INTRASPECIFIC AND INTERSPECIFIC STUDIES IN *NEMIPTERUS* (PISCES : PERCIFORMES : NEMIPTERIDAE) USING TRUSS NETWORK ANALYSIS AND PROTEIN GEL ELECTROPHORESIS

DISSERTATION SUBMITTED
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JULY 2000

DEDICATED TO MY
DEAR PARENTS

CERTIFICATE

Certified that the dissertation entitled "**Intraspecific and interspecific studies in *Nemipterus* (Pisces: Perciformes: Nemipteridae) using truss network analysis and protein gel electrophoresis**" is a bonafide record of work done by **Ms. Juliet Joseph** under our guidance at the Central Marine Fisheries Research Institute, Kochi, during the tenure of her **M. F. Sc (Mariculture) programme** of 1998-2000 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any other publication.

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I hereby declare that this thesis entitled "**Intraspecific and interspecific studies in *Nemipterus* (Pisces:Perciformes: Nemipteridae) using truss network and protein gel electrophoresis** " is based on my research and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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सारांश

चेन्नै और कोचीन से संग्रहित *नेमीटीरस मीसोप्रिओन* जीवसंख्या में हानेवाली अंतराजातीय विभिन्नता समझने के लिए इनमें ट्रस नेटवर्क विश्लेषण और प्रोटीन जेल इलक्ट्रोफोरसिस किए गए. ट्रस आकृतिमान अध्ययनों द्वारा यह दिखाया पडा कि जीवसंख्याएं आकार में सजातीय है. प्रोटीन इलक्ट्रोफोरटिक अध्ययनों ने व्यक्त किया कि दोनों जीवसंख्याओं की विकल्पी आवृत्तियों (एलील फ्रीक्वन्सी) में उल्लेखनीय विभिन्नता नहीं है. चेन्नै और कोचीन की *एन. मीसोप्रिओन* जीवसंख्याओं के औसत विषमयुग्मजता (हेटेरोज़ाइगोसिटी) मूल्य क्रमशः 0.643 और 0.718 है. *एन. मीसोप्रिओन* जीवसंख्या की तीन लोसियों में केवल एक ने हार्डी - वाइनबर्ग इक्विलिब्रियम में सार्थक विचलन दिखाया . जीवसंख्याओं के आनुवंशिक पहचान (I) और आनुवंशिक दूर (D) के मूल्य क्रमशः 0.9962 और 0.0038 थे . इलक्ट्रोफोरटिक परिणाम आकृतिक परिणामों के संरूप निकले .

एन . जापोनिकस और *एन . मीसोप्रिओन* की तुलना के लिए प्रोटीन जेल इलक्ट्रोफोरसिस किया गया . एन . मीसोप्रियोन में आर एफ मूल्य 60.49 वाला एक जाति विशिष्टता बैंड दिखाया पडा . *एन* . जापोनिकस और *एन . मीसोप्रियोन* ने मसिल प्रोटीन में क्रमशः 8 और 9 घटक दिखाए . परीक्षण की गई दोनों जातियों की लोसियों में उल्लेखनीय विभिन्नताएं दिखाई पडी . सिर्फ *एन . जापोनिकस* के लोकस 2 के प्रति हार्डी - वाइनबर्ग इक्विलिब्रियम ने संरूपण दिखाया . *एन . मीसोप्रियोन* और *एन . जापोनिकस* के तुलनात्मक आनुवंशिक पहचान और दूर मूल्य क्रमशः 0.836 और 0.181 थे जो इनके जातीय स्तर की पुष्टि की जाती है .

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INTRODUCTION

INTRODUCTION

Species of fish like most other organisms do not exist as one continuous or homogenous population. Rather, they consist of a collection of populations that can be recognised on the basis of a variety of characters (Ihssen *et al.*, 1981;Spanakis *et al.*, 1989). The recognition that a stock structure might have evolved and been maintained in species of fish of economic importance (Heincke, 1898) has led to the concept of 'stock' that has played a pivotal role in fisheries science (Spanakis *et al.*, 1989).

Ihssen *et al.* (1981) defined stock as an intraspecific group of randomly mating individuals with temporal or spatial integrity. A stock concept needs to be adopted by managers in order to introduce a genetic perspective, which must be integrated with ecological viewpoints before realistic decisions about approximate compromises between biological and socio-economic costs can be reached (MacLean and Evans, 1981).

Morphometric and anatomical measurements have traditionally been used to differentiate populations in general and stocks of fish in particular (Ihssen *et al.*, 1981). Humphries *et al.* (1981) developed a

method where transects are drawn systematically between key morphological features, known as a truss network (Beddow and Ross, 1996). Unlike conventional measurements, the truss network covers the whole body form evenly so that shape differences can be detected in both vertical and horizontal planes (Strauss and Bookstein, 1982).

Stock identification based on electrophoretically measured biochemical differences is being used by fisheries management and research agencies as a tool to estimate the stock composition in mixed stock fisheries (Milner *et al.*, 1981; Fournier *et al.*, 1984; Beacham *et al.*, 1985; Millar, 1987; Ryman and Utter, 1987). Of outstanding significance of electrophoresis is that it made it possible to differentiate between the heterozygotes and the homozygous and to quantify the number of individuals with different genotypes (Kirpichnikov, 1981).

Muscle myogen patterns show a high degree of species specificity when separated by starch gel electrophoresis (Tsuyuki *et al.*, 1962; Tsuyuki and Roberts, 1963). A remarkable degree of parallelism was found to exist between Berg's 1947 classification of fishes, particularly within families and the similarity of muscle myogen patterns.

The Nemipterids or threadfin breams are small to moderately sized fishes commonly found throughout the Indo-Pacific region (Eggleston, 1970). Lately these species have been receiving increasing attention because of their commercial importance, specifically in the manufacture of surimi and surimi- based products (Santos, 1993). Threadfin breams form an important demersal fishery resource along Indian West Coast. They are mainly exploited by small commercial trawlers in depths upto about 50 m. The existence of rich resources of threadfin breams on the continental shelf beyond 50 m depth, especially in the 75-100 m belt along different parts often form 75 % of the trawl catch. The two species, *Nemipterus japonicus* and *N. mesoprion* form an important constituent of the trawl catches of South-west coast, particularly during South-west monsoon. Some attempts have already been done to find out genetic variation in Nemipterids (Chakraborty, 1989; Santos, 1993).

Marine stock enhancement programmes should use information on population structure to optimize enhancement strategies and to design and implement operations so as to protect the genetic character and diversity of existing wild stocks, thereby maintaining their productivity and evolutionary potential (Shaklee and Bentzen, 1998). The present study is an attempt to find out intraspecific and

interspecific differences in *Nemipterus* spp. using truss network analysis and protein gel electrophoresis.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

A :Stock / population analysis :

A .I .Truss network analysis :

In different species of fishes, attempts were made to dilineate stock /strain structure using truss network analysis. Investigations on the distinctness and the interrelationships of six stocks of Common carp, *Cyprinus carpio* by canonical variate analysis showed that the stocks were morphologically distinct(Corti *et al* .,1988). Geographic differences in body shapes among eighteen populations of northern red belly dace, *Phoxinus eos* in three regions of Ontario were quantified with truss analysis (Toline and Baker, 1993). Truss morphometric characterisation of eight strains of Nile Tilapia, *Oreochromis niloticus* indicated few truss morphometric differences among the eight strains(Velasco *et al* .,1996).

A . II : Electrophoretic analysis :

Electrophoretic analysis of simple proteins and enzymes form an important tool in population genetic structure. Based on electrophoretic analysis of the protein esterase it was shown that atleast two different genetic stocks of skipjack tuna, *Katsuwonus pelamis*

existed in the Pacific (Richardson, 1978). Electrophoresis of soluble crystalline proteins of albacore, *Thunnus alalunga* in the North-east Atlantic concluded that there were three populations in that zone(Hue, 1979). Geographic populations of the Anemone fish, *Amphiprion clarkii* from six widely separated locations off the coast of Southern Japan were electrophoretically examined. Results indicated that genetic clines were absent(Bell *et al.*,1982). Malate dehydrogenase allele frquencies of walleyes, *Stizostedion vitreum vitreum* were assessed and the augmented cohorts and the unstocked cohorts were found to differ in MDH allele frequencies significantly (Murphy *et al.*, 1983). Forty eight loci were electrophoretically surveyed for protein variation in skipjack tuna, *Katsuwonus pelamis* from central and South-west Pacific and east-west clines in allele frequency was reported(Richardson, 1983).

