AN INVESTIGATION ON THE BIOCHEMICAL GENETICS OF THE INDIAN PEARL OYSTER, *Pinctada fucata* (GOULD)

THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN MARICULTURE OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
VERSOVA, MUMBAI - 400 061

BY

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INDIAN COUNCIL OF AGRICULTURAL RESEARCH
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
P.B. No. 1603, KOCHI - 682 014
INDIA

MAY 1998

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DECLARATION

I hereby declare that this thesis entitled **"An investigation on the biochemical genetics of the Indian pearl oyster, *Pinctada fucata* (Gould)"** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Kochi,

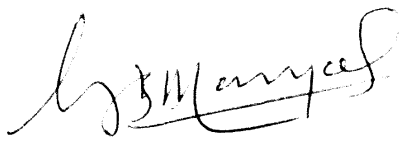
May, 1998.

V. SAPNA

CERTIFICATE

Certified that the thesis entitled **"An investigation on the biochemical genetics of the Indian pearl oyster, *Pinctada fucata (Gould)*"** is a bonafide record of the work carried out by **Ms. V. Sapna** under my guidance and supervision and that no part thereof has been presented for the award of any other degree, diploma or any other similar title.

Major Advisor



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1. *Introduction*

Since time immemorial, pearl obtained from the marine pearl oyster, one among the bivalves, has been highly appreciated as a fascinating gem by all. Its commercial and aesthetic values have also been expressed in different ways in folk songs, poetries, philosophies and even in various scriptures (Alagarswami 1991). The precious nature of the pearl has been vividly recorded in the Bible (Mathew, chapter 13, versus 45,46). However, the biological mechanism of pearl formation within the oysters remained a mystery until recent times.

In view of the high commercial value of pearls, scientific investigations have progressed to the extent, that, it has become a subject of genetic engineering, for improving the quality of the pearls formed through culture methods. Consequently, the culture of pearl has become a booming industry in the nations, like Japan and Australia. The world contribution of the pearls, mother of pearls and shells, for the year 1995, was 16,956 metric tonnes (FAO 1997). Except pearl oysters, no other organism is able to produce the gem quality pearls. So, the biological importance of the pearl producing oyster species, chiefly *Pinctada fucata* (Gould), *P. maxima* (Jameson) and *P. margaritifera* (Linnaeus) is immense. Since the pearl culture industry throughout the world depends on the traditional fisheries of the oysters for collection of broodstock or spats for the culture, a thorough knowledge on the biology and fishery of the resources is essential for their judicious exploitation.

It is quite interesting to note that the fishery investigations on the Indian pearl oyster, *P. fucata* began as early as 1835 and more than 200 reports on various aspects of its fishery and biology are available (CMFRI Bulletin No. 39: Pearl Culture). The pearl oyster species, *P. fucata* formerly known as *P. vulgaris*, has a wide distribution from Western Pacific Ocean, Australia, along the Indian coast and to the Persian Gulf. In India, *P. fucata* occurs in the Gulf of Mannar, where the oyster beds are popularly called as "Paars", and in the Gulf of Kutch, where the beds are called as "Khaddas". In addition, spats are collected from the wild in Vizhinjam. A comprehensive account of the earliest pearl fisheries of India was published in 1922 by James Hornell. Since then, the pearl fisheries of India, have undergone wide ranging fluctuations, and now *P. fucata* is categorised as a " vulnerable species" along the coast of India (James 1994). Declaration of the pearl oyster beds as marine parks and attempts to translocate the stocks to more congenial places are the welcome protective measures (Patel 1985). However, a thorough knowledge on the natural units of *P. fucata* fisheries is a prerequisite for planning and taking short and long term management measures for the exploitation as well as conservation of the resource, especially when it has been declared as a vulnerable species. Besides, a detailed knowledge on the genetic variability in the wild stock is essential before founding the base population for a hatchery. Genetic variation is the basic resource of any successful breeding programme (Allendorf and Ryman 1987).

Identification of the natural units of fisheries management depends on its accepted biological definitions. The concept of natural units within the species rests on the theory of evolution, which presupposes that every species may be undergoing micro and macro evolutionary changes. Such unknown changes might have caused the original species to gradually differentiate into heterogeneous populations/stocks or races or even subspecies, depending on the degree of evolutionary changes taken place (Ayala and Kiger 1980). In population dynamics, a unit stock of fisheries management is defined as "a relatively homogeneous and self contained population where losses by emigration, and successions by immigration, if any, are negligible in relation to the rates of growth and mortality (Anon 1960; Cushing 1981). The current concept of the units of fisheries management is based on a mendelian population. It is defined as a "reproductive community of sexual and cross fertilized individuals, among whom matings regularly occur and consequently, have a common gene pool" (Dobzhansky 1967). Naturally, a comparison of sample genes of populations of *P. fucata should* reveal the population genetic composition of the species. Significant differences in the gene frequencies at several loci can indicate the existence of genetically heterogeneous stocks within the species. Detection of differences in the gene frequencies is possible only when intraspecies genetic variability exists within the species.

Intraspecies genetic variability expressed by the gene controlled enzymatic and non-enzymatic proteins is a natural phenomenon in almost all

kinds of invertebrate and vertebrate species. Because of the gene- protein direct relationship, protein variability can be equated with genotype variability. In practical terms, the proteins and their variant forms are useful genetic markers or tags for the analysis of the population genetics of a species (Robertson 1972; Jamieson 1974).

The current popular method for separation, detection and identification of the biochemical genetic markers and their variant forms are gel electrophoresis and specific enzyme techniques developed by Smithies (1955) and Hunter and Markert (1957) (vide Smith 1968; Brewer 1970). The worldwide application of the gel electrophoresis and isoenzyme techniques have revealed not only intraspecies genetic variability in fishes and shell fishes of commercial importance, but also valuable informations on the population genetic stock structure which are essential for planning scientific management of the marine resources in all respects. The specific genotype informations gathered are also essential for planning to improve the quality of the broodstock through selective breeding programmes.

The doctoral thesis submitted here contains the results of an investigation undertaken for the first time to study the genetic variability and the population genetic stock structure of the commercially very important pearl oyster species of India, *Pinctada fucata* (Gould).

2. *Review of literature*

The discovery of the laws of Mendelian genetics and the gene-protein relationship have inspired the researches in various fields of biology to look for the methods or techniques for the detection or identification of genetic variations between individuals, populations, species and so forth. Thus, the introduction of zone electrophoresis for separation of soluble tissue proteins by Smithies (1955) and histochemical techniques for the visual identification of enzymes separated by zone electrophoresis by Hunter and Markert (1957) have enabled the biologists to discover and report a common phenomenon of inter and intraspecies genetic polymorphism in all kinds of organisms including drosophila (Lewontin and Hubby 1966; Ayala et al. 1971) and man (Harris 1966). The above pioneer works and the later publication of books on different methods of zone electrophoresis by Smith (1968) and isozyme techniques by Brewer (1970) have helped to apply these biochemical genetic techniques in various specialised fields of biology. The advantages of gel electrophoretic and isozyme techniques over the traditional phenotypic methods of genetic studies, involving dominant and recessive alleles were of two folds. First, the observed protein phenotype patterns could be equated with genotype patterns expressed as codominant allelic products (Jamieson 1974; Utter et al. 1987; Purdom 1993). Second, the attempts to estimate the level of genetic variations by traditional methods of genetic analysis were severely restricted and laborious, since they did not permit to measure the proportions of the genes which are polymorphic or the loci which are heterozygous in a sample population. On the otherhand, the biochemical genetic techniques allowed to screen larger number of enzyme loci for direct

estimation of genetic polymorphism and heterozygosity in a sample population (Ayala and Kiger 1980).

The application of gel electrophoresis for accurate identification of fish species and its subpopulation units has been reported since 1950s (Connel 1953; Utter 1991). Agar gel electrophoresis of blood haemoglobin in different marine fish species revealed inter and intraspecies haemoglobin polymorphism (Sick 1961). Species specific muscle protein and haemoglobins of marine and fresh water vertebrates were also reported (Tsuyuki et al. 1965). Later, Kirpichnikov (1981) reported protein variations in 100 species of fish.

The importance of application of biochemical genetic techniques in fisheries research and management was highlighted during a special meeting of the International Council for Exploration of Seas (ICES) on the biochemical and serological identification of fish stocks (de Ligny 1971). Meanwhile, the problems of defining and recognising units of fisheries management were discussed and reviewed by many fishery biologists (Marr 1957, Muzinic and Marr 1960). Realising the possible effects of environmental parameters on the biological parameters considered for the unit stock definition, a more effective definition was suggested on the basis of genetic characteristics of the populations (Moller 1971). The problems of the concept of unit stock and methods of stock identification were again elaborately discussed under the

stock concept in the symposium held in 1981 (Can. J. Fish Aqu. Sci., Special issue, Vol.38, No.12).

Consequently, the application of the refined biochemical genetic techniques resulted in an upsurge of studies on the levels of genetic variability and genetic stock structure differences within many commercially important species of fin fish and shell fish, both marine as well as fresh water. Many reviews were made on the reports of genetic variability in various fin fish (Utter et al. 1974; Allendorf and Utter 1979), molluscan shell fish (Koehn and Mitton 1972; Campbell et al. 1975; Wilkins 1975,1977; Tracey et al. 1975a; Fujino and Nagaya 1977a, 1977b; Buroker et al. 1979a, 1979b; Berger 1983; Fujio et al. 1983; Koehn 1983; Beaumont and Beveridge 1984; Benzie and Williams 1992, 1995, 1996; Beaumont et al. 1993; Beaumont and Pether 1996; Yokogawa 1997) and crustacean shell fish populations (Tracey et al. 1975b; Sbordoni et al. 1986; Li et al. 1993).

Among the commercially important molluscan shell fish subjected to genetic studies, lion's share is taken by the bivalve species of genus *Mytilus* (Boyer 1972; Tracey et al. 1975a; Koehn et al. 1976; Levinton and Koehn 1976; Lassen and Turano 1978; Gartner – Kepkay et al. 1980; Kartavtsev and Zaslavskaya1983; Buroker 1984; Grant and Cherry 1985; Varvio et al.1988; Humphrey and Crenshaw 1989; Frank et al. 1990; Koehn 1991; Gosling 1992; Grant et al. 1992). Others in the category are *Crassostrea* spp. (Buroker et al. 1975, Buroker 1979; Fujino and Nagaya 1977a, 1977b; Gosling 1982;

Buroker 1983; Foltz and Chatry 1986; Hedgecock and Sly 1990), *Saccostrea* spp. (Buroker et al. 1979 a, 1979b), *Chlamys* sp. (Beaumont 1982) and species of abalone (Fujino 1978 a , 1978b ; Fujino et al. 1980; Mgaya et al. 1995).

Application of various genetic tools can be viewed in plenty in the aquaculture of pearl oysters (Wada 1994). Among the economically important pearl oysters, the Japanese pearl oyster, *P. fucata* (Wada 1975b, 1975c, 1982,1984,1986c), *P. margaritifera* (Blanc et al. 1985; Blanc and Durand 1989; Durand and Blanc 1989; Durand et al. 1993; Benzie and Ballment 1994) , *P. maxima* (Johnson and Joll 1993), *P. albina* and *P. maculata* (Wada 1982) and *P. radiata* (Blanc, Personal communication) were investigated for the biochemical genetic variations.

The results of the biochemical genetic studies on bivalves may be reviewed as follows. As in other organisms, the level of genetic variability in the bivalves may vary from species to species (Wilkins 1975). Thus the genetic variability measured especially by the heterozygosity at many loci varied in different animals or situations, and in some instances even heterozygote deficiency was noted (Buroker et al. 1975; Tracey et al.1975a; Lassen and Turano 1978; Buroker et al. 1979a, 1979b; Gartner – Kepkay et al.1980; Beaumont 1982; Gosling 1982; Zouros and Foltz 1984; Singh and Green 1984). The influence of age (Flotz and Zouros 1984), or size (Beaumont 1982), or growth (Newkirk et al. 1977; Koehn and Shumway 1982; Mallet et

al. 1986; Rodhouse et al. 1986; Koehn and Gaffney 1984) on the degree of heterozygosity or how growth and other fitness related traits (Lannan 1980; Gaffney and Scott 1984; Rodhouse and Gaffney 1984; Mallet et al. 1985; Foltz and Chatry 1986; Saavedra and Guerre 1996) act accordingly in molluscs have been reported. The level of heterozygosity in wild and hatchery populations can also vary (Mgaya et al.1995). A statistical comparison of allele frequencies at many polymorphic loci is a means of measuring the homogeneity or heterogeneity of the populations existing at the time of investigation. Significant allelic frequency differences at many loci can mean genetically different stocks.

The primary objectives of most of these investigations on the pearl oysters were to measure the level of genetic variability within the species and between its different geographic populations. The secondary objectives were to measure the genetic differences between the wild and hatchery stocks and attempt to genetically improve the hatchery stock for the betterment of the quality of pearls produced by the pearl oysters (Wada 1975a; 1984, 1985, 1986a, 1986b, 1989, 1990, 1991, 1994,1995; Wada and Komaru 1991, 1994; Durand and Blanc 1989; Johnson and Joll 1993; Benzie and Ballment 1994).

Though detailed reports on the fishery, biology, hatchery techniques and induced pearl production of the Indian pearl oyster, *P. fucata* are available (Pearl culture, CMFRI Bulletin No. 39, 1987; Alagarswami 1991;

Gervis and Sims 1992; Victor et al. 1995), there are no reports on the genetic studies of Indian species. The thesis submitted here contains the results of the biochemical genetic studies conducted on Indian pearl oyster, *P. fucata* for the first time.

3. Materials and Methods

3. 1. Materials

3. 1. 1. Collection

Wild specimens of *Pinctada fucata*, were collected between February 1996 and December 1997, from Vizhinjam ($8^0$22'N, $76^0$56'E), Tuticorin ($8^0$48'N, $78^0$8'E) in the Gulf of Mannar and Sikka ($22^0$31'N, $69^0$49'E) in the Gulf of Kutch (Fig. 1, Plates 1& 2). Vizhinjam specimens were not directly collected from the bed, but from the Vizhinjam Centre of CMFRI where the spats collected from the ropes used for mussel culture were reared to adult stage at their farm. Tuticorin specimens were taken from the wild sample collection made by Tuticorin Centre of CMFRI and Sikka samples from the wild samples collected by Sikka Research Centre of Gujarat State Agricultural University. A total of 77 adult specimens were examined from the three sites selected for wild oyster collection. Hatchery produced spats and mother pearl oysters were supplied by Tuticorin and Mandapam pearl oyster hatcheries of CMFRI. Mandapam samples were the spats produced at Tuticorin, and then reared to juvenile stage at Mandapam. From the hatchery specimens, 54 adults, 25 juveniles and 53 spats were examined. Thus a grand total of 209 oyster specimens collected from the wild and hatchery were tested.

