in conformity with the report of Khuda- Bukhsh(1979).

Fishes exhibit chromosome variations from population to population or within a population of the same species. They even show chromosomal variability in different tissues of the same individual. (Ohno et al., 1965; Junxiu, 1983). Different individuals within a population showed chromosomal variability due to centric fusion as it is observed in the case of rainbow trout. But in the present study, the two populations of L. calcarifer from Cochin and Tuticorin did not show any difference in chromosome number. Only a single modal number at 48 could be observed in both the populations. Therefore, the possibility of intraspecific variation in chromosome number may not be indicated on the basis of the present study. The geographic separation does not seem to have created any variation in their chromosome number.

c) Karyotype analysis:

In normal fish species, each pair of homologous chromosomes is assumed to differ genetically from all other chromosome pairs in the same cell. Superficial manifestations of some of these differences

comprise the morphological "phenotype" or karyotype and include differences between chromosome pairs in relative size, shape and centromere position.

Many orders of fish species are relatively uniform in karyotype, although they were apart in palaentoilogical times scale by tens of millions of years. For example, the haploid(n) karyotype of 24 acrocentric chromosomes is found throughout several diverse orders of the subclass Teleostei (class Osteichthyes) and appears to be the predominant karyotype in the recently evolved Perciformes (Roberts 1964, 1967; Denton, 1973; Chairelli and Capanna, 1973). This has led to the suggestion that the 24 acrocentric chromosome complement may be ancestral to all modern fishes, and perhaps was possessed by the primordial teleost (Leptolepis) over 100 million years ago (Ohno, 1974). The occurrence of 24 pairs of acrocentric chromosomes is a general feature found in the order Perciformes. 48 acrocentric chromosomes are the basic karyotype reported for the species of the families centrarchidae (Roberts, 1964), Theraponidae (Subrahmanyam and Natarajan, 1970), Serranidae and Sillaginidae (Nogusa, 1960). The species of the family Mugilidae comprise 48 chromosomes. It includes 46 acrocentrics and 2 subtelocentric chromosomes (Cataudella et al., 1974). The basic karyotype of Percidae also is reported to be consisted of 48 acrocentric chromosomes. (Nygren et al., 1968; Mayr et al., 1987). This view that 48 acrocentric chromosomes constitute the primitive karyotype had a

biased origin because most of the early work had been on marine species which incidently had such type of diploid chromosome constitution quite commonly. Studies on some fresh water species (Manna and Prasad 1973a, b, 1974a; Manna and Khuda-Bukhsh 1977a, 1978) and the check list of chromosome numbers prepared by many workers (Denton, 1973; Manna and Khuda-Bukhsh, 1977b; Ojima, 1981) have revealed that the modal number of 48 acrocentric chromosomes was conditional and the morphology was variable (Manna and Prasad, 1971; Manna and Khuda-Bukhsh, 1977b).

Detailed observations have shown that the karyotype of L. calcarifer consists of 48 chromosomes which can be aligned into 24 homomorphic pairs comprising 1 pair of metacentrics, 4 pairs of submetacentrics and 19 pairs of acrocentric chromosomes. It has been reported by Khuda-Bukhsh (1979) that the chromosome complement of L. calcarifer consists of 1 pair of metacentrics, 3 pairs of submetacentrics, 1 pair of subtelocentrics and 19 pairs of acrocentric chromosomes. According to Levan et al. (1964) the chromosomes with terminal and subterminal centromeres are both described as acrocentric. Hence in the present study the subtelocentric chromosome pair is included under acrocentric type. Karyotypes containing biarmed elements (metacentrics and submetacentrics) are generally regarded to represent a drived or non-primitive condition within teleosts (Ohno et al., 1968; Ohno, 1970; Denton, 1973).

The chromosome numbers and types reported in the order Perciformes to show the karyological evolution are given in Table 11.

Since fishes have survived millions of years in the most diverse environments, it is expected that all known mechanisms of chromosomal changes took place in the evolution of the karyotypes. The wide range of values of fundamental arms (NF) in species of fishes with 2n = 48 has been observed in the order Perciformes and this has been ascribed to pericentric inversion (Manna and Prasad, 1971).

Intraindividual chromosomal polymorphism has been observed in rainbow trout (Ohno et al., 1965; Thorgaard, 1976). Intrapopulation chromosomal variations have been reported in Spicara, Mystus, white sea herring, African tooth carps (Kirpichnikov, 1981) and Mystus vittatus (Manna and Prasad, 1974). As the study was restricted to direct chromosome observations without using advanced differential banding techniques, the present study did not reveal any chromosomal variations between the two populations of L. calcarifer from Cochin and Tuticorin. But the possibility of intraspecific variation or differences due to geographic separation cannot be ruled out. Although an attempt has been made to clearly visualise centromeric positions by adopting C- banding technique, the result was very poor due to lack of sophisticated laboratory facilities for tissue culture and also non-availability of suitable methodology or standardization.

Table 11: Chromosome numbers and types reported in the order Perciformes.