Attempts were made to delineate stocks of tile fish, *Lopholatilus chamaeleonticeps* along US east coast and in Gulf of Mexico. Electrophoretic data consistently supported a separate Mid-Atlantic Bight stock and also suggested that South Atlantic Bight and Gulf of Mexico samples belonged to a separate single stock (Katz *et al.*, 1983). Stock separation studies on blue whiting, *Micromeristius poutassou* in North-East Atlantic gave support to the hypothesis that separate stocks existed there(Bussmann, 1984). Based on the

electrophoretic separation of water soluble eye lens proteins, three major stocks have been recognised from the migratory population of East coast stripped bass, *Morone saxatilis* (Fabrizio, 1986).

Genetic characterisation of *Oreochromis niloticus* populations in Philippines indicated well established introgression with *O.mossambicus*. Genetic differentiation of the *O.niloticus* stocks, measured by Nei's genetic distance was highly correlated with *O.mossambicus* gene content(Macaranas *et al* .,1986). A survey of the protein genes in Atlantic mackerel populations, *Scomber scombrus* revealed that the species had not evolved into genetically divergent stocks in the Northeast Atlantic (Jamieson and Smith, 1987). Studies on genetic relationship and postglacial dispercal of Northern pike, *Esox lucius* populations in North America showed that all populations from the drainages in Western Canada and Missouri rivers were genetically identical and Mississippi river populations were unique (Seeb *et al* .,1987). Three populations each of *Oreochromis aureus* and *O.niloticus*, one each of *O.mossambicus* and *O.urolepis hornorum*, and two each of red tilapia derived from the hybridisation of *O.urolepis hornorum* females and *O.mossambicus* males were compared for the electrophoretic mobilities of their enzymes at 27 enzyme loci. Variation was sufficient to differentiate the species, but not all of the populations surveyed (Brummett *et al* .,1988).

Hairtails, *Trichiurus haumela* from East China sea were classified into 4 groups by polyacrylamide disc and slab gel electrophoresis and isoelectric focussing (Keling and Lanying, 1988). The variations in the electrophoretic patterns of the total eye lens proteins from *Micropogonias furnieri* were determined and 4 types of patterns were characterised(Vazzoler and Phan, 1989). Studies on the population structure of *Acanthopagrus latus* suggested that there is only one stock in the Shalt al- Arab river and Khur al –Zubair area of Iraq, ruling out any idea about subpopulation differentiation (Hassan, 1990). Genetic differences were reported among and within populations of diadromous and lacustrine smelt, *Retropinna retropinna* from Waikato basin by analysing 4 polymorphic enzyme loci (Mitchell *et al* .,1993).

The amount and pattern of genetic variation expressed by barramundi perch, *Lates calcarifer* throughout Queensland was investigated and 7 genetically distinct stocks were identified (Shaklee *et al* .,1993). Allozyme variation used to investigate genetic stucture of *Lutjanus sebae, Lethrinus nebulosus*, *Lethrinus chorrhynchus* and *Epinephelus multinotatus* showed little genetic subdivision in all the species (Johnson *et al* .,1993). An electrophoretic survey of walleye *Stizostedion vitreum* and sauger *S.canadensi* from Ohio river showed that levels of variation in sauger were low and significant differences

were there among walleye populations (White and Schell, 1995). Electrophoretic variability in 34 protein coding loci in *Sardina* and five Indian - Pacific populations of *Sardinops* indicated that *Sardinops* consisted of a single species with widely scattered subpopulations (Grant and Leslie, 1996).

Isozyme analysis was used to characterise samples of three populations of *Oreochromis niloticus* at 10 enzyme loci that were known to be polymorphic in some of it's populations. Results showed introgression of *O.aureus* genes in the selected strain of *O.niloticus* and in *O.niloticus.niloticus* (Capili and Skibinske, 1996). Isozyme electrophoresis of horse mackerel *Trichurus symmetricus* populations in Chile indicated the existence of only one population (Gonzalez *et al* .,1996). Genetic variation studies in pumpkinseed *Lepomis gibbosus* populations from 4 East - Central Ontario watersheds showed 3 distinct groups (Fok *et al* .,1997).

A .III : Truss network and electrophoretic analysis :

The degree of differentiation between Capelin, *Mallotus villosus* populations in the estuary and Gulf of Lawrence evaluated using truss and electrophoresis differentiated the sample sites along an east-west axis (Roby *et al* .,1991). Four populations of Blunt snout bream *Megalobrama amblycephala* from three lakes and one branch of

Changjiang river were morphologically and biochemically compared. Discriminate analysis indicated morphometric differences among populations whereas biochemical data showed very little variation among populations (Li *et al* .,1993). Allopatric populations of Australian freshwater eel-tailed cat fish *Tandanus tandanus*, compared morphologically and electrophoretically indicated absence of morphological differentiation among all populations and fixed allele differences revealing three discrete gene pools (Musyl and Keenan, 1996).

B .Species characterisation by protein / enzymes :

Electrophoretic studies of *Sebastes spp.* and *Heliconus sp* based on enzymes revealed enzymes diagnostic for the two species (McGlade *et al* .,1983). Species specific electrophoretic patterns were found in haemoglobins and parvalbumins of *Oreochromis mossambicus* and *O. urolepis hornorum* (Uribe *et al.*,1989). Gene products of 40 loci in fifteen species of the Cichlid genera *Chetia, Hemichromis, Oreochromis, Pharynogochromis, Serranochromis* and Tilapia were examined. Genetic variation within species was observed at 18 loci and average heterozygosities ranged between 0 multiplied by 021 and 0 multiplied by 047 (Van-der-Bank *et al* .,1989). Electrophoretic investigations of the isozymes of 3 species of the family Myctophidae showed high levels of polymorphisms in all

species, close to the highest values known for fish (Afanas *et al.*, 1990). MDH electrophoretic mobility patterns were used as a standard against which other field methods were compared to separate the beaked red fish species, *Sebastes fasciatus* and *S.mentella* (Rubec *et al* .,1991).

O- and I- group red fish (Genus *Sebastes*) were taxonomically identified by analysing 4 enzymes using electrophoresis (Nedreaas and Narudal, 1991). Electrophoretic studies of nonenzymic proteins between species of *Gobio* genus proved myogenic esterase and MDH to be species specific (Dobrovolov, 1994). Electrophoretic analysis of muscle tissues from *Thunnus thynnus* and *T.maccoyii* found 2 out of the 33 enzyme loci with species specific gel phenotypes (Smith *et al.*, 1994). Studies on biochemical genetic structure and identification of hair tail fish populations in Chinese coastal waters suggested that they should be classified into 3 different species (Keling *et al* .,1994). The highly stable parvalbumin prevalent in fish white muscle tissue was suggested to have potential applications as species specific biomarkers in adult snook (Ross *et al* .,1997). Species specific electrophoretic patterns were found in PGI and PGM in red mullet *Mullus barbatus* and stripped red mullet *M.surmutetus* populations from Mediterranean (Mamuris *et al* .,1997).

Biochemical genetic studies have been done in Indian fishes which were mainly attempts to find out species specific or inter species protein differences. Eye lens and blood sera of 2 species of sardine, *Sardinella fimbriata* and *Sardinella longiceps* showed no marked difference in the number and positions of different protein fractions in the eye lenses of the two species but the sera showed a clear difference in the number and position of different protein components of the two species (Menezes, 1975a). Blood sera and eye lenses of two species of flat fishes namely *Brachiurus orientalis* and *Pseudorhombus arsius* showed clear difference in the number, amount and position of the different protein components (Menezes, 1975b). Electrophoresis of eye lens proteins of oil sardine and mackerel showed separation of proteins into 3 and 4 components respectively, indicating the heterogenous nature of the population (Rao and Dhulkhed, 1976). Electrophoretic studies on serum proteins revealed the existence of genetically different groups of oil sardine and mackerel on the south-west coast of India (Dhulkhed and Rao, 1976).

Electrophoretic separation of nuclear eye lens proteins in the mullet *Mugil cephalus* revealed 5 pattern types reflecting heterogeneity (Bhosle, 1977). The effect of different gel concentrations on the resolutions of the muscle myogens of the

Bombay duck were studied (Kurian, 1977). Investigations on eye lens proteins of three species of flat fishes *Psettodus erumei, Brachiurus orientalis* and *Pseudorhombus arsius* indicated a pattern which was characteristic of the species (Menezes, 1979). Electrophoretic variation of soluble protein fractions in tissues of different size groups of *Channa stewartii* and *Danio dangila* were studied (Bhattacharya and Alfred, 1982). Species specific muscle protein pattern was found in *Penaeus monodon* (Puthran Prathibha, 1984). LDH (LACTATE DEHYDROGENASE) patterns of *Etroplus suratensis, Liza macrolepis* and *Mystus gulio* were analysed (Rao *et al* .,1985).