3. 1. 2. Transportation

Except spats which were transported live in airfilled polythene bags having seawater filled to 50% capacity, pearl oysters were otherwise

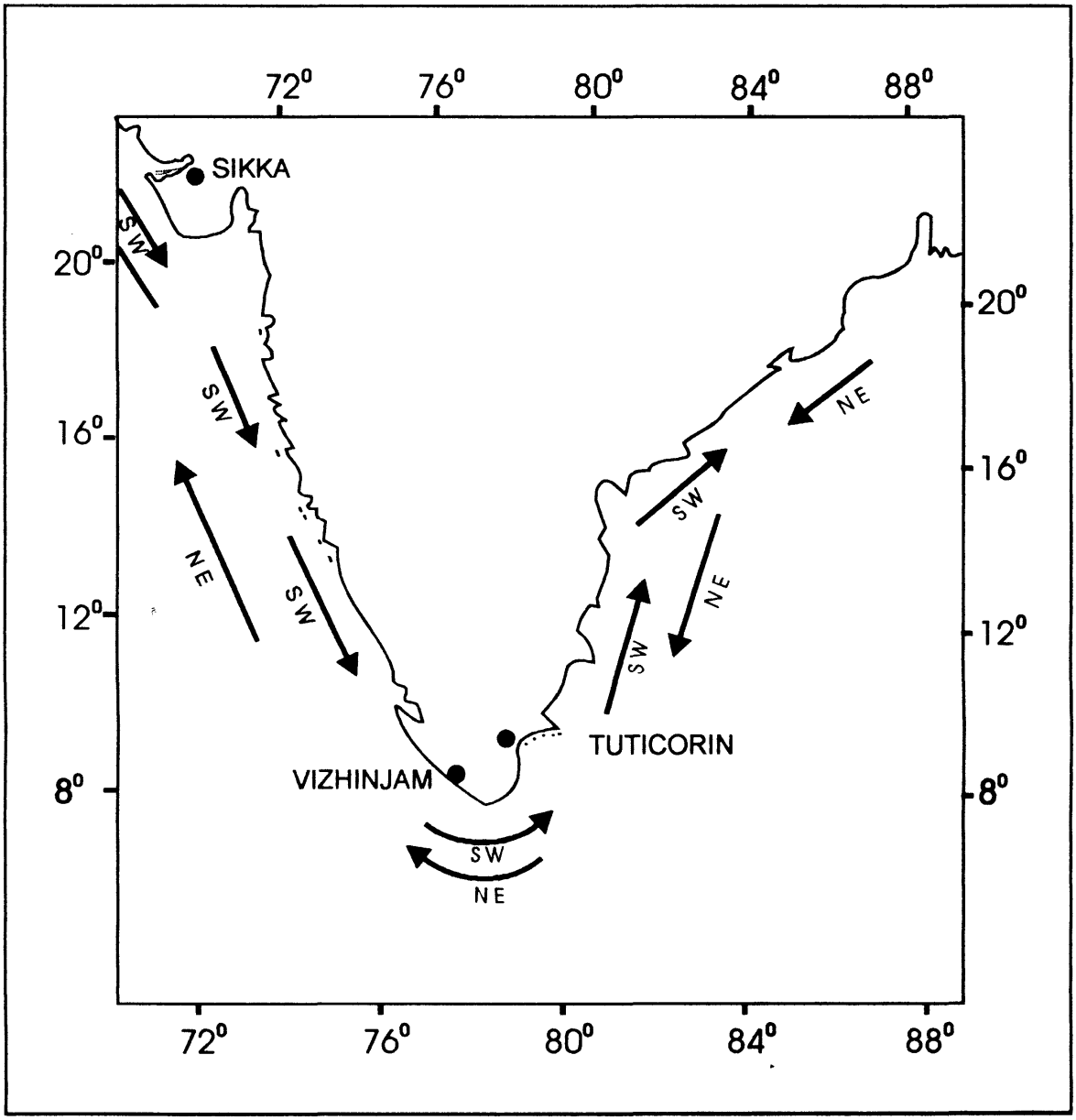

Fig 1. Collection sites for *Pinctada fucata* and the general surface currents along the coast of India

SW: Current direction during South West Monsoon.
NE: Current direction during North East Monsoon.

Plate 1. *P. fucata* from the Gulf of Kutch.

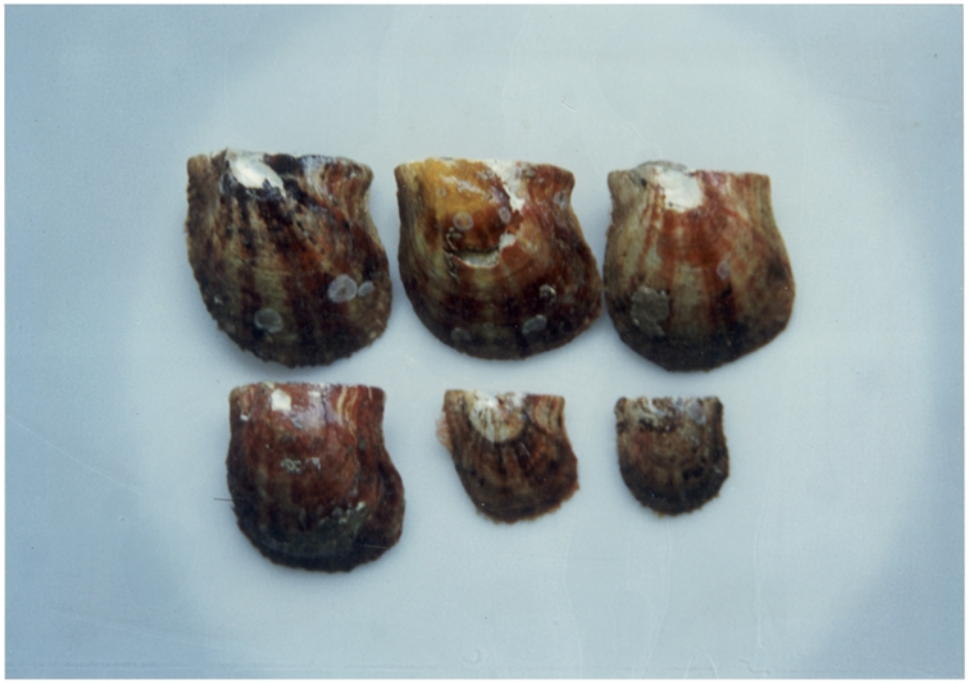

Plate 2. *P. fucata* from the Gulf of Mannar.

Plate 3. Prismatic layer colour variation in *P. fucata*
(Top Gulf of Kutch strains
Bottom Gulf of Mannar strains)

Plate 4. Nacre colour variation in *P. fucata*
(Top Gulf of Kutch strains
Bottom Gulf of Mannar strains)

transported live in moist condition to Cochin. Wild samples from the Gulf Kutch were air lifted from Jamnagar to Mumbai and upon reaching there, animals were transferred to seawater collected from the Gulf of Kutch, and aeration was provided. The next day, pearl oysters were again kept in moist condition and air lifted to Cochin. Total transportation time taken was 17 hours, including the overnight halt at Mumbai. In the lab, animals were transferred to aerated seawater having salinity 30~33ppt and temperature 28^{0}C. After the initial stress faced during transportation was over, animals were fed with *Chaetoceros sp.* the next day. Dead animals were removed at once from the tank, and were not used since in most of the cases they were detected only when the tissues showed extreme stages of deterioration. Weak animals were immediately stored at –20^{0}C, till analysis.

3. 2. Methods

3. 2. 1. Electrophoretic Analyses

3. 2. 1. 1. Standardisation of the methodology

3. 2. 1. 1a Sample Preparation

Tissue samples from adductor muscle, digestive diverticula and mantle were taken from each of the sacrificed animals. Each of these were weighed and immediately kept below 0^{0}C. This procedure was repeated initially for five individuals at a stretch. Later, each of the tissues was minced under cold conditions and then separately homogenised in selected media at selected tissue-medium ratios. The homogenising media tried were double distilled

water, 0.2 M sucrose solution and 0.05 M Tris/HCl, pH 7 buffer. The ratios at which these media were utilised for homogenising the three selected tissues were 1:1 (w/v), 1:2 (w/v) and 2:1 (w/v). Mechanical homogenisor (Remi) and a manual glass homogenisor were employed. Homogenisation was invariably conducted under cold conditions. Spats were homogenised in a precooled mortar using a pestle. Homogenates were taken in Eppendorf tubes. Centrifugation was done at speeds ranging from 3,000 rpm to 12,000 rpm for periods ranging from 5 minutes to 30 minutes, at 4^0C. (These conditions were selected based on the experience of previous workers on other bivalves). Supernatant obtained was drawn and transferred to another set of labelled Eppendorf tubes, and stored at -20^0C till they were analysed the next day.

3. 2. 1. 1b Electrophoresis

Electrophoresis was done in vertical mini gel electrophoretic unit (Hoeffers, U.S.A.) Medium used was Polyacrylamide gel. Various proportions of acrylamide (30% stock) and Bisacrylamide (2.4% stock) solutions were tried to maximise the resolution and separation of bands. However, for any gel percentage, the proportion of Bisacrylamide was 5% of the total acrylamide concentration (Gordon 1980). For one gel, the total solution prepared was 15 ml and when two gels were simultaneously prepared 25 ml of solution was used. The amount of buffer, Ammonium Per Sulphate (APS) and Tetramethylethylene diamine (TEMED), in any percentage of gel was kept constant at 3 ml, 105 µl, and 15 µl respectively for 15 ml of gel

Table 1. Average values of morphometric variables studied for *P. fucata*

Morphometric Variable	Wild Populations			Hatchery Population		
	Sikka	Tuticorin	Vizhinjam	Adults	Juveniles	Spats
Hinge length (mm)	55.21 (±1.62)*	40.31 (±0.97)	52.83 (±1.19)	43.69 (±0.50)	26.28 (±0.50)	12.41 (±0.46)
Dorsoventral Length (mm)	60.03 (±2.07)	46.47 (±1.09)	63.04 (±0.66)	48.27 (±0.62)	28.14 (±0.69)	11.30 (±0.37)
Shell Width (mm)	19.53 (±0.37)	17.72 (±0.42)	23.78 (±0.28)	21.16 (±0.30)	10.19 (±0.26)	3.25 (±0.21)
Total Weight (gm)	37.90 (±3.20)	13.62 (±1.96)	39.51 (±1.41)	22.01 (±1.20)	3.25 (±0.18)	0.231 (±0.04)
Shell Weight (gm)	24.60 (±1.41)	7.29 (±1.12)	17.63 (±0.68)	11.59 (±0.42)	1.38 (±0.08)	0.066 (±0.007)
Shell Convexity	0.144 (±0.002)	0.154 (±0.002)	0.174 (±0.009)	0.187 (±0.002)	0.158 (±0.002)	0.118 (±0.004)

*Standard error values in parenthesis

preparation and, 5 ml, 0.35 ml and 25 µl respectively for 25 ml of gel preparation. The composition of the buffers used (Table 2) and gels (Table 3) are given. The proportions tried for sample and loading buffer were 1:1 (v/v), 2:1 (v/v), 3:1 (v/v), 1:2 (v/v) and 1:3 (v/v). Sample amounts used per well were 5µl, 10 µl, 15 µl, 20 µl and 25 µl. Loading buffer consisted of 1 ml of 0.5% Bromophenol Blue, 2 ml of Glycerol and 7 ml of double distilled water. Electrophoresis was stopped when the marker dye reached the anodal end, which usually occurred within 2 hours. When the electrophoretic run was over, the gel was taken from the cassette and stained for proteins.

The best tissue giving maximum activity, number of bands and showing band variation for a given set of electrophoretic conditions, producing the best separation and resolution of bands, was selected for protein analysis.

The method standardised for protein separation was utilised for further standardisation of the enzyme systems tried here (Table 4). The staining method of Harris and Hopkinson (1976) described by Aebersold et al. (1987) were adopted for detection of the enzymes. The details of staining recipes with modifications made to suite the local conditions are given below. Agar overlay was used only for GPI and PGM. The electrophoretic banding patterns obtained after the staining were recorded as well as photographed for further reference and analysis.

Table 2. Electrophoresis buffers tried for activity and resolution of the different enzyme/protein loci examined in *P. fucata*

Buffer	Electrode Buffer		Gel Buffer	
	Components per 500 ml	pH	Components per 25 ml	pH
Tris[a] Borate, pH 8.3 (TB) (From Wada, 1975)	0.1M Tris 6.06 gm 1mM $MgCl_2$ 0.10 gm (Adjust the pH with 1M Borate)	8.3	0.1M Tris 0.30 gm 1mM $MgCl_2$ 0.005 gm (Adjust the pH with 1M Borate)	8.3
Tris Citrate Borate, pH 8.7 (TCB) (Modified from Waller and Harris, 1961)	0.3M Borate 9.27 gm 60mM NaOH	8.1	0.5M Tris 1.51 gm (Adjust the pH with 2M Citrate)	8.7
Tris Citrate Borate, pH 6.8 (TCB(1)) (Own Modification)	0.3M Borate 9.27 gm 60mM NaOH	8.1	0.4M Tris 1.21 gm (Adjust the pH with 2M Citrate)	6.8
Tris Citrate, pH 7 (TC) (Shaklee and Keenan, 1986)	135 mM Tris 8.18 gm 43 mM Citrate 4.52 gm.	7.0	9.6 mM Tris 0.03 gm 3mM Citrate 0.016 gm	7.0
Tris EDTA[b] Borate, pH 8.4 (TEB) (Benzie and Ballment, 1994)	150 mM Tris 9.09 gm 3 mM EDTA 0.56 gm 117 mM Borate 3.62 gm	8.4	48 mM Tris 0.15 gm 1 mM EDTA 0.009 gm 37 mM Borate 0.06 gm	8.4
Tris EDTA Citrate, pH 7.7 (TEC) (Modified from Benzie and Ballment, 1994)	0.23 M Tris 13.93gm 40 mM EDTA 7.44gm (Adjust the pH with 2M Citrate)	7.9	0.1 M Tris 0.30 gm 2.5 mM EDTA 0.02 gm (Adjust the pH with 2M Citrate)	7.7
Tris Glycine, pH 8.2 (TG)	0.2 M Glycine 7.51 gm (Adjust the pH, with 2M Tris)	8.2	1.8 M Tris 5.45 gm (Adjust the pH with 3N HCl)	8.7

Tris Glycine, pH 8.0 (TG(1)) (Own Modification)	0.2M Glycine 7.51 gm (Adjust the pH with 2M Tris)	8.0	1M Tris 3.03 gm (Adjust the pH with 3N HCl)	8.0
Tris Maleate, pH 7.4 (TM) (Benzie and Ballment, 1994)	100 mM Tris 6.06 gm 100mM Maleic acid 5.80gm 10 mM EDTA 1.86 gm 10 mM $MgCl_2$ 1.02 gm 125 mM NaOH 2.5 gm	7.4	10 mM Tris 0.03 gm 10mM Maleic acid 0.03 gm 1 mM EDTA 0.009 gm 1 mM $MgCl_2$ 0.005 gm 125 mM NaOH 0.125 gm	7.4

a Tris (hydroxy methyl) aminomethane

b Ethylene Diamine Tetra Acetic Acid

Table 3. Composition of the gels used in the electrophoretic analysis in *P. fucata*

Gel Composites	Gel Composition			
	8% Gel		9% Gel	
	For 15 ml	For 25 ml	For 15 ml	For 25 ml
Acrylamide (30%)	3.8	6.3	4.3	7.1
N,N' – Methylene Bisacrylamide (2.4%)	2.5	4.2	2.8	4.7
Buffer	3.0	5.0	3.0	5.0
Double Distilled Water	5.6	9.3	4.8	8.0
N,N,N',N' – Tetramethylethylene diamine (TEMED)	15*	25*	15*	25*
Ammonium Per Sulphate (APS, 5%)	105*	175*	105*	175*

*In microlitre

Table 4. Enzymes used in screening for activity and resolution, and their names as recommended by the International Union of Biochemistry's Nomenclature Committee (IUBNC 1984)

Abbreviation	Enzyme	E.C*. Number
AAT	Aspartate aminotransferase	2.6.1.1
ADH	Alcohol dehydrogenase	1.1.1.1
AO	Aldehyde oxidase	1.2.3.1
EST	Carboxylesterase	3.1.1.1
DIAPH	Diaphorase	1.8.1.4
ESD	Esterase - D	3.1.1.1
G6PD	Glucose – 6 – phosphate dehydrogenase	1.1.1.49
GPI	Glucose phosphate isomerase	5.3.1.9
IDH	Isocitrate dehydrogenase	1.1.1.42
LAP	Leucine aminopepetidase	3.4.11.1
LDH	Lactate dehydrogenase	1.1.1.27
MDH	Malate dehydrogenase	1.1.1.37
ME	Malic enzyme	1.1.1.40
ODH	Octanol dehydrogenase	1.1.1.73
PGM	Phosphoglucomutase	5.4.2.2
SOD	Superoxide dismutase	1.15.1.1.
PROT	General protein	-

*Enzyme Commission

3. 2. 1. 2. Enzyme Staining Recipes

1	Alcohol Dehydrogenase	ADH
	Dimer	E.C.1.1.1.1
	0.2 m Tris/HCl, pH 8.0	15 ml
	NAD+ 0.5%	0.5 ml
	Ethyl alcohol (Absolute)	0.08 ml
	* MTT 0.5%	0.5 ml
	*PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

2	Aspartate aminotransferase	AAT
	Dimer	E.C. 2.6.1.1
	0.1 M Tris, 0.02 M Citric acid	
	24 mM LiOH, 12 mM Borate	
	0.22% α - ketoglutarate (Sigma)	
	0.5% L - Aspartate (Sigma)	
	(Adjust pH with 3M NAOH)	15 ml
	*Fast Blue BB salt (Sigma)	17 mg

Incubate at 37^{0}C in dark until sufficient activity is present.