Species	Sex	Tissue	Karyotype	NF	Locality	Reference
Order. Perciformes Suborder: Mugiloidei						
Family Mugilidae.						
Mugil cephalus	F,M	K	48, 48a	(48)	Roma etc., Italy	Cataudella et al., 1974.
Chelon labrosus	F,M	K	48, 2st + 46a	(50)	Roma etc, Italy	Cataudella et al., 1974
Liza aurata	F,M	K	48, 2st + 46a	(50)	Lesina Italy	Cataudella et al., 1974.
Liza ramada	F,M	K	48, 21st + 46a	(50)	Roma etc, Italy	Cataudella et al., 1974.
Liza saliens	F,M	K	48, 2st + 46a	(50)	Lesina, Italy	
Mugil parsia	F,M	K	48, 48a	(48)	India	Chatterjee and Majhi (1973)
Oedalechilus labeo	F,M	K	48, 2st + 46a	(50)	Roma, Italy	Cataudella et al., 1974.
Family Centrarchidae.						
Acanthaschus pomotis	M	cT	48, 48a	48		Roberts, 1964
Ambloplites rupenstris	M	cT	48, 48a	48		Roberts, 1964
Centrarchus macropeterus	M	cT	48, 48a	48		Roberts, 1964
Chaenobryttus guloses	M	cT	48, 48a	48		Roberts, 1964

Contd.....

Species	Sex	Tissues	Karyotype	NF	Locality	Reference
Elassoma zonatum	M	cT	48, 48a	48		Roberts 1964
Enneacanthus gloriosus	M	cT	48, 48a	48		Roberts 1964
Lepomis gibbosus	M	cT	48, 48a	48		Robert 1964
Lepomis auritus	M	cT	48, 48a	48		Robert 1964
Micropterus dolomieui	M	cT	48, 48a	48		Robert 1964
Family Cichlidae						
Cichlosoma citrinella	-	G	48, 36sm + 12a	(84)		Nishikawa et al., 1973.
Family: Blennidae						
Blennius pavo	-	G,K	48,8st + 40a	(56)		Cataudella 1975.
Family: Percidae.						
Perca fluviatilis	M	K,T	48, 48a	(48)	L. Malaren, Sweden.	
Lucioperca lucioperca	M	T	48, 48a	(48)	L. Malaren, Sweded	Nygren et al., 1968
Acerina cernua	M	T	48, 48a	(48)	L. Malaren, Sweden	Nygren et al., 1968
Family Centropomidae						
Lates calcarifer	Indistinguishable	K,T	48,1m+3sm+1st+19a	55	Calcutta, India.	Khuda-Bukhsh, 1979.
Lates calcarifer	"	K,T	48,1m+4sm+19a	55	Cochin and Tuticorin, India	Present study

Among about 1400 species of fishes cytologically examined, only in about 100 species belonging to 48 families the sex chromosomes have been claimed to have been identified (Manna, 1984). Heteromorphic sex chromosomes have been identified in fishes (Passakas and Kelkowski, 1973; Park and Kang, 1979). However, heteromorphic sex chromosomes could not be identified in the present study on L. calcarifer, as reported by Khuda-Bukhsh(1979).

Generally fish chromosomes are smaller in size than chromosomes in most vertebrates. The length of the "average" fish chromosome is between 2 and 5 /um. Many species possess numerous small chromosomes of 2 /um or less. Very large chromosomes of 15-30 /um in length are found in the lung fish Lepidosiren paradoxa. Extremely small chromosomes (microchromosomes) have been reported in a few species. Ohno et al. (1969) found between 26 and 48 microchromosomes in the karyotype of very primitive species like Hydrolagus colliei and Lepisosteus productus. Chromosomal lengths in L. calcarifer varied from 1.78 /um in the smallest pair to 3.75 /um in the largest pair. Based on the overall studies on fish chromosomes it can be suggested that L. calcarifer chromosome lengths fall into the general pattern observed.

In this context it may be concluded that the chromosome constitution of L. calcarifer is similar to the general pattern found in the order

Perciformes. The geographic separation does not seem to have created any variations in the two populations of L. calcarifer from Cochin and Tuticorin on the basis of the present study. Detailed investigations using advanced chromosomal banding techniques have to be carried out regarding the population cytology of L. calcarifer for indentifying, conserving and maintaining its relatively limited stocks.

SUMMARY

The experiments and observations made are summarised as follows:

1. The chromosome preparations were made from 14 Lates calcarifer specimens from Cochin and 10 from Tuticorin.

2. Selected methods of fish chromosome preparations were tried.

3. The method of Kligerman and Bloom (1977) was standardised for the chromosome preparations of L. calcarifer.

4. chromosome spreads were made from kidney and gill tissues.

5. The diploid chromosome number was determined for the L. calcarifer from Cochin and Tuticorin separately. In both the populations, the modal diploid number was found to be 48. No other modes were observed.

6. The karyotypes were constructed for Cochin and Tuticorin specimens separately.

7. The karyotype consisted of 24 homomorphic pairs comprising one pair of matacentrics, four pairs of submetacentrics and 19 pairs of acrocentric chromosomes. Morphological differences could not be observed in chromosomes between specimens from Cochin and Tuticorin.

8. Sex chromosomes were morphologically unidentified.

9. The morphometric analysis of the chromosomes were made. The total length of chromosomes was ranging between 1.7875 /um to 3.7504 /um in the specimens from Cochin and 1.9471 /um to 3.7319 /um in the specimens from Tuticorin.

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