Soluble eye lens nuclei proteins of the Indian mackerel *Rastrelliger kanagurta* studied by cell gel electrophoresis revealed a distinct pattern characterised by the number of bands, mobility and staining intensity (Menezes, 1986). Studies on Indian cichlids revealed variation with age in protein pattern of liver and haemoglobin of *Etroplus suratensis* and liver of *Etroplus maculatus.* Polymorphism was observed for LDH and Est (ESTERASE) in *E.suratensis* and for Est and Acph in *E.maculatus.* Significant difference in observed and expected frequency were found for LDH and Est in *E. Suratensis* and for Est in *E . maculatus* (Mahobia, 1987). LDH patterns in developmental stages and in different tissues of Grass carp *Ctenopharyngodon idella* were studied by Padhi and Bukhsh (1989). Muscle and eye lens

proteins of 3 fishes belonging to family Nemipteridae, *Nemipterus japonicus, N.mesoprion* and *N.delagoae* were studied. Densitometer scanning revealed species specific pattern for eye lens proteins whereas muscle protein pattern was uniform for all the 3 species (Chakraborty, 1989).

The difference in the number of protein fractions, their mobility pattern and the staining intensity in the muscle and eye lens proteins of 3 species of Sciaenids indicated species specificity (Chakraborty, 1990). Biochemical genetic divergence studies in 3 Carangid species from Andaman sea revealed that they were clearly divided into 2 groups (Menezes, 1990). Biochemical genetics of *Mugil cephalus* from Kochi, Chennai and Orissa revealed significant differences among 3 populations at 14 out of 21 loci (Vijayakumar, 1992). Significant allele frequency differences at 14 out of 21 enzyme loci suggested that the populations of *Sardinella longiceps* tested were genetically different stocks (Venkita Krishnan, 1993). Biochemical genetic polymorphism in the Indian mackerel *Rastrelliger kanagurta* from the Mangalore region was studied (Verma *et al* ., 1994). Biochemical genetic profiling of Indian mackerel, *Rastrelliger kanagurta* during mud bank and post mud bank period suggested that the samples had closely comparable genetic profile(Verma *et al.*, 1996).

Cytogenetic, biochemical genetic and morphological studies of oil sardine, *Sardinella longiceps* indicated the heterogeneity of the 4 populations studied (Mohandas, 1997). The basic isoelectric focussing eye lens profiles of Lucknow populations of *Channa punctatus* revealed species specific proteins (Srivastava and Ponniah, 1998). Tissue specificity and degree of polymorphism of 5 enzyme systems of *Labeo rohita* from river Yamuna have been studied (Chaudhary and Krishna, 1998). Biochemical genetic studies on the chocolate mahseer, *Neolissochilus hexagonolepis* concluded that the fish populations studied were heterogenous for the two genes which coded for sorbitol dehydregenase (SenGupta and Chatterjee, 1998).

MATERIALS AND METHODS

MATERIALS AND METHODS

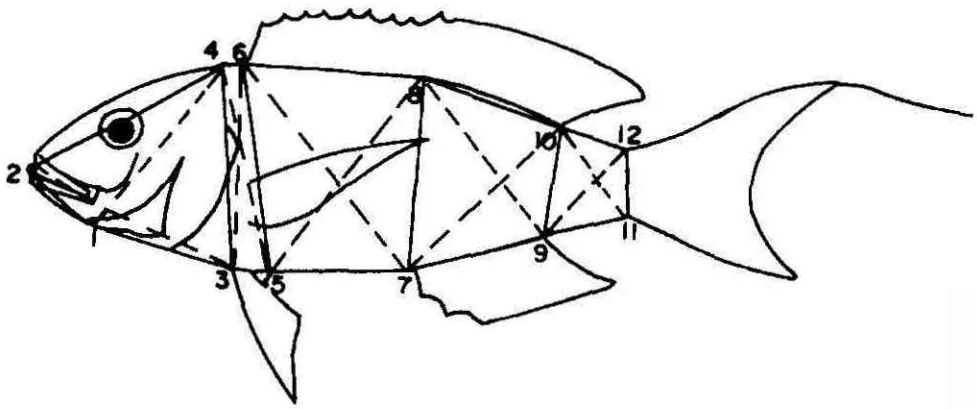
Truss network analysis:

Hundred specimens each of *N. mesoprion* were collected from commercial catches at Thoppumpady fishing harbour, Kochi (West coast) and Kashimedu fishing harbour, Chennai (East coast) during March – April, 2000. The fish was placed on a water-resistant paper and the body postures and the fins were teased into a natural position. Around the outline of the fish form, 12 landmarks that were distinct and homologous from specimen to specimen were identified (FIG .1). Each landmark was identified by making a hole with a dissection needle in the water-resistant paper along its respective location. These points were transferred to a graph sheet. The X - and Y co-ordinates were then calculated using the relationship

$$\sqrt{(x_1-x_2)^2 + (y_1-y_2)^2}$$

Principal component analysis of these 26 truss network distances was carried out (Morrison, 1990). PC-I and PC-II scores were plotted as XY scatters with PC-I on the X-axis and PC-II on the Y-axis. The clusters were further analysed by Sheared principal component analysis (Humphries *et al.*, 1981). Sheared PC-I and PC-II scores were then plotted as XY scatter diagram with PC-I on the X-axis and PC-II on the Y-axis.

FIG. I. TRUSS NETWORK OF 12 LANDMARK POINTS ON THE BODY OUTLINE OF *N.mesoprion.*



THE LANDMARKS REFER TO:

1. POSTERIOR TIP OF MAXILLA.
2. TIP OF SNOUT
3. ANTERIOR TIP OF PELVIC FIN.
4. POSTERIOR POINT OF NEUROCRANIUM.
5. *POSTERIOR END OF PELVIC FIN.*
6. ORIGIN OF DORSAL FIN.
7. ORIGIN OF ANAL FIN.
8. POINT BETWEEN SPINOUS AND SOFT DORSAL.
9. POSTERIOR END OF ANAL FIN.
10. POSTERIOR END OF DORSAL FIN.
11. LOWER INSERTION OF CAUDAL FIN.
12. *UPPER INSERTION OF CAUDAL FIN.*

Electrophoretic analysis:

The samples for the study (*N.mesoprion* and *N.japonicus)* (Plates 1 & 2) were collected from Thoppumpady fishing harbour, Kochi & Kashimedu fishing harbour, Chennai. Threadfin breams were mainly exploited by small commercial trawlers in depths upto about 50 m. The samples collected soon after landings were transported to lab in ice box packed with crushed ice. Initially white muscle and eye lens were dissected out from the samples for analysis. Later white muscle alone was used since it gave more clear bands. Care was taken to remove red muscle while preparing tissue samples. Approximately 1 g of the tissue was taken and stored at -20^0 C prior to homogenisation.

Protein extraction from sample:

Protein was extracted using double distilled water and 0.2 Molar sucrose medium at different ratios of the sample and homogenizing media (W/V) (1:1, 1:2) and 1:1 was found to be ideal. Homogenisation was done using a manual glass homogeniser, kept in a beaker containing chilled water (4°c). The homogenized samples were transferred to eppendorf tubes and were centrifuged at 4^0 C at 10 K for 20 minutes. The supernatant obtained was transferred to another eppendorf tube and stored at 4^0 C.

Plate 1. ***Nemipterus japonicus***

Plate 2. ***Nemipterus mesoprion***

Reagents for PAGE: (POLYACRYLAMIDE GEL ELECTROPHORESIS)

Gel buffer:

Two types of gel buffers were used- Separating gel buffer and Stacking gel buffer.

Separating gel buffer:

Tris buffer of 0.5 M and pH 8.9 was used.

Composition:

Tris buffer : 21.8 g

TEMED : 250 microlitres.

This was then made upto 100 ml with double distilled water and the pH of the solution was adjusted to 8.9 using 2 N HCl. The solution was then stored in refrigerator.

Stacking gel buffer:

Tris buffer of 0.5 M and pH 6.8 was used.

Composition:

Tris buffer : 6.04 g

This was made upto 100 ml using double distilled water. The pH of the solution was adjusted to 6.8 using 2 N HCl. The solution was then stored in a refrigerator.

Tank buffer:

Composition:

Tris glycine :36 g

This was dissolved in 2.5 l of double distilled water. The pH of the solution was adjusted to 8.3 by adding 2 M Tris buffer stock.

30% Acrylamide-Bis-Acrylamide Stock:

Composition:

Acrylamide : 29.1 g

Bis-acrylamide : 0.9 g

Double distilled water : 100 ml

The solution was filtered through Whatman No.l filter paper and was stored in refrigerator in amber coloured screw capped bottles.

10% Ammonium per sulphate stock:

10% APS stock was always prepared afresh by dissolving 0.1 g of APS in 1 ml of distilled water.

Sample buffer:

Composition:

Glycerol : 2 ml

Bromophenol dye (0.5%) : 1 ml

Stacking gel buffer : 7 ml

This was then stored at 4^0C.