3	Aldehyde Oxidase	AO

	Dimer	E.C. 1.2.3.1
	0.2 M Tris/HCl, pH 8.0	15 ml
	Benzaldehyde	0.5 ml
	* NBT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

4	Carboxylesterase	EST
	Subunit structure variable	E.C.3.1.1.1
	0.1 M Phosphate, pH 7.0	15 ml
	* α - Naphthyl acetate (Sigma)	3 mg
	(Dissolve first in 2 ml of acetone)	
	* Fast Blue BB salt (Sigma)	9 mg

Incubate at 37^{0}C in dark until sufficient activity is present.

5	Diaphorase	DIAPH
	Subunit structure uncertain	E.C.1.8.1.4
	0.2 M Tris/HCl, pH 8.0	13 ml
	2,6, Dichlorophenol indophenol 0.02%	2 ml
	(Sigma) in 0.2 M Tris/HCl, pH 8.0	
	* FAD	2 mg
	* NADH	7.5 mg

	* MTT 0.5%	2 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

6	Esterase – D	ESD
	Subunit structure variable, but detects a unique dimeric locus.	E.C.1.3.3.1.-
	0.1 M Phosphate, pH 7.0	15 ml
	* 4 – methylumbelliferyl acetate (Sigma) (Dissolve first in 0.5 ml acetone)	1.5 mg

Incubate at room temperature in dark until sufficient activity is present Only visible by UV light.

7	Glucose 6 Phosphate Dehydrogenase	G6PD
	Dimer	E.C.1.1.1.49
	0.2 M Tris/HCl pH 8.0	15 ml
	NADP + 0.25%	0.5 ml
	1M $MgCl_2$	0.25 ml
	Glucose 6 Phosphate	50 mg
	* MTT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

8	Glucose Phosphate Isomerase (Modified)	GPI
	Dimer	E.C.5.3.1.9
	Fructose 6 Phosphate (20 mg/ml) (Sigma)	1 ml
	NADP+ 0.25%	260 µl
	$MgCl_2$ (20 mg/ml)	0.5 ml
	Glucose 6 Phosphate Dehydrogenase (Sigma)	10 UNITS
	* NBT (8 mg/ml)	0.1 ml
	* PMS 0.5%	340 µl
	Agar overlay	2% agar in 7.7 ml of 0.2 M Tris/HCl, pH 8.0

Incubate at room temperature in dark until sufficient activity is present.

9	Isocitrate Dehydrogenase	IDH
	Dimer	E.C.1.1.1.42
	0.2 M Tris/HCl, pH 8.0	15 ml
	NADP+ 0.25%	0.5 ml
	1 M $MgCl_2$	0.25 ml
	Isocitric acid (Sodium salt)	36 mg

* MTT 0.5%	0.5 ml
* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

10	Lactate Dehydrogenase	LDH
	Tetramer	E.C.1.1.1.27
	0.2 M Tris/HCl, pH 8.0	13 ml
	NAD+ 0.5%	0.5 ml
	0.5 M Lactic acid (1 M NaOH), pH 8.0	2 ml
	* MTT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

11	Malate Dehydrogenase	MDH
	Dimer	E.C.1.1.1.37
	0.2 M Tris/HCl, pH 8.0	13 ml
	NAD+ (0.5%)	0.5 ml
	0.5 M Malic acid/NaOH (~1 M), pH 7.0	2 ml
	* MTT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

12	Malic enzyme	ME
	Tetramer	E.C.1.1.1.40
	0.2 M Tris/HCl, pH 8.0	13 ml
	NADP+ 0.25%	0.5 ml
	1.0 M $MgCl_2$	0.5 ml
	Oxaloacetic acid	9 mg
	0.5 M Malic acid NaOH (~ 1.0 M) pH 7.0	2 ml
	* MTT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

13	Octanol Dehydrogenase	ODH
	Dimer	E.C.1.1.1.73
	0.05 M Tris/HCl, pH8.5	50 ml
	95% Ethanol	1 ml
	Octanol	0.2 ml
	NAD+ 1%	1.25 ml
	* NBT 1%	1 ml
	* PMS 1%	0.5 ml

Incubate at 37^{0}C in dark until sufficient activity is present.

14	Phosphoglucomutase (Modified)	PGM
	Monomer	E.C.5.4.2.2
	Glucose 1 Phosphate (with 1% glucose 1,6 diphosphate (Sigma) 50 mg/ml	125 µl
	NADP+ 0.25%	260 µl
	$MgCl_2$ (20 mg/ml)	1 ml
	Glucose 6 Phosphate Dehydrogenase (Sigma)	10 Units
	* NBT 0.5%	0.2 ml
	* PMS 0.5%	0.3 ml
	Agar overlay	2% agar in 0.2M Tris/HCl, pH 8.0

Incubate at 37^{0}C in dark until sufficient activity is present.

15	Superoxide dismutase	SOD
	Dimer	E.C.1.15.1.1
	0.2 M Tris/HCl, pH 8.5	15 ml
	* MTT 0.5%	0.5 ml
	* PMS 0.5%	0.5 ml

Incubate at 37^{0}C in dark until activity just begins to appear and then set in indirect light until sufficient activity is present.

* To be added, only at the time of staining.

16	General Protein	PROT
	Monomer	
	Coommasie Blue (Sigma)	1.25 g
	Methanol	230 ml
	Double Distilled Water	230 ml
	Glacial Acetic Acid	40 ml

Filter the solution. Stain the gel in dark for 90 minutes and wash. Transfer to destaining solution containing 150 ml of methanol, 70 ml of Acetic acid and 780 ml of water.

3. 2. 2. Morphometric Analyses

Each individual was measured for Hinge length (HL), Dorsoventral Length (DVL) and Shell Width (SW) (Fig. 2). Shell convexity was calculated as SW/(HL+DVL+SW). Individual weight of live animals with the shell, and later shell weight alone were taken. The prominent colour of nacre in the selected area on the left valve of adult oysters (Fig. 3), was observed in a room having only a single source of monochromatic light. Care was taken to maintain uniform angle of observation of the shells.

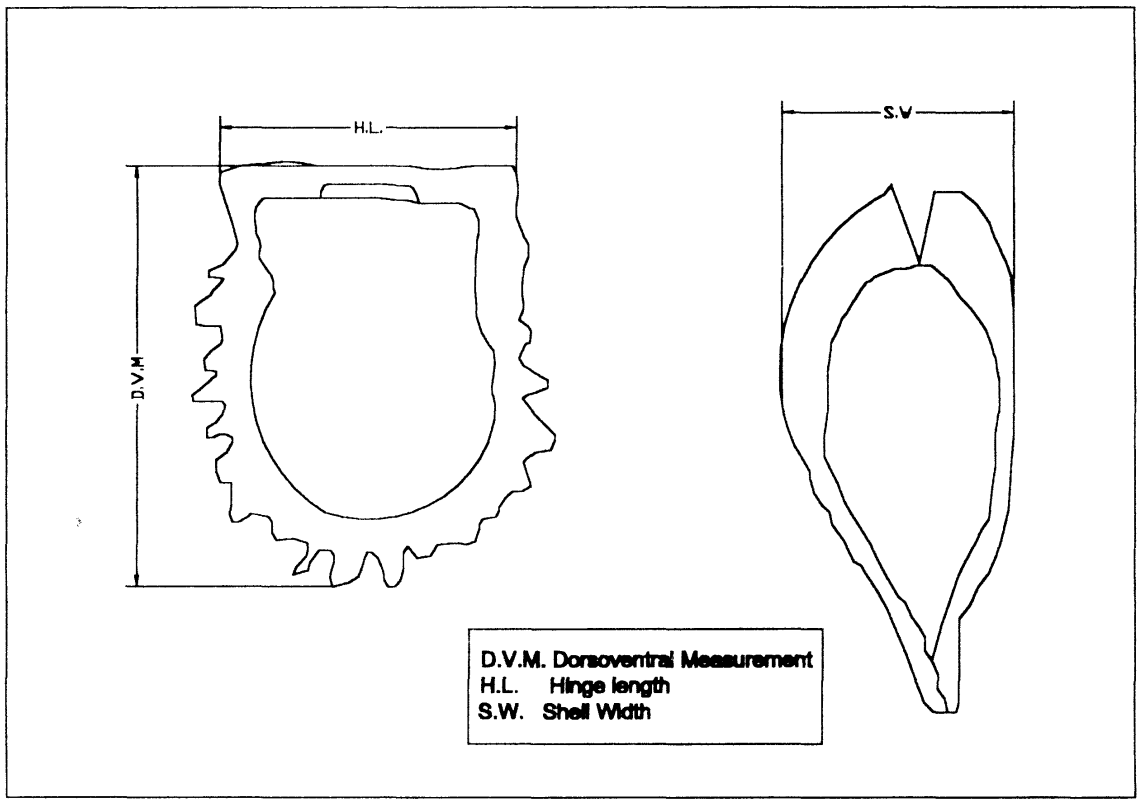

Fig. 2. Various morphometric measurements taken for *P. fucata*

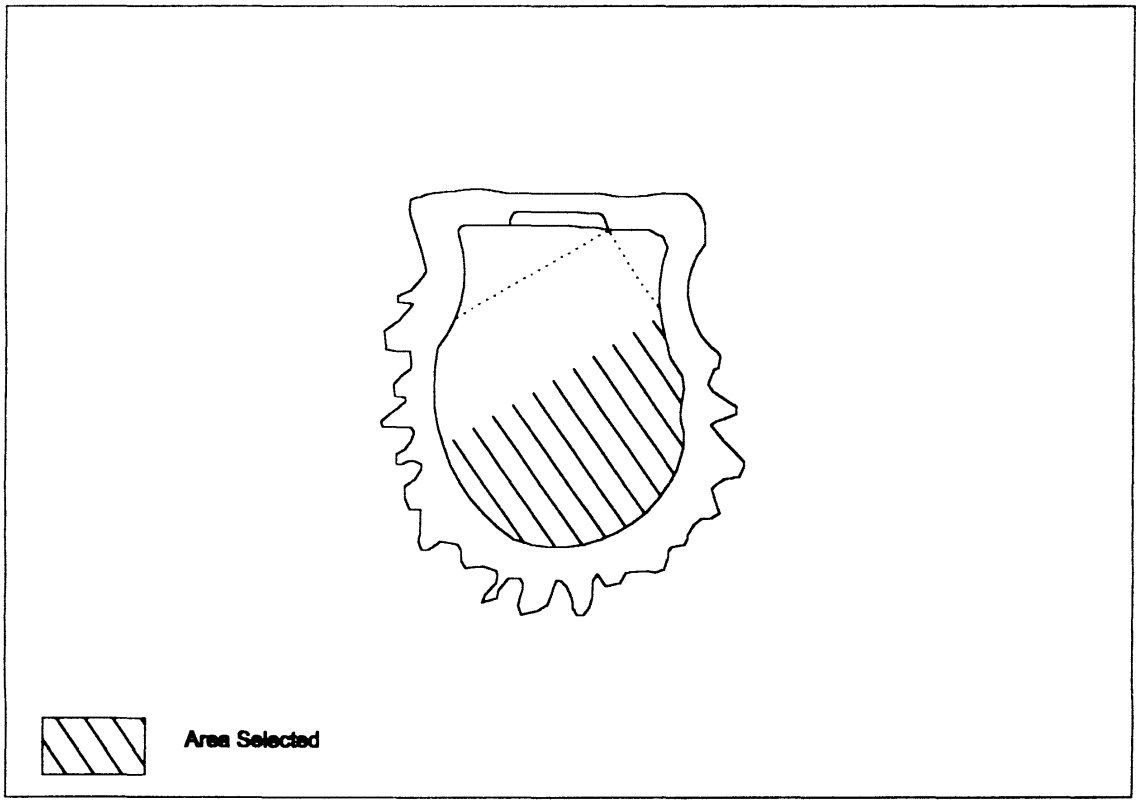

Fig. 3. The area selected for nacre colour observation on the left valve of *P. fucata*

3. 2. 3. Statistical Analysis

3. 2. 3. 1. Biochemical Genetics

The distance migrated by each band was measured. The most common band amongst others for each individual enzyme system was noted as allele 100. Depending on the expected monomeric or dimeric structure, remaining band or bands, were assigned additional allelic status. These bands or alleles were labelled according to the distance migrated by them relative to the allele 100. The observed electrophoretic banding patterns (phenotypes) were classified into single banded slow or fast homozygotes depending on their migration differences and double or triple banded heterozygotes depending on the expected monomeric or dimeric enzyme structure. From these assumed genotypes, genotype frequencies and allelic frequencies were calculated for each locus in each sample. The significance of deviation of the observed genotype frequencies from that of the expected under Hardy-Weinberg equilibrium was calculated using a Chi-Square test. Degrees of freedom was calculated using the formula, d.f = n(n-1)/2, where n is the number of alleles observed for a locus. Examination of heterogeneity in allelic frequencies among the populations was carried out using the contingency Chi-Square test of Workman and Niswander (1970).

3. 2. 3. 1a Population F-Statistics

F- Statistics for the analysis of population structure were calculated as suggested by Weir and Cockerham (1984). F_{ST}, gives the correlation of genes of different individuals in the same population (Coancestory), F_{IS}, gives the correlation of genes within individuals within populations. Using Chi-Square method (Waples 1987) the significance of the F-Statistic values was analysed.