Standardisation of methodology:

Standardisation of gel concentration was done with 3 concentrations such as 7 %, 7.5 % and 8%. Resolution was better for 7.5% gel (Table.1). Initially eye lens and white muscle tissue was taken for electrophoretic analysis. Later white muscle tissue alone was taken for electrophoretic analysis since it gave better electrophoretic patterns.

Horizontal Polyacrylamide Gel Electrophoresis:

The muscle tissues were subjected to PAGE (Smithies, 1955).

TABLE I. Gel composition used in electrophoretic analysis (7.5 % Gel concentration)

Gel Composites	Separating Gel	Stacking Gel
30% Acrylamide-Bis-Acrylamide	12.5 ml	3 ml
Tris buffer (pH8.9)	6.25 ml	-
Tris buffer (pH6.8)	-	6.25 ml
Double distilled water	6.25 ml	15.75 ml
APS(10%)	400*	100*
TEMED	-	25*

* Microlitres.

Staining procedure for protein:

The separating gel was stained in Coomassie brilliant blue (0.15%). Coomassie brilliant blue of 0.15 % was prepared by adding 0.75g of Coomassie brilliant blue, 230 ml of double distilled water, 230 ml of methanol and 40 ml glacial acetic acid. The stain was poured onto the gel and kept to take stain for $1^1/_2$ -2 hours.

Destaining:

After $1^1/_2$ –2 hrs, the gel kept in staining solution was transferred to the destaining solution which was prepared by mixing 150 ml methanol, 70 ml glacial acetic acid and 780 ml double distilled water. The gel was then kept in destainer overnight to remove excess stain.

ANALYSIS OF GEL:

The band patterns were observed in a transilluminator over visible light. Zymogram of each gel was recorded carefully on a graph sheet for species wise and population wise comparisons. Relative frequency was determined.

$$\text{Relative frequency} = \frac{\text{Distance moved by the fraction}}{\text{Total distance moved by the marker dye}} \times 100$$

Interpretation of electrophoretic data:

The protein / enzyme banding patterns or the phenotypes were compared between individuals at a particular gel area. Phenotype variants observed between the individuals in terms of the differences in the distance travelled by the particular band at that particular gel area were designated as slow moving (S) in one individual, fast

moving (F) in another individual and slow-fast moving (SF) when a combination of these 2 occur in yet another individual. Thus individuals having S, F and SF genotypes were scored as slow moving and fast moving homozygotes and slow-fast moving heterozygotes respectively. As a standard practice, these protein phenotypes are persumed as genotypes produced by co-dominant alleles at a particular genetic locus.

Allelic frequencies:

Allelic frequencies were calculated directly from genotype frequencies. Genotype frequencies are proportions of each genotype in total number of individuals tested for each locus.

Thus frequency of S allele = Frequency of SS genotype +Half the frequency of SF genotype.

Frequency of F allele = Frequency of FF genotype + Half the frequency of SF genotype.

Allelic frequency can also be calculated using the formulae,

$((Ho \times 2) + He) / 2N)$

Where Ho is the observed number of a particular homozygote

He is the observed number of a particular heterozygote

And N is the total number of individuals tested.

Expected genotype frequencies:

Expected genotype frequencies, as per Hardy-Weinberg law were calculated using the binomial expansion, $(a+b)^2=a^2+2ab+b^2$ when 2 variant alleles were involved and multinomial expansion when more than 2 variant alleles were considered.

Chi-square values for determining the significance of deviation between the observed and expected genotype frequencies were calculated using the formulae, $\chi^2=\sum(O-E)^2/E$.

The degrees of freedom for determining P value was calculated from the number of phenotypes minus 1, minus the number of alleles minus 1. Thus the degrees of freedom to be considered in diallelic 3 phenotype system is 1.

Heterozygosity:

Heterozygosity was directly estimated from the number of heterozygotes present in the total number of individuals tested. Average heterozygosity in the species was calculated by estimating the heterozygosity for each locus in each population, followed by their

averages for the total loci for each population, followed by the average for the number of populations tested. Both polymorphic and non-polymorphic loci were considered for calculation.

Genetic Identity (I) and Genetic distance (D):

$$I = \frac{\sum XiYi}{\sqrt{\sum Xi^2 \sum Yi^2}}$$

Where Xi and Yi are the frequencies of specific alleles in the populations X and Y respectively.

D= -ln (I), for a single locus.

RESULTS

RES ULTS

A .Truss network analysis:

Principal component analysis (PCA) of 26 truss network distances gave principal component (PC) scores PC I to PC 26. PC I and PC II could explain 78.1% of the total cumulative variation. In the present study with *N.mesoprion* the clusters in the X-Y scatter diagram obtained by PCA were mixed up. (Graph-I , Table-II)

Sheared Principal Component Analysis (SPCA) was done to obtain sheared PCA scores. Sheared PC-I and PC-II scores accounted for 72.87 % of the total cumulative variation. In the X-Y graph obtained by sheared PCA, there was no clustering, indicating morphological homogeneity of the tested populations. (Graph-II, Table-III)

GRAPH.I. PRINCIPAL COMPONENT ANALYSIS.

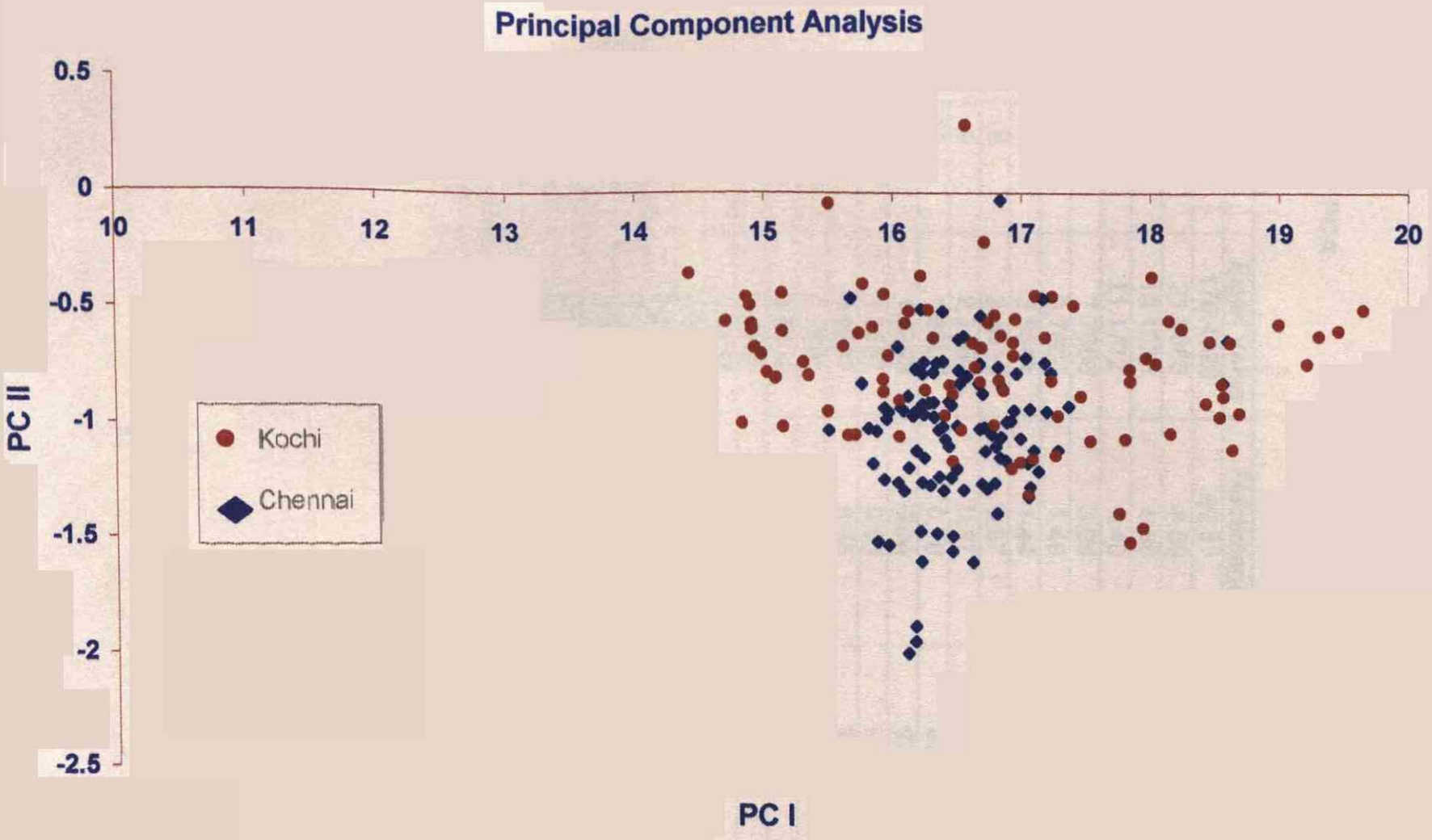

Principal Component Analysis
0.5
0
-0.5
-1
-1.5
-2
-2.5
10
11
12
13
14
15
16
17
18
19
20
PC II
PC I
Kochi
Chennai

Table II .PCA

PC#	Eigen Value	Percentage	Cum.Percentage
1	176.4320	69.51	69.51
2	21.8085	8.59	78.10
3	15.1230	5.96	84.06
4	11.1772	4.40	88.46
5	7.7566	3.06	91.52
6	3.7807	1.49	93.01
7	3.6641	1.44	94.45
8	2.4516	0.97	95.41
9	2.2163	0.87	96.29
10	1.9807	0.78	97.07
11	1.6343	0.64	97.71
12	1.3357	0.53	98.24
13	0.9237	0.36	98.60
14	0.7681	0.30	98.90
15	0.5439	0.21	99.12
16	0.4936	0.19	99.31
17	0.4231	0.17	99.48
18	0.3066	0.12	99.60
19	0.2387	0.09	99.70
20	0.2315	0.09	99.79
21	0.1730	0.07	99.85
22	0.1451	0.06	99.91
23	0.0977	0.04	99.95
24	0.0757	0.03	99.98
25	0.0366	0.01	99.99
26	0.0142	0.01	100.00

GRAPH.II. SHEARED PRINCIPAL COMPONENT

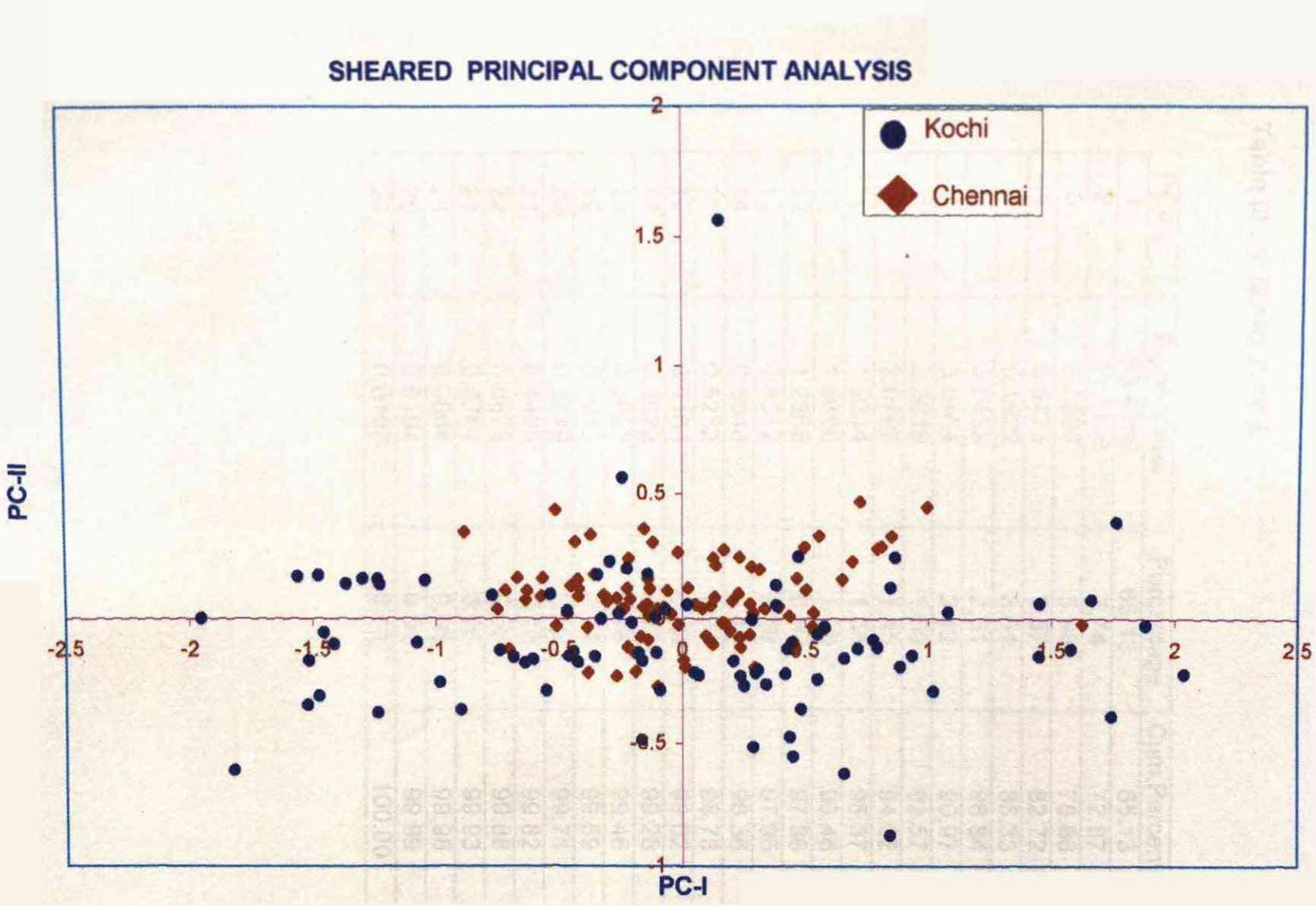

SHEARED PRINCIPAL COMPONENT ANALYSIS
Kochi
Chennai
PC-II
PC-I
2
1.5
1
0.5
-0.5
-1
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5

Table III . SHEARED PCA

PC#	Eigen Value	Percentage	Cum.Percentage
1	93.4756	65.13	65.13
2	11.1116	7.74	72.87
3	8.5866	5.98	78.86
4	5.5478	3.87	82.72
5	5.1822	3.61	86.33
6	3.6039	2.51	88.84
7	3.0464	2.12	90.97
8	2.3019	1.60	92.57
9	2.0793	1.45	94.02
10	1.9344	1.35	95.37
11	1.5690	1.09	96.46
12	1.2868	0.90	97.36
13	0.8512	0.59	97.95
14	0.5948	0.41	98.36
15	0.5282	0.37	98.73
16	0.4164	0.29	99.02
17	0.3622	0.25	99.28
18	0.2627	0.18	99.46
19	0.1949	0.14	99.59
20	0.1719	0.12	99.71
21	0.1490	0.10	99.82
22	0.0873	0.06	99.88
23	0.0784	0.05	99.93
24	0.0664	0.05	99.98
25	0.0194	0.01	99.99
26	0.0102	0.01	100.00

B .Analysis of electrophoretic data :

B.I. Interspecies comparison:

In the interspecies comparison studies between *N.mesoprion* and *N.japonicus* collected from Thoppumpady fishing harbour, Kochi, all the *N.mesoprion* samples showed the same electrophoretic pattern. But *N.japonicus* showed three patterns, A, B & C. 53.85 % of the samples collected were of type A, 23.08 % each of types B and C. (Plate 3, Fig –2)

Basically *N. mesoprion* and *N.japonicus* differed in 1 band / protein fraction. The band with Rf value of 60.49 was present only in *N. mesoprion*. *N. japonicus* showed 8 components in muscle proteins whereas *N.mesoprion* showed 9 components. The staining intensity of the components 7 and 8 were more in both *N.japonicus* and *N.mesoprion*. The difference remained constant showing remarkable stability and species specificity.

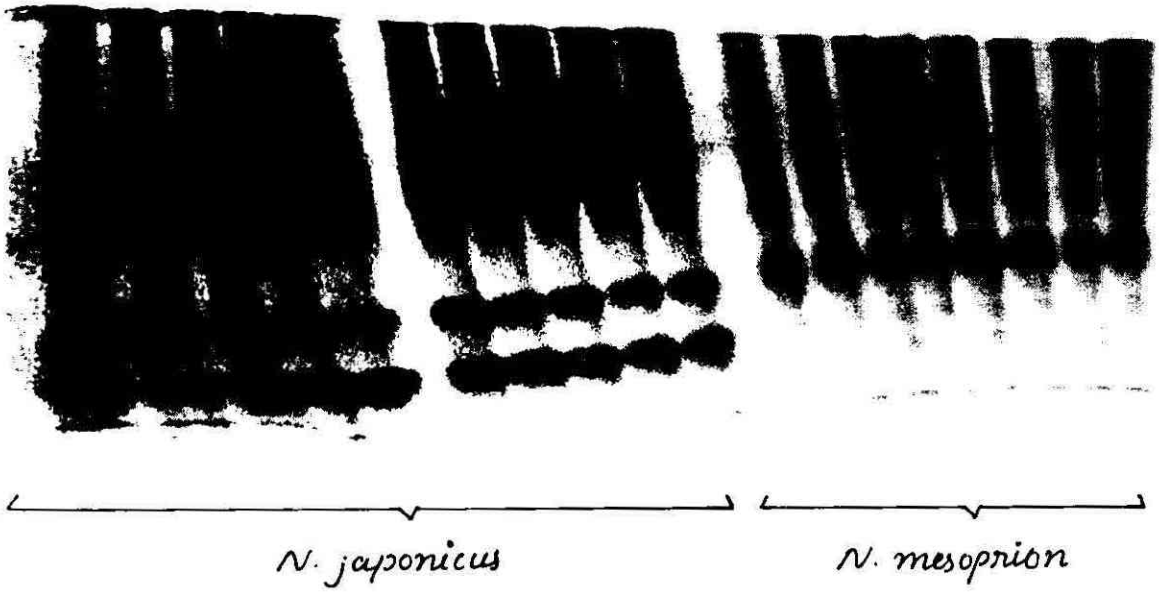


Plate 3. Sarcoplasmic protein patterns observed in *N. mesoprion* and *N. japonicus*