3. 2. 3. 1b Genetic Identity

For a locus, genetic identity between two populations was calculated using the following formula:

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

Where Xi and Yi are the frequencies of the i^{th} allele in populations X and Y respectively. The mean genetic identity for the populations was calculated by

$$\overline{I} = \frac{I_{XY}}{\sqrt{I_X . I_Y}}$$

Where I_{XY}, I_X I_Y are the arithmetic means over all loci of $\sum XiYi$, $\sum Xi^2$ and $\sum Yi^2$ respectively.

3. 2. 3. 2. Morphometrics

Each of the morphometric measurement – hinge length, shell convexity, shell height, shell depth and shell weight – was averaged for each sample. Student's t-test was used to find the significance of variation in shell convexity for pairwise comparisons of Sikka, Tuticorin and Vizhinjam samples.

4. Results

4. 1. ELECTROPHORETIC ANALYSES

4. 1. 1. Standardisation of methodology

The general details of the results of tissues tested, buffer systems tried and the remarks made are given in the table 5. The specific details of the results of standardisation made for individual enzymes are described under each enzyme heading. To select a suitable homogenising medium, double distilled water, 0.2M sucrose solution and 0.05M Tris/HCl, buffer(pH7), were tested and the results were compared. Tris/HCl, buffer (pH7) at 1:1 (w/v) ratio, followed by centrifugation at 11,000 rpm for 20 minutes at 4^0 C gave satisfactory results. The polyacrylamide gel percentage standardised for protein and for all but two enzymes was 8%. For carboxyl esterase and esterase-D 9% gel produced better results.

4. 1. 2. Enzyme systems studied

The list of enzyme loci studied, tissues giving maximum enzyme activity and resolution and the electrophoretic conditions suited for each enzyme system are given in table 5. Though 17 enzymes were tested for activity and resolution, only nine (AAT, EST, ESD, GPI, MDH, ME, PGM, SOD and PROT) were found suitable for population structure analysis of *P.fucata* (Table 6). Others were showing, poor (ADH, AO), or no activity (LAP, LDH, ODH) or inconsistent activity (DIAPH and IDH) or poor resolution (G6PD). From the nine systems, 12 loci, one each for AAT, GPI, ME and PGM, two for ESD

Table 5. Enzymes Studied for Activity and Resolution in *P. fucata*

Locus	Tissue used	Buffer Used	Remarks	
			Activity	Resolution
AAT	Ad.	TBC	Good	Good
ADH	Ad.	TG	Poor	Poor
AO	Ad/D.D.	TG	Poor	Poor
EST	D.D.	TBC	Good	Good
DIAPH	D.D	TBC (1)	Inconsistent	Poor
ESD	D.D.	TG	Good	Good
G6PD	Ad.	TG	Good	Poor
GPI	Ad.	TBC	Good	Good
IDH	Ad.	TBC	Good	Inconsistent
LAP	-	-	No activity in any system tried	-
LDH	-	TG	No activity in any system tried	-
MDH	Ad.	TG	Good	Good
ME	Ad.	TG	Good	Good
ODH	-	-	No activity in any system tried	-
PGM	Ad.	TG (1)	Good	Reasonable
SOD	Ad.	TG	Good	Good
PROT	Ad.	TG	Good	Good

Table 6. Enzyme loci examined for genetic variation in the populations of *P.fucata*

Locus	Polymorphism	No: of Alleles
AAT	Monomorphic	1
EST-1	Polymorphic	2
EST-2	Polymorphic	2
ESD-1	Monomorphic	1
ESD-2	Polymorphic	2
GPI	Polymorphic	2
MDH-1	Monomorphic	1
MDH-2	Polymorphic	3
ME	Monomorphic	1
PGM	Polymorphic	4
SOD-1	Monomorphic	1
SOD-2	Monomorphic	1
PROT-1	Monomorphic	1
PROT-2	Monomorphic	1
PROT-3	Polymorphic	3
PROT-4	Monomorphic	1
PROT-5	Monomorphic	1

and MDH, two for EST and SOD, were scored and five loci from general proteins in were analysed. Monomorphism was exhibited at AAT, ESD-1, MDH-1, ME, SOD-1 and SOD-2, PROT-1, PROT-2, PROT-4 and PROT-5 loci. Six enzyme loci, EST-1, EST-2, ESD-2, GPI, PGM, MDH-2 and one protein locus (PROT-3) were polymorphic in all the tested samples. Hatchery reared adult population from Tuticorin hatchery exhibited variation at both the SOD loci. The details of the results obtained under each enzyme systems are given below.

AAT (Aspartate aminotransferase)

The enzyme activity was tested in TC, TCB and TG buffers. Its activity was detected in buffers TC and TCB. However, the TC buffer caused a warping. TCB gave better resolution. Though adductor muscle, digestive diverticula and mantle showed the enzyme activity, maximum activity was shown by the adductor muscle. The zone of activity was located towards the cathodal end as a fine sapphire coloured blue band with a red background colour throughout the gel. Occasionally, an inconsistent faster moving second zone of activity was observed in some samples (Fig. 4, Plate 6). There was no other variation in the band pattern in any of the populations sampled. Thus single banded monomorphic phenotypes were recorded at AAT locus in Sikka, Tuticorin, Vizhinjam and hatchery populations.

ADH(Alcohol dehydrogenase)

The enzyme activity could be detected only in adductor muscle and TG buffer system. Since its activity was weak, it was not used in the population analysis. Increasing the substrate concentration in the staining recipe also did not improve its activity.

AO (Aldehyde oxidase)

None of the buffers (TC, TCB, TEB, TEC, TG) tried produced sufficient activity and hence it was not tested further in the studies.

DIAPH(Diaphorase)

Its weak activity was seen in the digestive diverticula of some samples and a comparatively better activity was produced by TCB(1). However, the resolution was not achieved to the desired extent for analysis.

EST (Carboxyl Esterase)

Intense activity of EST was observed in TCB and TG buffers, giving good resolution. For convenience, TG was employed in the present study. Enzyme was active in adductor muscle, digestive diverticula and mantle (Fig. 5, Plate 7).

Fig. 4. Zymogram for AAT in adductor muscle for *P. fucata*

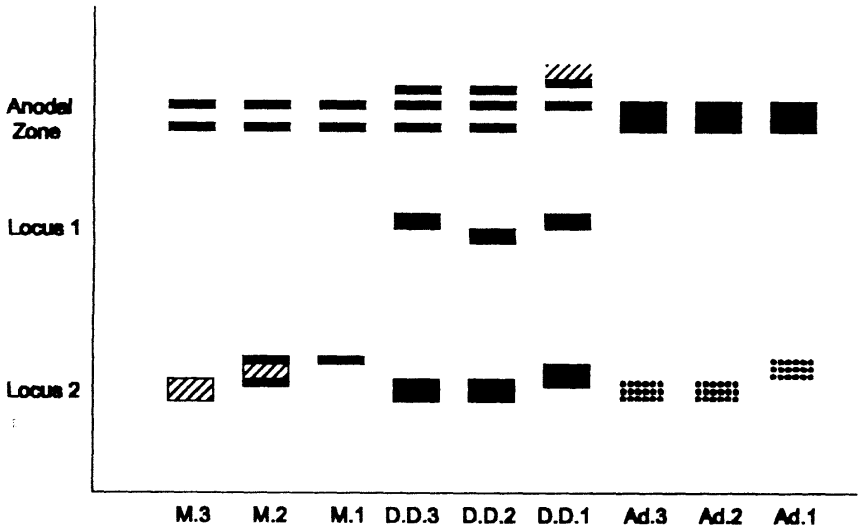

Fig. 5. Zymogram of EST in various tissues of *P. fucata*
(Ad: Adductor muscle; D.D: Digestive diverticula; M: Mantle)
(Faint band)

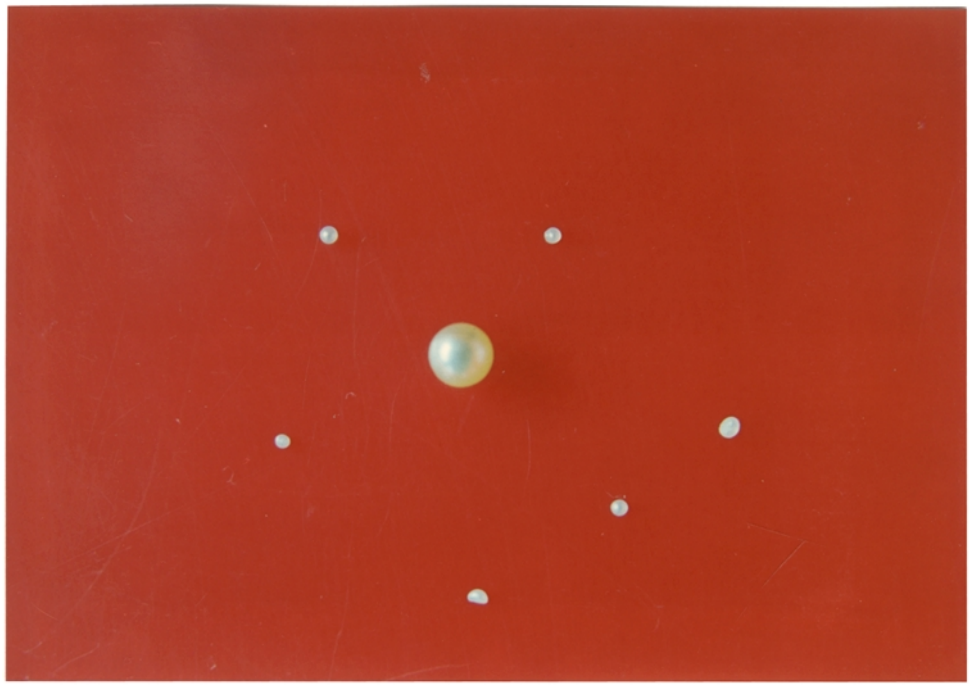

Plate 5. Collection of natural pearls obtained from *P. fucata*

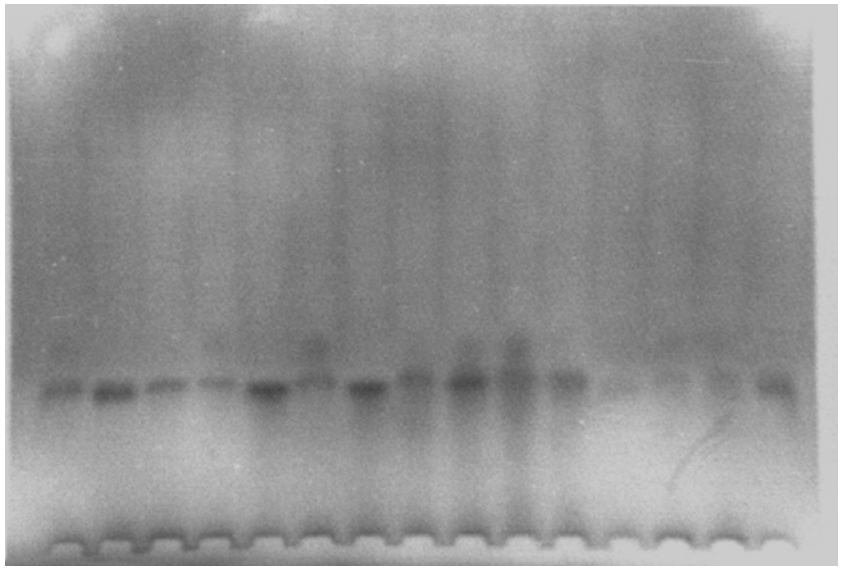

Plate 6. Enzyme pattern observed for AAT in adductor muscle of *P. fucata*

Three zones of activity (anodal, cathodal and an intermediate) were observed in digestive diverticula. The anodal zone was however showing uninterpretable banding patterns ranging from two to four bands and all with equal intensities. Hence, this zone was discarded from the analyses. EST-1 or the intermediate zone and EST-2 or the cathodal zone exhibited genetic variations. EST-1 was not observed in adductor and mantle tissues. The EST-2 was weakly active in the adductor and mantle tissues. The esterase in the digestive diverticula was analysed for the studies. Single banded and double banded individuals were observed at EST-1 and EST-2 loci (Fig. 6, Plate 8). The alleles 100 and 94 were present at EST-1 locus. The alleles 100 and 87 were present at EST-2 locus. The EST-1 was too weak for scoring the banding patterns in Sikka and Vizhinjam. The single and double banded individuals suggested a monomeric subunit structure for both the loci examined. Single banded individuals were considered to be homozygotes and double banded as heterozygotes.

ESD(Esterase - D)

The enzyme was active in TC and TCB buffers. The enzyme activity was detectable only at pH 7 or below. The resolution of the enzyme was poor in TC buffer but fairly good in TCB. The enzyme activity was observed within seconds of adding the substrate. However, the enzyme-substrate complex formed was very unstable since the bands got diffused rapidly. So the banding patterns were scored immediately after staining. All three tissues

showed the enzyme activity in the TCB buffer (Fig. 7, Plate 9). Digestive diverticula was selected because the enzyme was most active in comparison to other two tissues. Three main zones of activity were seen as in the case of carboxyl esterase. The intermediate zone or locus-2 showed single and double banded phenotypes (Fig. 8, Plate 10). The alleles 100 and 103 were present. The phenotypes of anodal zone or locus-1 were monomorphic. The cathodal zone did not show resolved patterns. There were also some minor zones of weak enzyme activity between the intermediate and anodal zones.

G6PD(Glucose - 6 - phosphate dehydrogenase)

Intense activity of the enzyme was present in all the three tissues at the cathodal region. All attempts to resolve the enzyme were to no avail. Varying the buffer constitutions or the gel percentages gave no better results. Hence, this locus was not utilised in further studies.