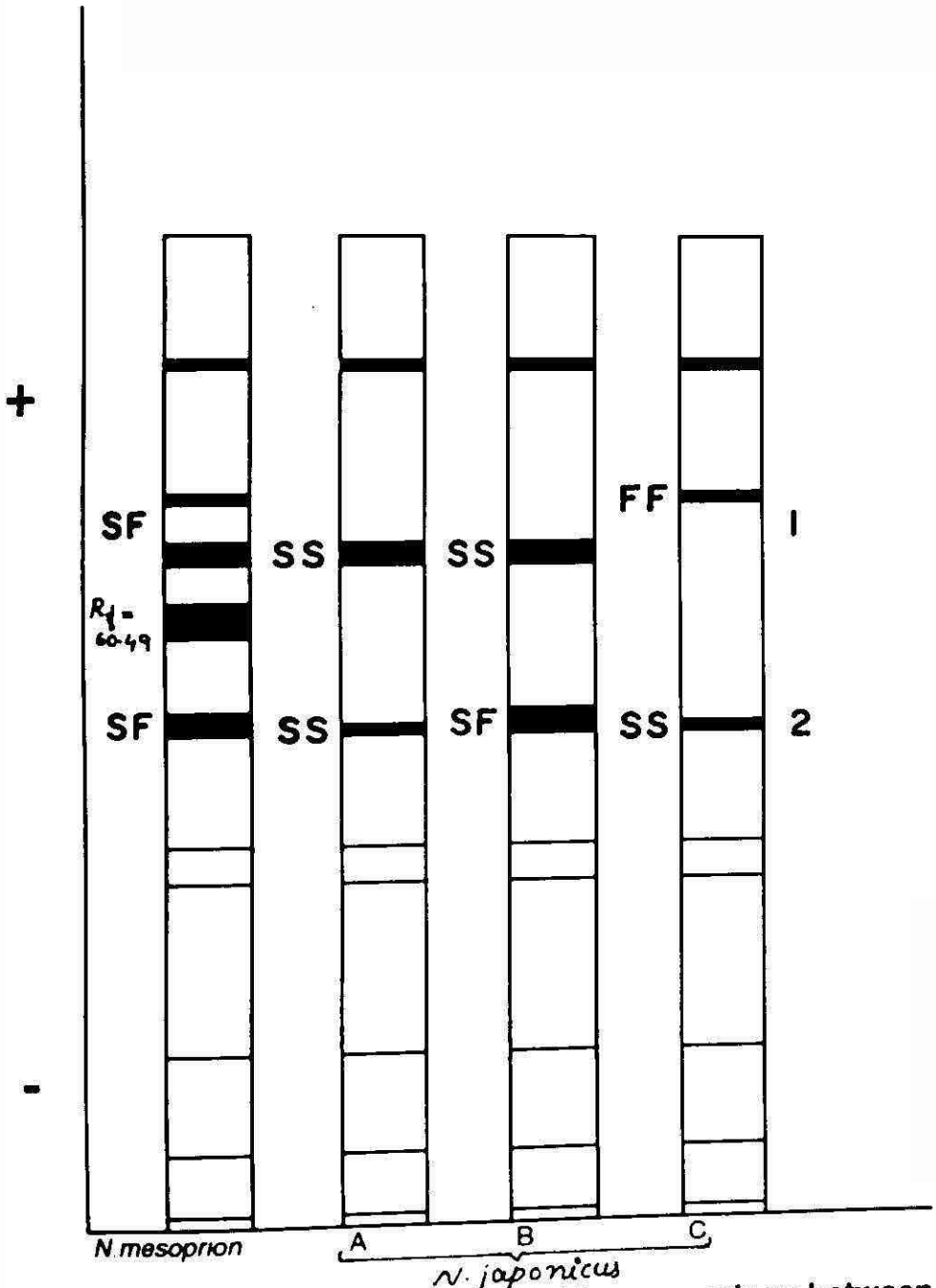


FIG. 2 Zymogram showing interspecific comparison between *N.mesoprion* and *N.japonicus*

Table IV. ALLELE FREQUENCIES AT 2 LOCI IN *N.japonicus* AND *N.mesoprion* FROM KOCHI.

Locus	Allele	*N.japonicus*	*N.mesoprion*
1	100	0.769	0.5
	107.27	0.231	0.5
2	100	0.885	0.5
	102.38	0.115	0.5

Table V. OBSERVED AND EXPECTED GENOTYPE FREQUENCIES IN *N.japonicus* AND *N.mesoprion* POPULATIONS FROM KOCHI.

LOCUS	GENOTYPE	*Nemipterus japonicus*		*Nemipterus mesoprion*	
		OBSERVED (EXPECTED)	χ^2	OBSERVED (EXPECTED)	χ^2
	100/100	10 (7.69)		0 (3)	
1	100/107.27	0 (4.62)	12.9762 *	12 (6)	12*
	107.27/107.27	3 (0.694)		0 (3)	
	100/100	10 (10.18)		0 (3)	
2	100/102.38	3 (2.646)	0.2225	12 (6)	12*
	102.38/102.38	0 (0.172)		0 (3)	

*Significant at 5 % ($p<0.05$)

LOCUS I:

In *N. japonicus*, the observed heterozygosity was 0 and in *N.mesoprion* it was 1. The expected heterozygosity was 0.355 in *N.japonicus* and 0.5 in *N.mesoprion*. Genetic Identity (I) value for *N.japonicus / N.mesoprion* comparison was 0.880 and the Genetic distance (D) 0.128.

LOCUS II:

In *N.japonicus*, the observed heterozygosity was 0.231 and in *N.mesoprion* it was 1. In *N.japonicus* the expected heterozygosity was 0.2036 and in *N.mesoprion* it was 0.5. Genetic Identity (I) was 0.792 for *N.japonicus / N.mesoprion* comparison and genetic distance (D) 0.233. Only locus 2 in *N.japonicus* showed genotype frequencies as per Hardy-Weinberg equilibrium.

Average heterozygosity was 0.116 for *N. japonicus* and 1 for *N. mesoprion*. Average comparative genetic identity value for *N.* japonicus */ N.mesoprion* was 0.836 and genetic distance 0.181.

B.II. INTRASPECIES / POPULATION COMPARISONS OF *N.mesoprion*:

The samples of *N.mesoprion* collected from Chennai showed 5 electrophoretic patterns - A, B, C, D and E. 35.71 % showed pattern A, 21.43 % each showed pattern B and C, 14.29 % showed pattern D and 7.14 % pattern E. Samples of *N.mesoprion* collected from Kochi showed 4 electrophoretic patterns - F, G, H and I. 46.15 % showed F pattern, 30.77 % showed G pattern, 15.38 % H pattern and 7.69 % I pattern. (Plates 4 & 5, Fig .3)

Table VI. ALLELE FREQUENCIES AT 3 LOCI IN *N.mesoprion* POPULATIONS FROM KOCHI AND CHENNAI .