GPI(Glucose phosphate isomerase)

The enzyme was most active in the adductor muscle. Only one zone of activity towards the cathodal side was observed (Fig. 9, Plate 11). Since TG buffer gave good results the other buffers were not tried. Single banded and three banded phenotypes were exhibited by the samples. The alleles 100 and 73 were accounted for the observed one and three banded phenotypes

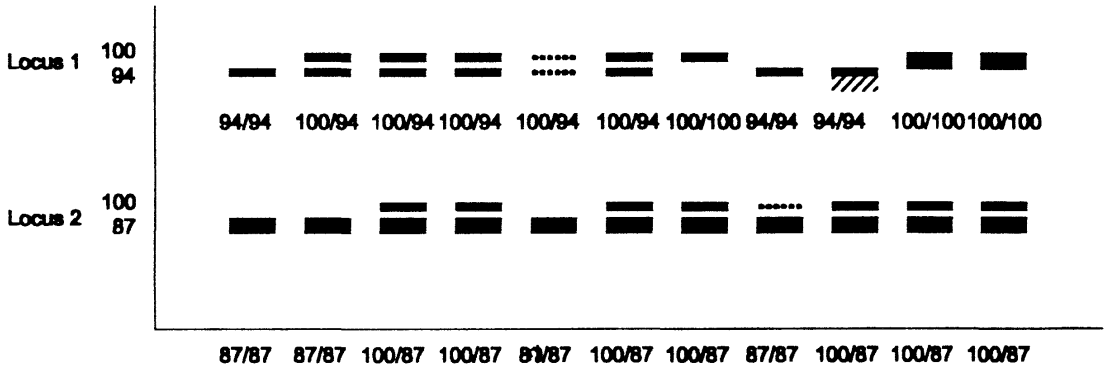

Fig. 6. Zymogram of EST in digestive diverticula of *P. fucata*

(⁘ Faint band)

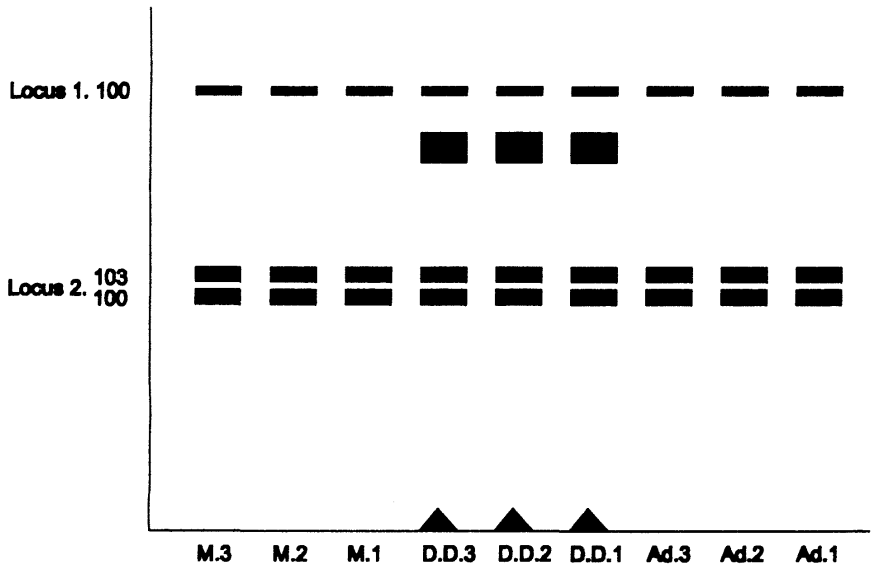

Fig. 7. Zymogram for ESD in various tissues examined for *P. fucata*
(Ad: Adductor muscle; D.D: Digestive diverticula; M: Mantle)

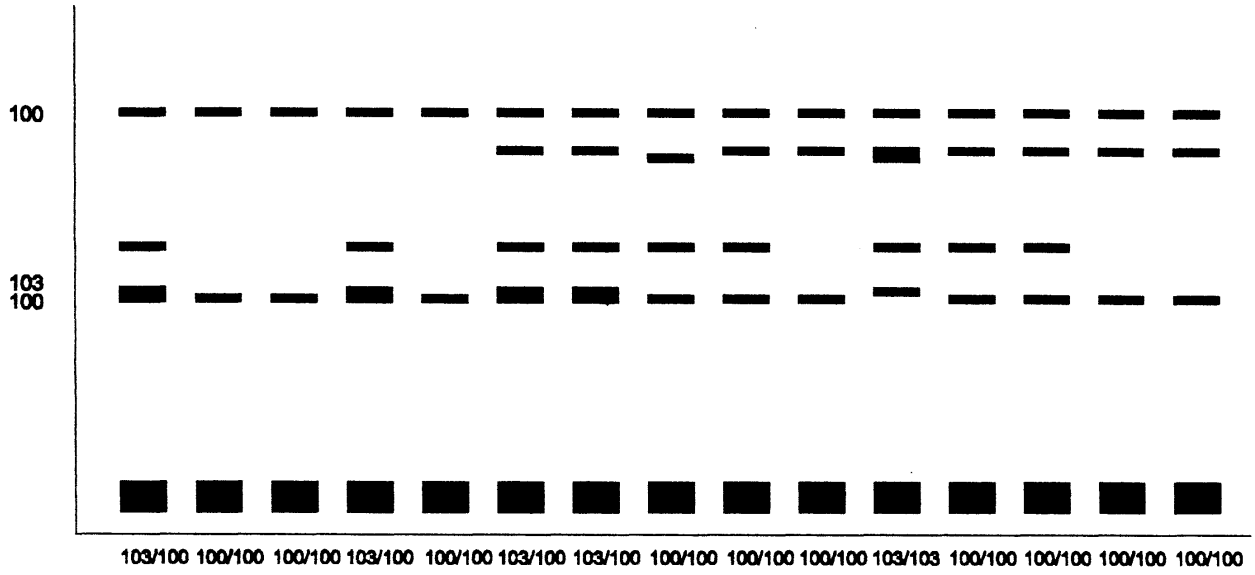

Fig. 8. Zymogram for ESD - 2 in digestive diverticula of *P. fucata*

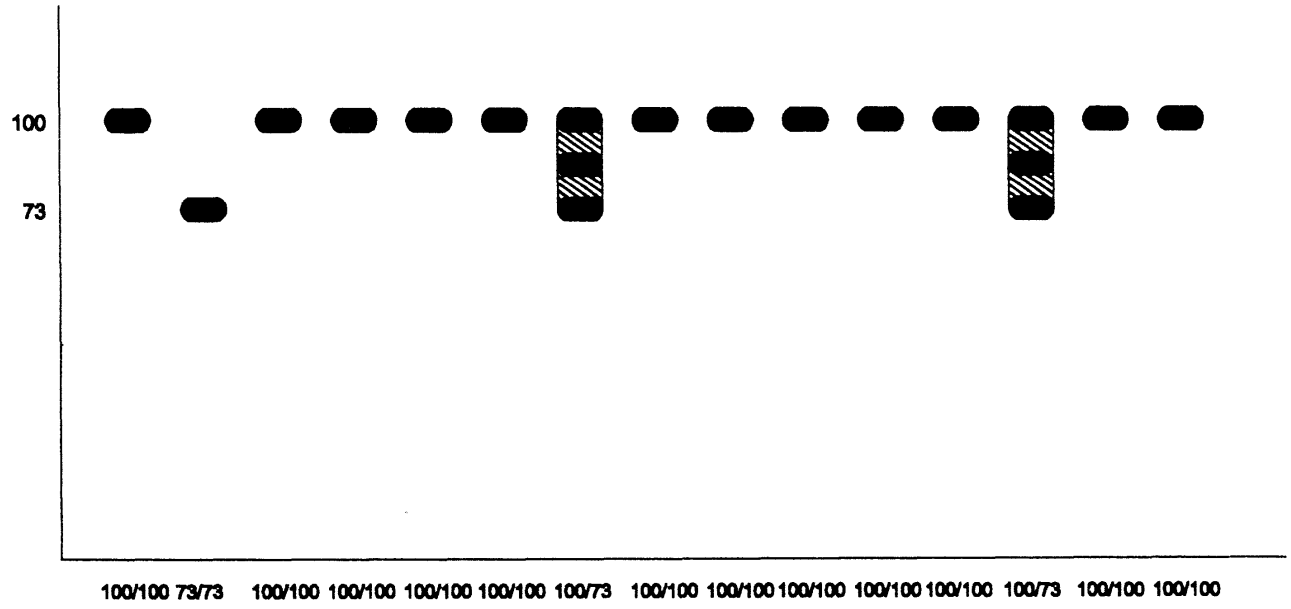

Fig. 9. Zymogram for GPI in adductor muscle of *P. fucata*

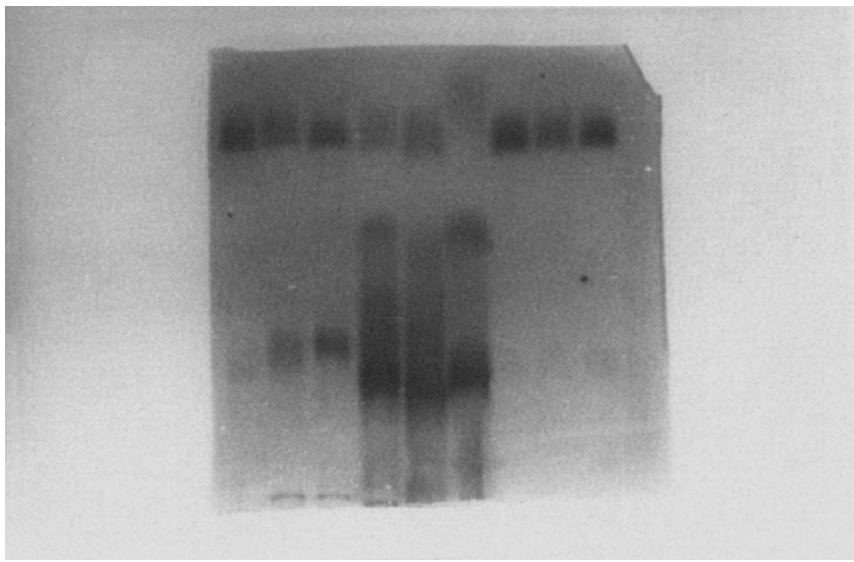

Plate 7. Enzyme pattern observed for EST in different tissues of *P. fucata*

(L to R : Lanes 1 to 3 : Mantle, Lanes 4 to 6 : Digestive di verticula, Lanes 7 to 9: Adductor muscle)

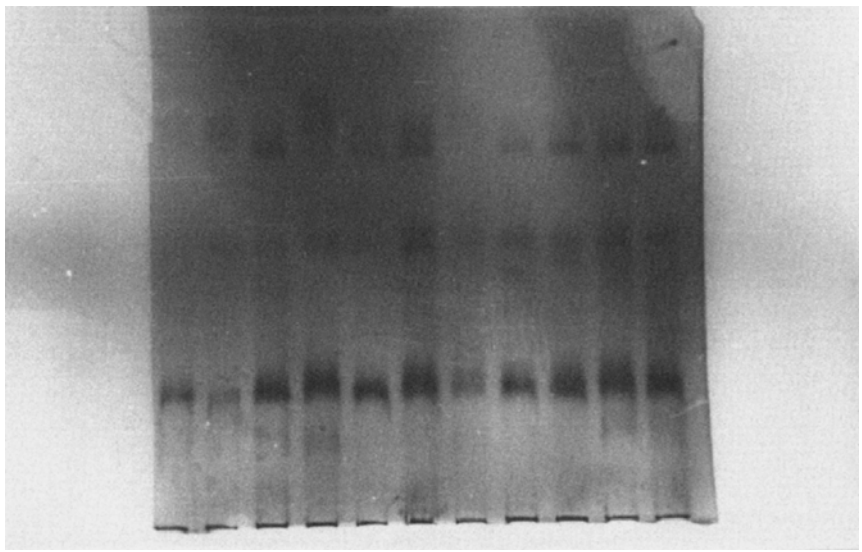

Plate 8. Enzyme pattern observed for EST in digestive diverticula of *P. fucata*

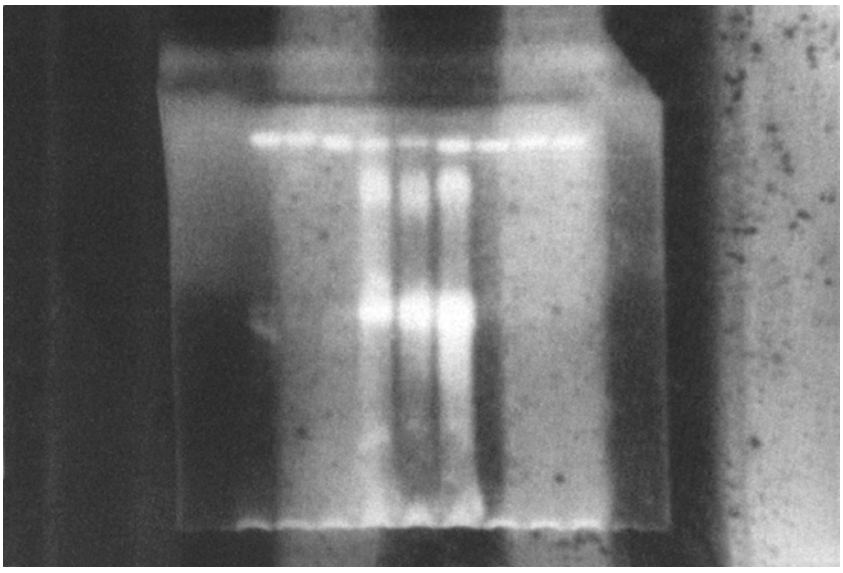

Plate 9. Enzyme pattern observed for ESD in different tissues of *P. fucata*

(L to R : Lanes 1 to 3 : Mantle, Lanes 4 to 6 : Digestive di verticula, Lanes 7 to 9: Adductor muscle)

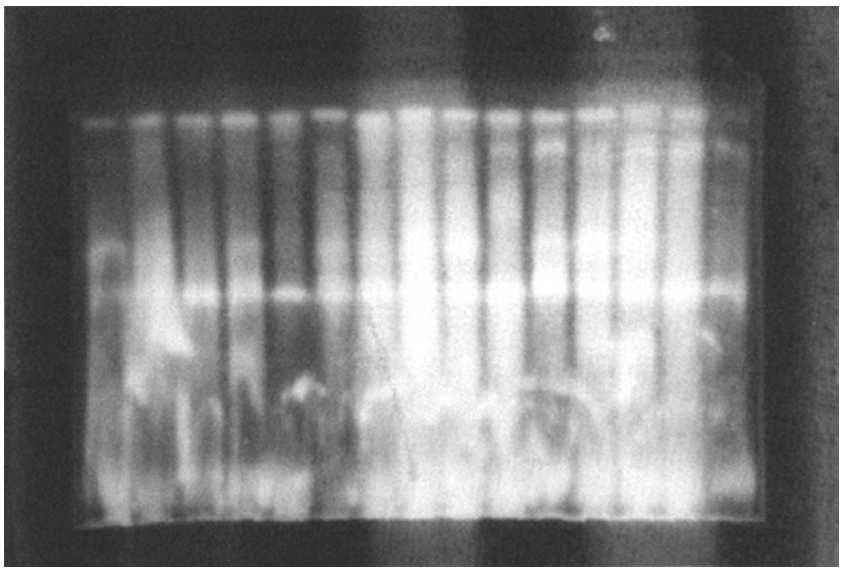

Plate 10. Enzyme pattern observed for ESD in digestive diverticula of *P. fucata*

suggesting a dimeric structure for the enzyme. These two alleles were present in all the sampled populations.

IDH (Isocitrate dehydrogenase)

The enzyme was just active in Citrate buffers (TC, TEC and TCB). Only two banded individuals were observed. Due to inconsistent activity and resolution it was not used in the population survey.

LAP (Leucine aminopeptidase)

LAP was not showing any activity in any of the buffer systems tried here. Even, employing superior quality substrate did not give the required results.

LDH (Lactate dehydrogenase)

The LDH enzyme could not be detected in different electrophoretic conditions attempted in the study. Modification of the recipe by increasing substrate concentration also did not help. Hence this was also not included for further analysis.

MDH (Malate dehydrogenase)

MDH was active in all the three tissues tested and it was well resolved in TG buffer (Fig. 10, Plate 12). Other buffers were not tried. The tissues adductor muscle, digestive diverticula and the mantle showed comparable patterns of enzyme activity. Hence only adductor muscle was analysed. A single anodal zone of enzyme activity was observed. It consisted of two to five banded phenotypes. However, a close scrutiny of the banding patterns at the zone revealed that it was formed of a faster moving single banded monomorphic and one to three banded polymorphic phenotypes. The faster moving monomorphic zone was designated as MDH-1 and the polymorphic as MDH-2. The polymorphic locus showed three kinds of phenotypes - one faster moving single banded homozygous phenotype, followed by a fast moving three banded heterozygous phenotype and a slow moving three banded heterozygous phenotype. The alleles 100 and 84 accounted for ~~for~~ the fast moving three banded heterozygous phenotype. The alleles 100 and 68 accounted for the slow moving three banded heterozygous phenotype. The allele 100 was common for both three banded heterozygous phenotypes (Fig. 11, Plate 13). These two kinds of triple banded phenotypes were observed in Sikka population only. Tuticorin and Vizhinjam populations possessed only the fast triple banded phenotype formed with 100 and 84 alleles. The single banded homozygous and triple banded heterozygous patterns agree with the dimeric structure of MDH enzyme.

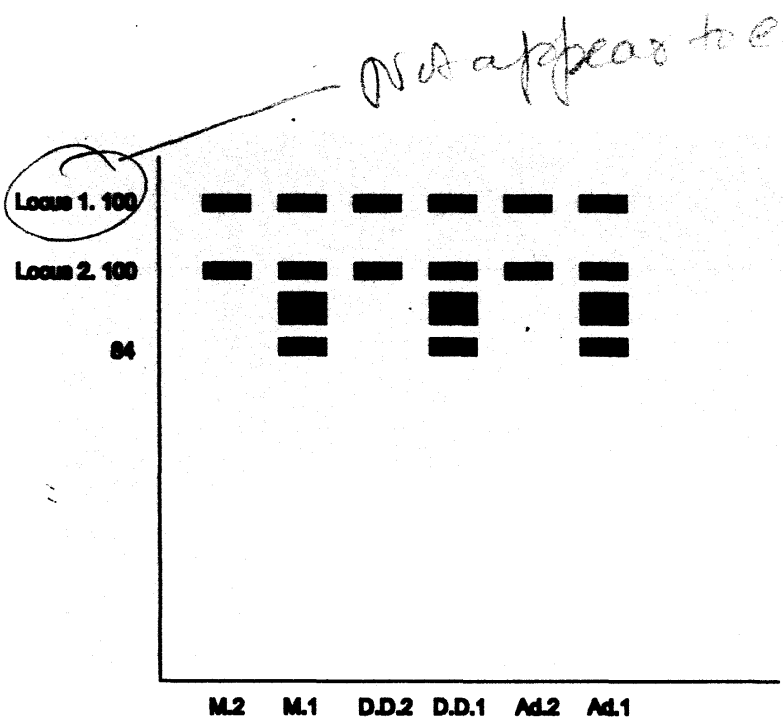

Fig. 10. Zymogram for MDH in the tissues examined for *P. fucata*
(Ad: Adductor muscle; D.D: Digestive diverticula; M: Mantle)

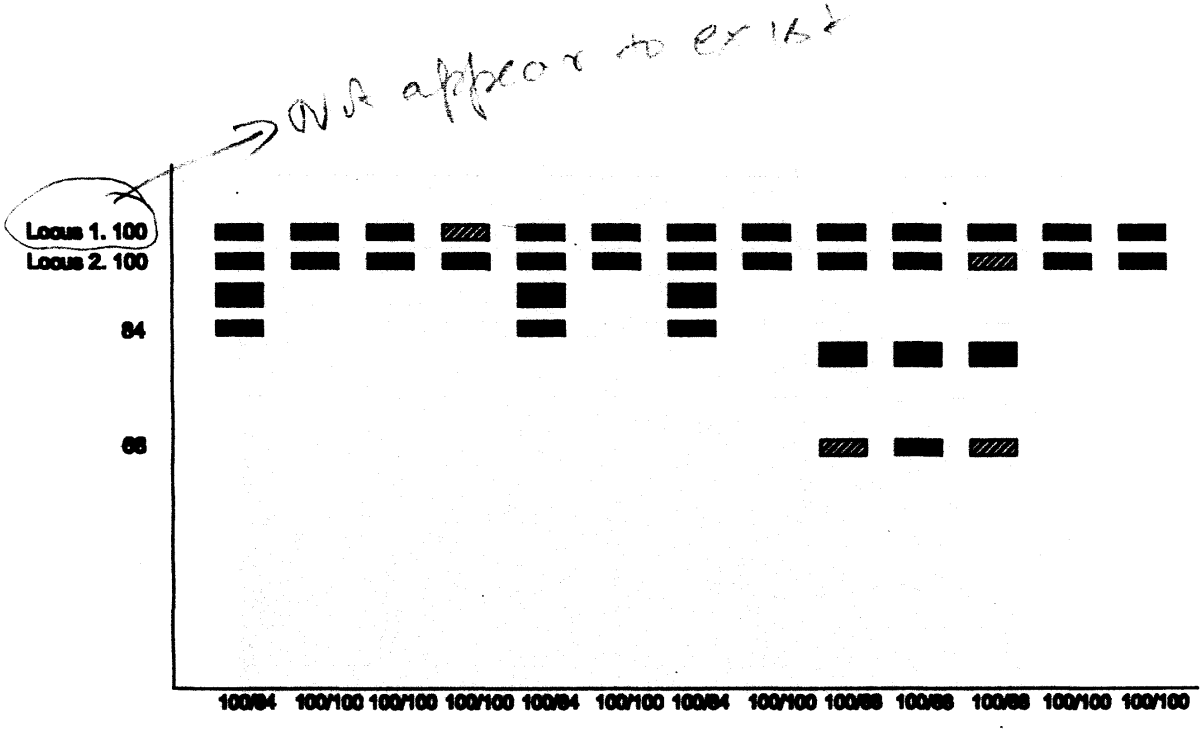

Fig. 11. Zymogram for MDH in adductor muscle of *P. fucata*
▨ Faint band

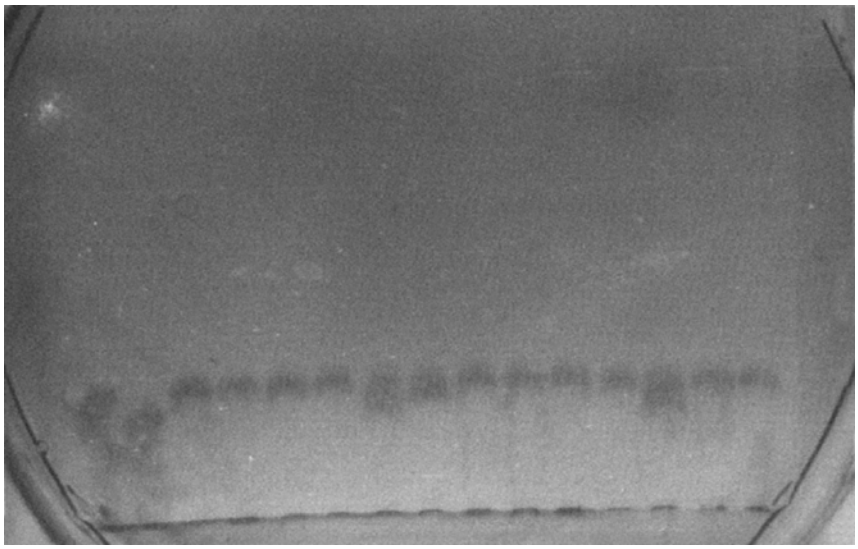

Plate 11. Enzyme pattern observed for GPI in adductor muscle of *P. fucata*

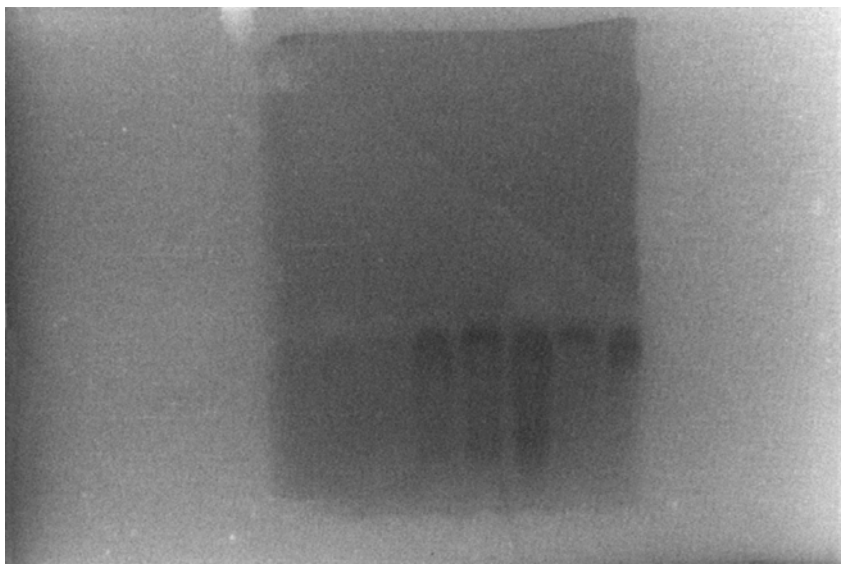

Plate 12. Enzyme pattern observed for MDH in different tissues of *P. fucata*

(Lanes 1 & 2 : Mantle Lanes 3 & 4:Digestive diverticule Lanes 5 & 6: Adductor muscle)

ME (Malic enzyme)

ME showed fairly good activity and resolution in TG buffer. The continuous buffers did not provide good resolution. It was active in all the tissues. Only single banded non-variant phenotypes were noted in the samples tested (Fig. 13).

ODH (Octanol dehydrogenase)

The ODH enzyme activity could not be detected in any of the buffer systems and tissues tried. Staining recipe modification also did not make any difference to the above result.

PGM (Phosphoglucomutase)

The PGM enzyme was the most variable locus in *P. fucata* (Fig. 14, Plate 15). It was also found to be highly pH sensitive. TG gave better activity as well as resolution between the two discontinuous buffers tried. To produce better resolution the pH of the TG buffer was reduced to 8. The enzyme activity was seen in all the tissues at the anodal end. The single locus observed lied just below the dye front. Hence, constant monitoring was required when the front neared the anodal end. Two single and five double banded phenotypes were observed These phenotypes were categorised according to their relative mobilities in respect of the most common single

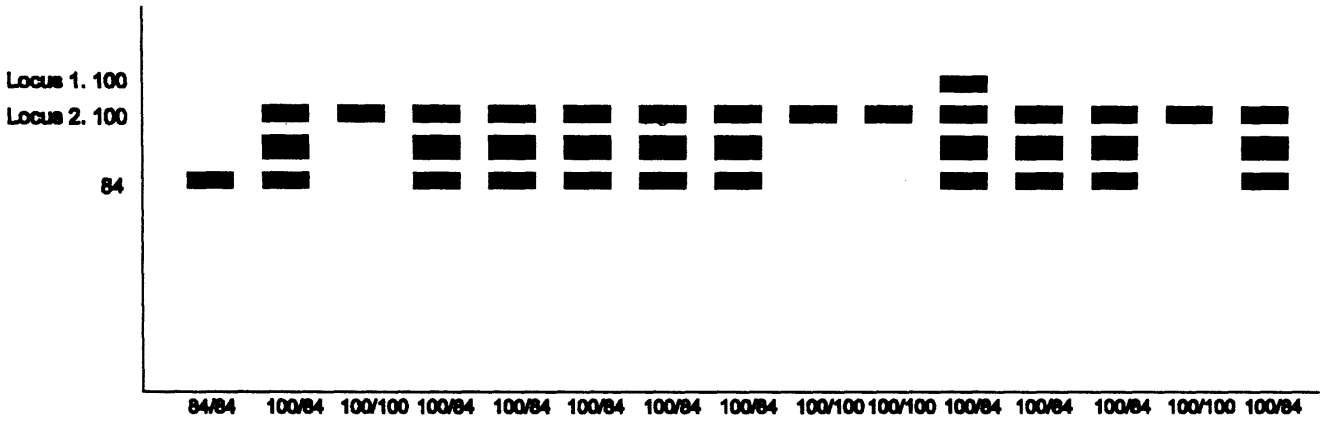

Fig. 12. Zymogram for MDH in adductor muscle of juveniles of *P. fucata*

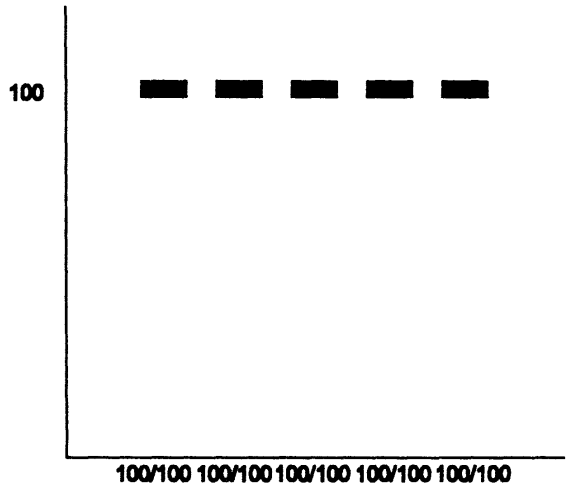

Fig. 13. Zymogram of ME in adductor muscle of *P. fucata*

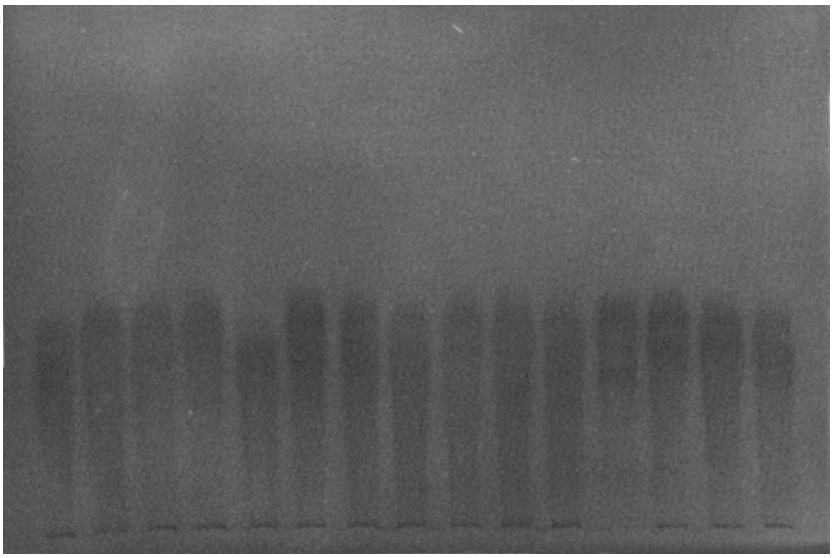

Plate 13. Enzyme pattern observed for MDH in adductor muscle of *P. fucata* from the Gulf of Kutch

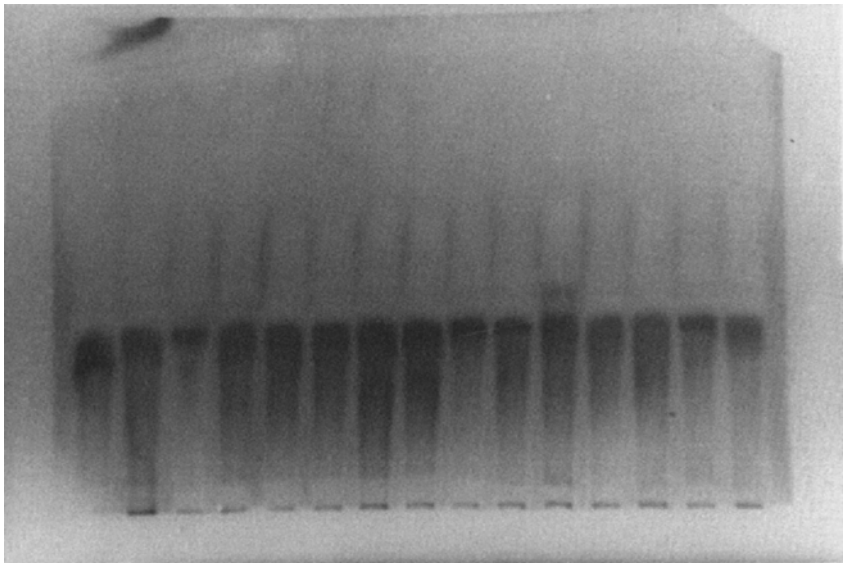

Plate 14. Enzyme pattern observed for MDH in adductor muscle of juveniles of *P. fucata*

banded phenotypes scored as 100. The single banded phenotypes were represented by bands scored as 96 and 100. The five kinds of two banded phenotypes were represented by bands scored as 96/86, 96/90, 100/86, 100/90 and 100/96. Thus four alleles 86, 90, 96 and 100 were responsible for the observed seven kinds of phenotypes - two homozygous and five heterozygous genotypes. The banding patterns suggest that the enzyme is monomeric in structure. Like ESD and GPI, the enzyme had to be recorded immediately when sufficient activity was reached, otherwise the bands got diffused and deformed to complicated patterns.