Locus	Allele	Kochi	Chennai
1	97.22	0.5	O.464
	100	0.5	0.536
2	100	0.692	0.679
	105.26	0.308	0.321
3	100	0.731	0.821
	102.38	0.269	0.179

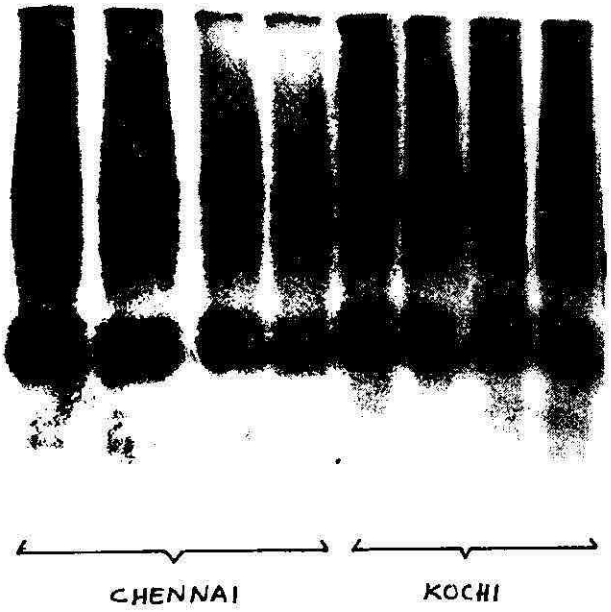


Plate 4. Sarcoplasmic protein patterns in *N. mesoprion* populations from Chennai and Kochi

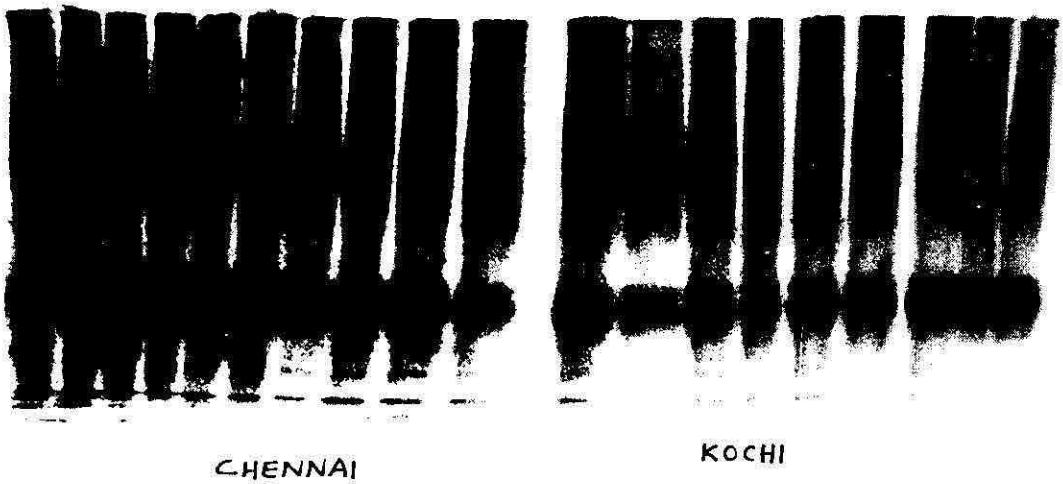


Plate 5. Sarcoplasmic protein patterns in *N. mesoprion* populations from Chennai and Kochi

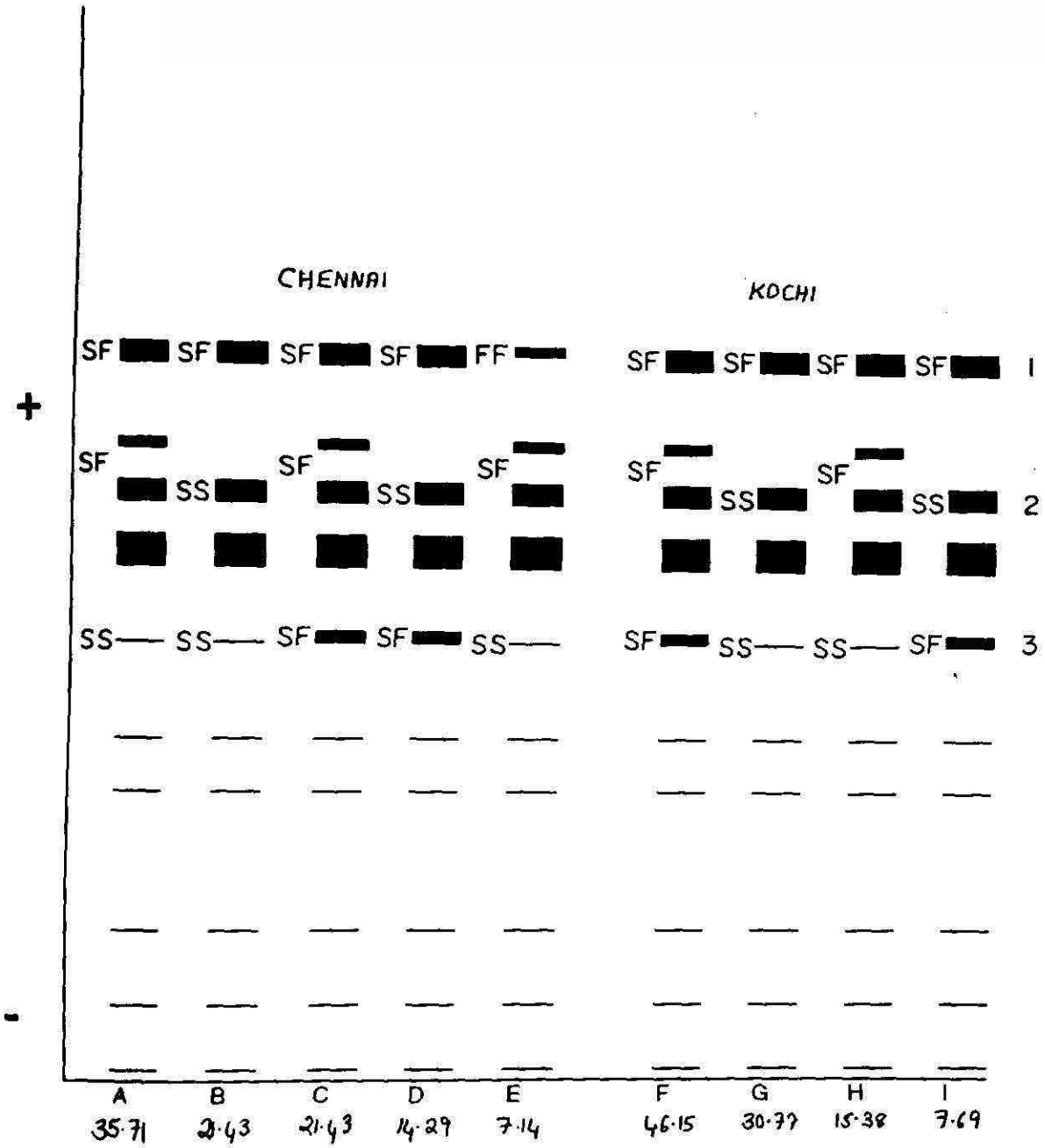


FIG. 3 Zymogram showing intraspecific comparison of *N.mesoprion* samples from Chennai and Kochi

Table VII. OBSERVED AND EXPECTED GENOTYPE FREQUENCIES IN *N.mesoprion* POPULATIONS FROM KOCHI AND CHENNAI.

LOCUS	GENOTYPE	KOCHI		CHENNAI	
		OBSERVED (EXPECTED)	χ^2	OBSERVED (EXPECTED)	χ^2
	97.22/97.22	0 (3.25)		0 (3.014)	
1	97.22/100	13 (6.5)	13*	13 (6.96)	10.5263*
	100/100	0 (3.25)		1 (4.022)	
	100/100	5 (6.225)		5 (6.45)	
2	100/105.26	8 (5.5415)	2.565	9 (6.10)	3.1472
	105.26/105.26	0 (1.233)		0 (1.4425)	
	100/100	6 (6.947)		9 (9.436)	
3	100/102.38	7 (5.1126)	1.766	5 (4.115)	0.658
	102.38	0 (0.9406)		0 (0.448)	

*Significant at 5 % (p, 0.05)

LOCUS I.

In *N. mesoprion* samples collected from Kochi, the observed heterozygosity was 1 and the expected 0.5. The Chennai samples showed an observed heterozygosity of 0.929 and expected 0.497. The genetic identity and distance values for Kochi / Chennai comparisons were 0.998 and 0.002 respectively. The genotype

frequencies did not conform to Hardy-Weinberg equilibrium in both the populations.

LOCUS II.

The observed heterozygosity for *N.mesoprion* samples collected from Kochi was 0.615 and the expected 0.426 .For Chennai samples, the observed and expected heterozygosities were 0.643 and 0.436 respectively.The genetic identity and distance values for Kochi / Chennai comparisons were 0.9995 and 0.0005 respectively.The genotype frequencies were in conformity with Hardy-Weinberg equilibrium.

LOCUS III.

The *N.mesoprion* samples showed an observed heterozygosity of 0.538 from Kochi and 0.357 from Chennai. The expected heterozygosity for Kochi and Chennai samples were 0.393 and 0.294 respectively. Genetic identity and distance values for Kochi and Chennai comparisons were in accordance with Hardy-Weinberg equilibrium law.

The average heterozygosity values for *N.mesoprion* samples from Kochi and Chennai were 0.718 and 0.643 respectively. Hence the average heterozygosity for *N.mesoprion* was 0.681. The average

genetic identity and distance values for Kochi / Chennai comparison of *N.mesoprion* were 0.9962 and 0.0038 respectively.

DISCUSSION

DISCUSSION

Electrophoretic data provide valuable information for the evaluation of interspecific and intraspecific genetic variability. However, complementary data from other sources are needed for a comprehensive view of population differentiation (Winans, 1984). Multivariate morphometry represents an appropriate tool to assess distinctness and phenetic relationships between closely related taxa as in the study of geographic variation and racial affinities (Thorpe, 1976).

Truss morphometrics is a better tool than traditional methods alone for probing evolutionary processes or elucidating relationships among populations (Winans, 1984). Morphometric studies by statistical methods were based on a set of traditional measurements, which were providing uneven and biased aerial coverage of the entire body form of the specimen. Truss network provides a more systematic and geometric characterisation of fish shape (Sathianandan, 1999). Morphometric errors in theory, radiates through repeatedly used conventional data much more readily than through truss data, as any uncertainity in the exact position of conventional points, such as snout tip, causes errors that radiate

throughout the result. Truss points are much easier to pinpoint as they are anatomical landmarks rather than body extremes, the position of which may not be homologous throughout the population (Strauss and Bookstein, 1982). Roby *et al.* (1991) observed more detailed shape description of capelin, *Mallotus villosus* using truss.

Analysis of truss network measurements in the present study was done by principal component analysis (PCA) and sheared principal component analysis (SPCA). PCA does not require any prior information about the groups in the analysis of truss data. The first component factor of PCA is interpreted as size component and subsequent component factors are designated as shape variables. The percentage of variation explained by these factors should be considered before conclusions are made. In the present study, PC-I and PC-II accounted for 78.1 % of the total cumulative variation.

Sheared PCA was done to remove size component from the PC scores computed for each specimen. Sheared PC-I and PC-II accounted for 72.87 % of the total cumulative variation.

The present study revealed that the *N. mesoprion* populations from Chennai and Kochi were morphologically homogenous. Truss morphometric characterisation of 8 strains of Nile tilapia indicated few

truss morphometric differences (Velasco *et al.*, 1996). Harris (1975) suggested that if the number of individuals minus the number of variables measured is more than 30, then the sample size could be considered as adequate. The sample size in the study of Nile tilapia, was inadequate. The sample size in the present study was adequate as per Harris's suggestions.

Tsuyuki *et al.* (1965) have discussed the value of muscle myogen in phylogenetic studies and in intraspecific protein variation as diagnostic character of stock analysis. In the present study, species specific electrophoretic pattern was found in muscle tissue of *N.mesoprion* and *N.japonicus*. Electrophoretic study of muscle and eye lens proteins of *N.mesoprion, N.japonicus* and *N.delagoae* revealed 8 components in the muscle protein in all the 3 species (Chakraborty, 1989). There were differences in the mobility of these components but that was not enough to establish species specificity. Eye lens proteins revealed species specific patterns. The present study revealed an extra band in the muscle tissue of *N.mesoprion*. The differences in the results of the two studies could be due to the differences in procedure or sample location.

In biochemical genetic investigation, it is important to report whether the distribution of observed genotypes at each locus in each

population is according to the expected genotype distribution as per Hardy-Weinberg equilibrium (Utter, 1987). In the present study involving interspecies comparison between *N.mesoprion* and *N.japonicus*, genotype frequencies at both loci in *N.mesoprion* did not conform to Hardy-Weinberg equilibrium. But in intraspecific studies involving *N.mesoprion* populations from Chennai and Kochi, only 1 locus out of 3, showed significant variation. The deviation in both cases were mainly due to excess of heterozygosity. The occurrence of high heterozygosity were reported in *Mugil cephalus* (Peterson and Shehadeh, 1971; Vijayakumar, 1992), lake white fish, *Coregonus clupeaformis* (Imhof *et al.,* 1980), anchovies (Daly and Richardson, 1980), Atlantic salmon, *Salmo salar* (Stahl, 1987; Verspoor, 1988) and oil sardine (Venkitakrishnan, 1993).

The possible reasons given by the above workers for the occurrence of excess of heterozygotes in populations of different species appear to be presumptive rather than based on experimental evidence. It may be suggested that some form of heterogenous advantage (survival, adaptability or growth) existed in *N.mesoprion* samples causing the observed unequilibrium in the genotype distribution in the present study. The heterozygote advantage is sometimes equated to heterosis, particularly produced by two different strains (Strickberger, 1968).

Nei (1976) estimated that for local races of a species, genetic distance, D, ranges from nearly 0 to 0.05, for subspecies 0.02 to 0.2 and for full species 0.10 to 2.0. Average D value for *N.mesoprion* / *N.japonicus* comparison was 0.181 that confirms the species status for these species. Average D value for comparison of *N.mesoprion* populations from Chennai and Kochi was 0.0038, which was within the range 0-0.005, which was suggested for local races of a species. In Sparid fishes, the average D value between congeneric species was reported as 0.115 and between conspecific subpopulation as 0.002(Taniguchi *et al.*, 1986). In Sciaenidae, the average D value varied between 0.0017 and 0.039 for conspecific subpopulation and 0.092 between two species of Nibea (Menezes and Taniguchi, 1988).

Average heterozygosity observed in *N.japonicus* in the present work was 0.116. In *N.mesoprion* populations from Chennai and Kochi, the average heterozygosity values were 0.643 and 0.718 respectively. The average heterozygosity values reported for *N.mesoprion* in the present study were higher than that reported for other tropical and subtropical marine fishes (Smith and Fujio, 1982; Vijayakumar, 1992; Venkitakrishnan, 1993; Menezes and Parulekar, 1998).

The present study could not reveal any marked differentiation between two populations of *N.mesoprion* from Chennai and Kochi. Lower levels of genetic divergence have been detected among marine fishes (Gyllensten, 1985; Smith *et al.*, 1990; Ward *et al.*, 1994). The relative lack of physical barriers and high incidence of extensive larval dispersal in marine systems generally result in little intraspecific genetic divergence, even over considerable geographic distances (Gyllensten, 1985). Also, electrophoretic characters may be too evolutionary conservative to demonstrate genetic differences among natural population (Lester and Pante, 1992). Gene flow does not have a swamping influence on processes such as selection and drift that lead to differentiation (Speiss, 1977) but it has atleast these three effects in subdivided populations. It introduces new alleles, it increases the effective population size and allows the local population to adjust to environmental change by utilizing new variability or by sifting it out if it lowers fitness.

The resolution of protein electrophoresis is not always adequate for detecting differences between populations or individuals. Because of the redundancy in the DNA code that dictates protein sequences, all changes in a gene may not result in a change in the overall charge of the protein expressed; thus many genetic variants are not detected by protein electrophoresis. Furthermore, protein electrophoresis is

limited to detecting genetic changes that affect genes that actively express proteins detectable with a histochemical stain (Hunter and Markert, 1957;Morizot and Schmidt, 1990). These genes constitute only a small percentage of the whole genome of an animal. DNA level markers are able to detect more variation because the sequences are being assayed more directly. Recent advances in molecular techniques such as RFLP analysis of mitochondrial DNA, hybridisation – based and PCR based analysis of nuclear DNA have added tools for stock identification in addition to serological, immunological and morphometric techniques. Population genetic analysis of Nemipterids from Indian waters using DNA level markers is suggested to elucidate their stock structure.

It is worth stating that though more variability is detectable with DNA methods, the existing data for proteins / isozymes in many fish species represent huge wealth of information that should not be disregarded. Indeed until a substantial amount of DNA data has been collected for a particular species, the existing protein / isozyme database often represents a more practical source of genetic information.

SUMMARY

. Truss morphometric studies on two populations of *N.mesoprion* from Chennai and Kochi showed that the two populations were morphologically homogenous.

. Electrophoretic studies on muscle proteins revealed no marked differences in the allele frequencies between *N.mesoprion* populations from Chennai and Kochi.

. Average heterozygosity value for Chennai and Kochi populations of *N.mesoprion* were 0.643 and 0.718 which were very high indicating that heterozygosity was favoured.

. Comparative genetic identity (I) and genetic distance (D) values for Chennai and Kochi populations of *N.mesoprion* were 0.9962 and 0.0038, which showed that the populations were homogenous.

. Protein gel electrophoretic study done to compare *N.mesoprion* and *N.japoniocus* identified a species-specific band (Rf 60.49) in

N.mesoprion. *N.mesoprion* showed 9 components in the muscle protein whereas *N.japonicus* showed 8 components.

. There was marked difference in the allele frequencies in both the loci tested in *N.japonicus and N.mesoprion.*

. Hardy-Weinberg equilibrium was conformed only in locus 2 of *N.japonicus*. Deviation occurred mainly due to excess of heterozygosity.

. Average observed heterozygosity for *N.japonicus* was 0.116 and for *N.mesoprion* it was 1.

. Comparative average genetic identity and genetic distance values for *N.mesoprion and N.japonicus* comparisons were 0.836 and 0.181 respectively.

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