SOD (Superoxide dismutase)

The enzyme activity was detected as bleached areas under bluish background. The enzyme was active in all the three tissues and exhibited similar band pattern in all tissues. The adductor muscle which showed good resolution was used for further analyses. TG buffer gave better resolution compared to other buffers like TC or TCB. For easy scoring, the contrast between the bleached areas of enzyme activity and the blue background was increased by staining the gel for a longer duration under indirect light. Two zones of enzyme activity were noted (Fig. 15, Plate 16) in the wild samples. The fast zone was sometimes inconsistent in activity. This zone was not scored. The second slow zone was more prominent and composed of a fast moving small bands closely followed by broad bands. These two subzones of the second zone were non-variable and were scored as independent

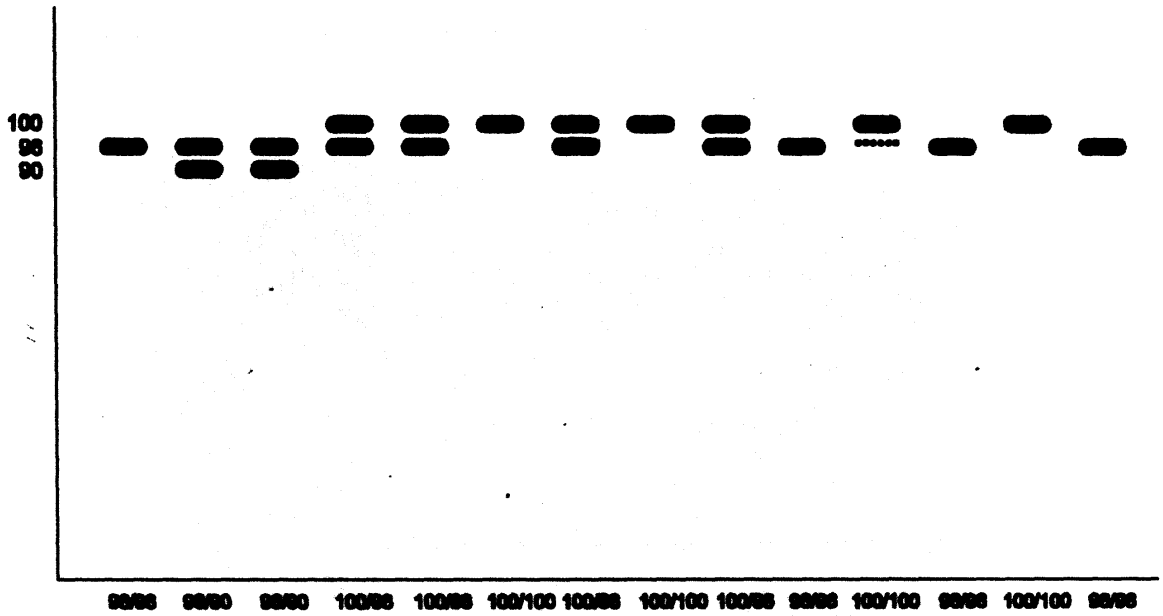

Fig. 14. Zymogram for PGM in adductor muscle of *P. fucata*

..... Faint band

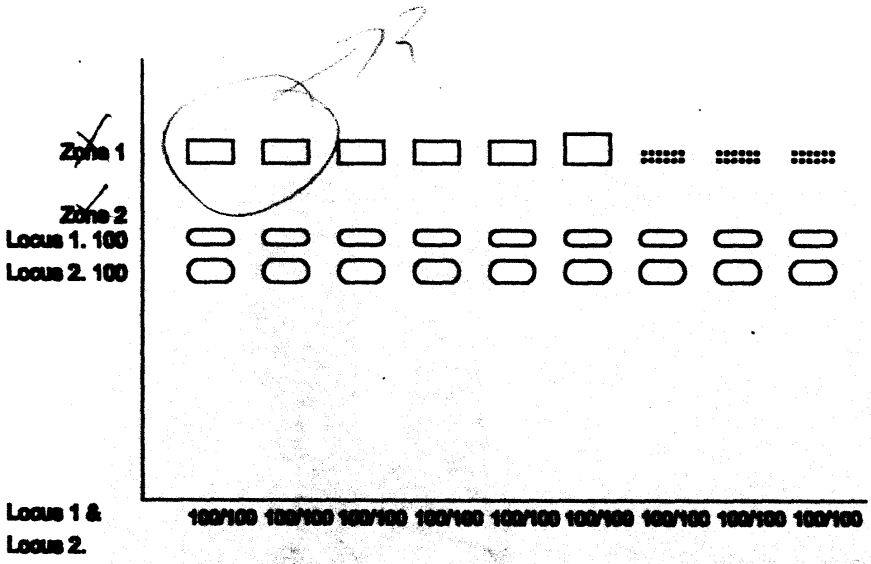

Fig. 15. Zymogram for SOD in adductor muscle of wild samples of *P. fucata*

..... Faint band

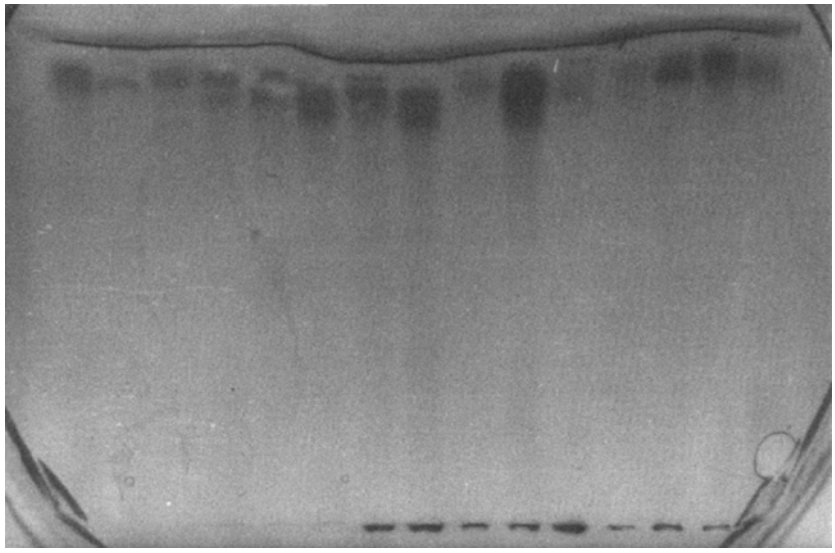

Plate 15. Enzyme pattern observed for PGM in adductor muscle of *P. fucata*

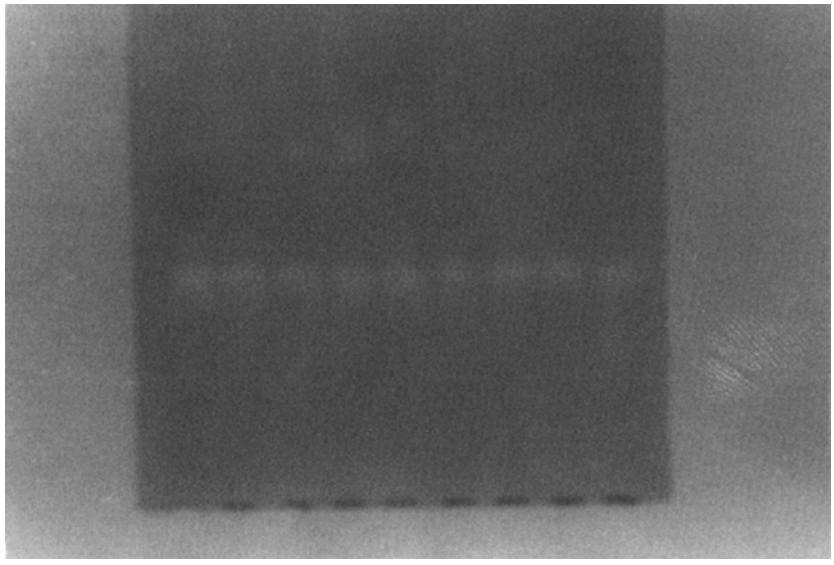

Plate 16. Enzyme pattern observed for SOD in adductor muscle of *P. fucata*

monomorphic loci (SOD-1, SOD-2). But interestingly, the slow zone showed single and triple banded phenotypic variations in the hatchery population of Tuticorin (Fig. 16 &17, Plate 17).

PROT

The non-enzymatic proteins (general proteins) present in the adductor muscle, digestive diverticula and mantle were got separated and resolved well in TCB and TG discontinuous buffer systems. The number and the position of major and minor bands differed among the tissues showing tissue specific nature of the proteins (Fig. 18, Plate 18). All tissues showed more than six bands each. But adductor muscle tissue contained maximum number of bands (Fig. 18, Plate 18). As the number of major and minor bands were many and moving closely, it was difficult to assume the exact number of loci responsible for all the observed banding patterns. The adductor muscle proteins may be under the control of about five loci (Fig. 18, 20). A number of loci showed additional apparently inconsistent variations. The loci PROT-1, 2, 4 and 5 were scored as monomorphic. However, the third locus revealed consistent phenotype variations and it was scored as polymorphic locus. The polymorphic phenotypes were formed of equally and strongly stained single and double banded patterns. However, the occurrence of a light band in the place of a strongly stained single band suggests that it may be the phenotypic expression of another locus whose products have apparently the same rate of migration but differed in staining intensity. These phenotypes get superimposed by the heterozygous strongly stained double bands. The

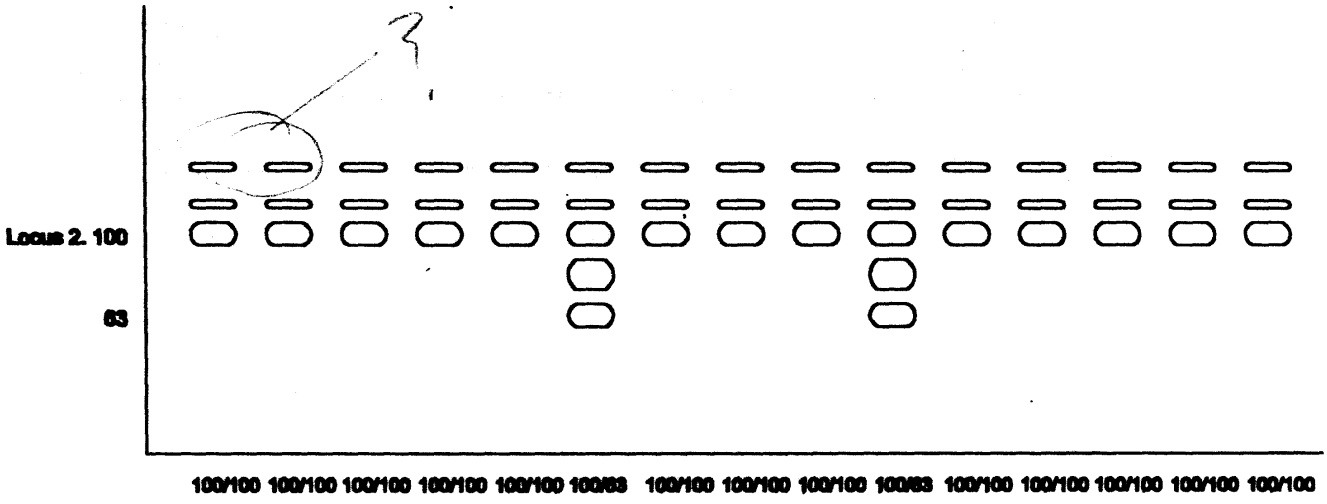

Fig. 16. Zymogram for SOD in adductor muscle of hatchery produced *P. fucata*

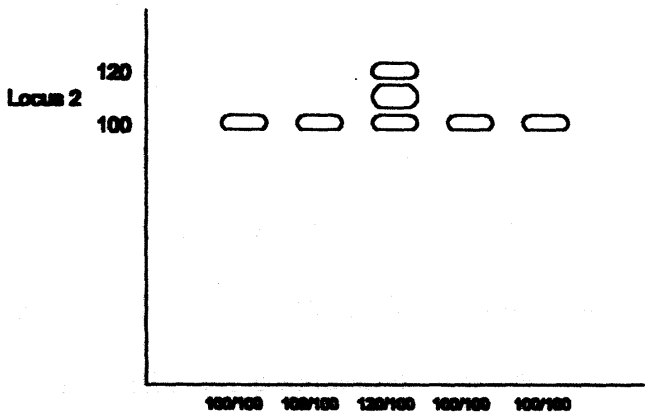

Fig. 17. Zymogram of SOD in adductor muscle of hatchery reared adults of *P. fucata*
(Faint band)

underlying light band appears visible only when the superimposing strongly stained band is homozygous in nature. The single and double banded phenotypes were assumed and scored as homozygotes and heterozygotes respectively. The fastest common band at locus-3 was designated as allele 100 and the remaining two bands, depending on their relative mobilities, were designated as allele 86 and allele 73. Two kinds of heterozygous genotypes, 100/86 and 100/73 (Fig. 19, Plate 20) were observed at locus-3. Allele 86 was not present in Sikka population (Fig. 21, Plate 21), while the other two alleles, 100 and 73 were present in Sikka. Vizhinjam and Tuticorin at varying frequencies. The different genotypes obtained at the protein locus (PROT-3) were, 100/100, 100/86, 100/73 and 86/86.

4. 2. Genetic Structure of Wild Oyster Populations

4. 2. 1. Genetic Variations

To evaluate genetic nature of the observed phenotype variations at seven polymorphic loci, their frequencies were compared with that of expected according to Hardy-Weinberg equlibrium condition (Table 8). The loci EST-2 in Tuticorin and GPI in Sikka and Vizhinjam and ESD-2 and PROT-3 in Vizhinjam populations showed significant deviations from the expected genotype frequencies ($P<0.05$). On the basis of ten monomorphic and seven polymorphic loci studied, the estimated mean observed and expected heterozygosities were 0.13($\pm$0.05) and 0.14($\pm$0.05) respectively for Sikka, 0.10 ($\pm$0.04) and 0.11($\pm$0.02) respectively for

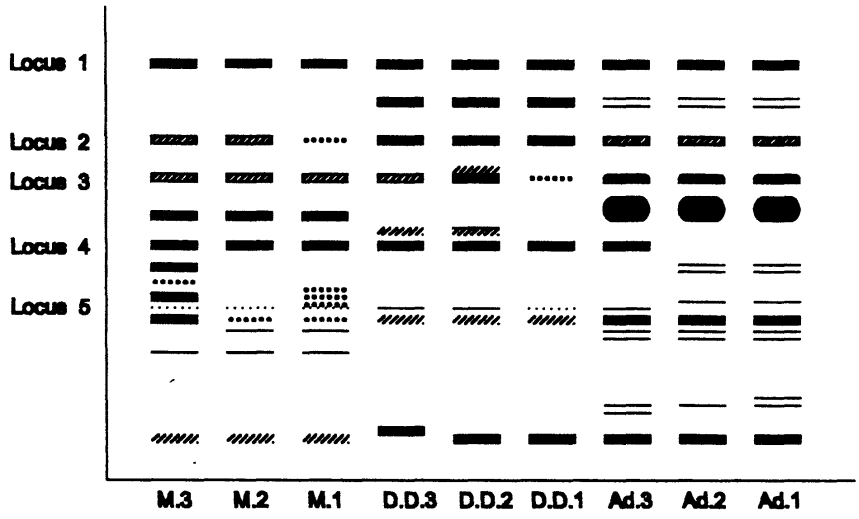

Fig. 18. Zymogram for PROT in various tissues of *P. fucata*
(Ad: Adductor muscle; D.D: Digestive diverticula;
M: Mantle)

Faint band ····· Breakdown band

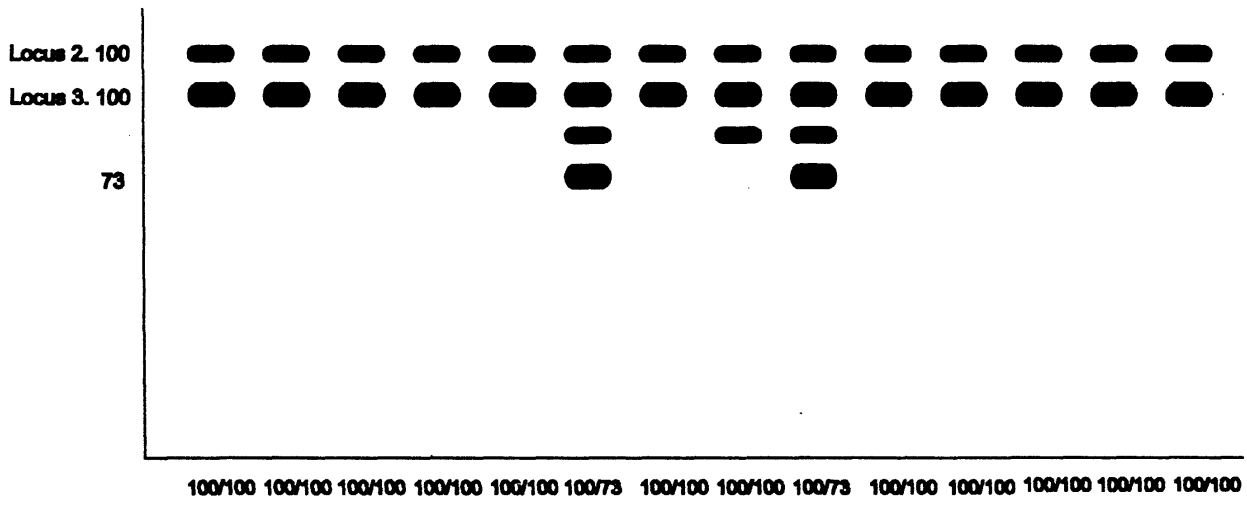

Fig. 19. Zymogram for PROT in adductor muscle of *P. fucata* from Sikka

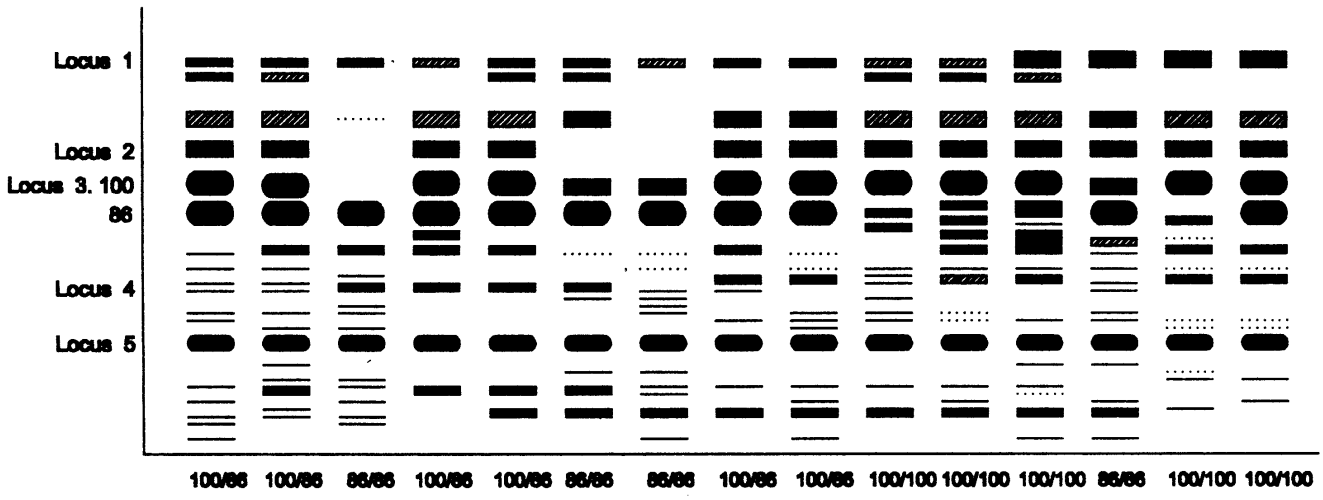

Fig. 20. Zymogram for PROT in adductor muscle of *P. fucata*

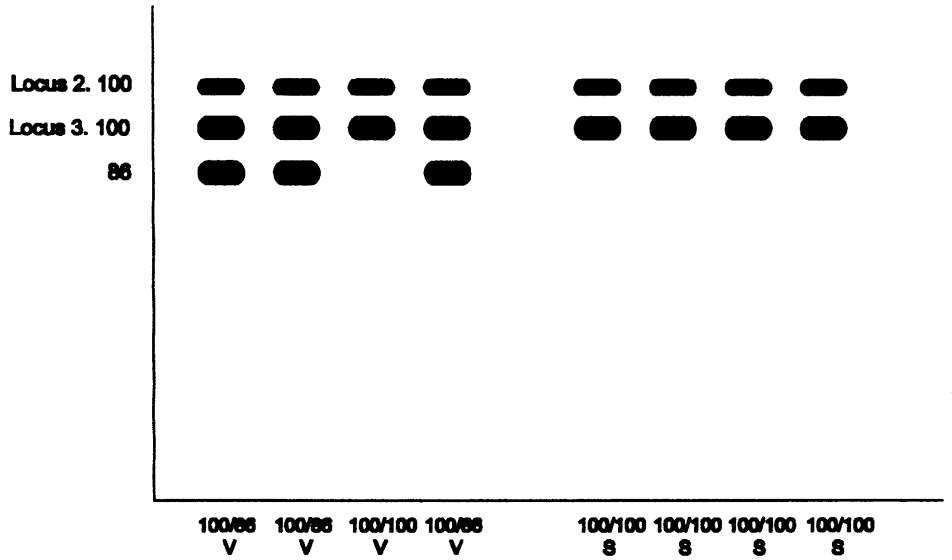

Fig. 21. Zymogram for PROT in adductor muscle of *P. fucata* from Vizhinjam (V) and Sikka (S)

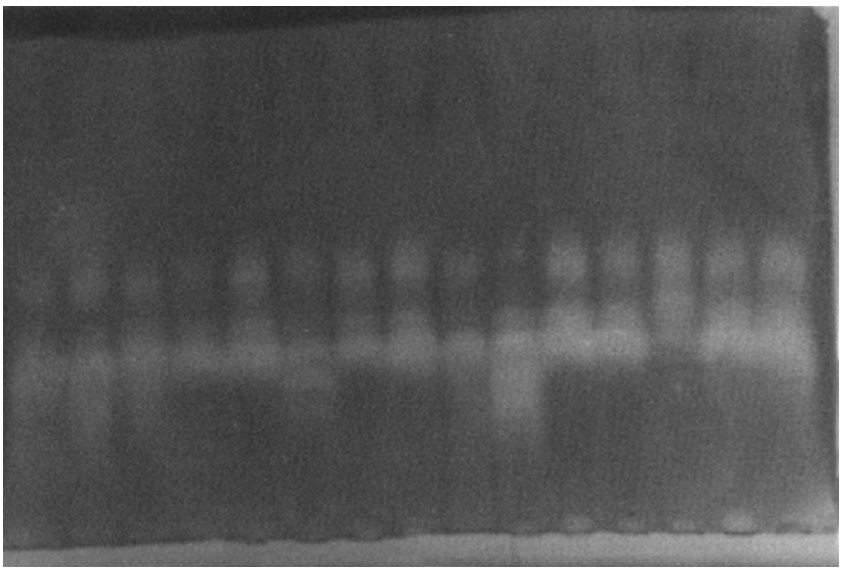

Plate 17. Enzyme pattern observed for SOD in adductor muscle of hatchery reared adults of *P. fucata*

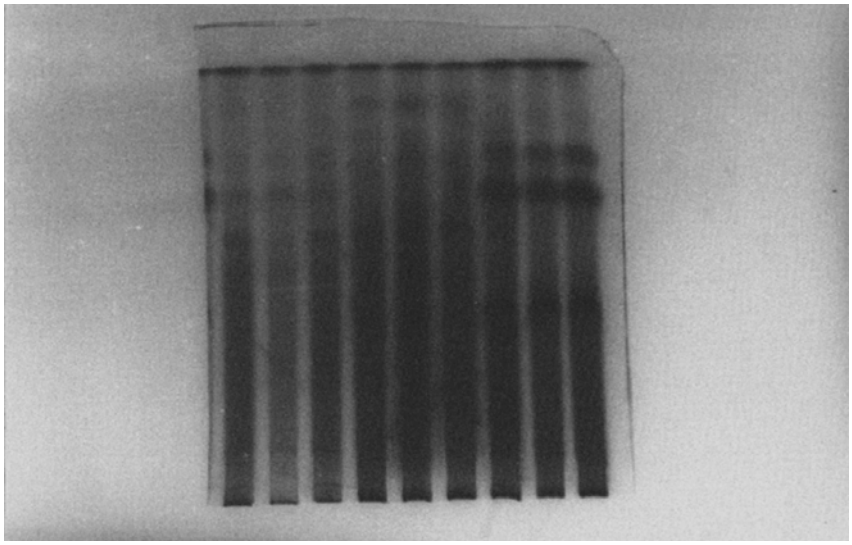

Plate 18. Pattern observed for general proteins in different tissues of *P. fucata*

(L to R : Lanes 1 to 3 : Mantle, Lanes 4 to 6 : Digestive di verticula, Lanes 7 to 9: Adductor muscle)

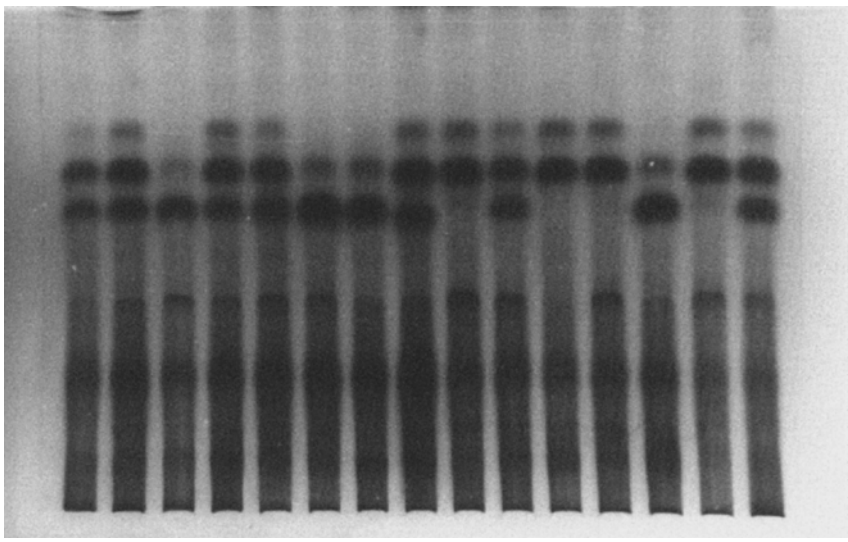

Plate 19. Pattern observed for general proteins in adductor muscle of *P. fucata*

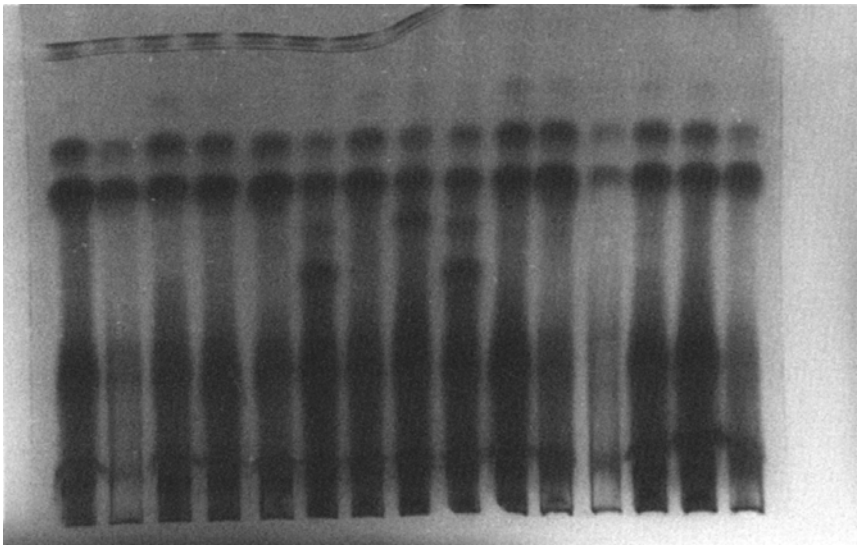

Plate 20. Pattern observed for general proteins in adductor muscle of *P. fucata*

Tuticorin and 0.16 (±0.06) and 0.16 (±0.05) respectively for Vizhinjam. The mean heterozygosity for the species was 0.13. The mean number of alleles per locus (N_a) was 1.56, 1.4 and 1.5 for Sikka, Tuticorin and Vizhinjam respectively and the mean effective number of alleles per locus ($N_{eff}= 1/(x_i^2$, where x_i is the frequency of the i^{th} allele) was 1.24, 1.18 and 1.29 respectively for Sikka, Tuticorin and Vizhinjam populations. The above values suggest that the observed phenotype variations were genetic in nature and the genetic variability is high in the species.

4. 2. 1a Allelic frequencies

Table 7 details the estimated allelic frequency distribution at 17 loci examined in the samples from the three regions. A comparison of the allele frequencies at seven polymorphic loci shows that the values are closely similar at all loci except at PROT-3 locus. The frequency of the PROT-3 allele, 86, differed much between Sikka and Vizhinjam. The allele PROT-3/86 was totally absent in Sikka while it had a frequency of about 0.44 in Vizhinjam. On the contrary, the rare MDH allele 68 was present only in Sikka population. Some amount of allelic heterogeneity occurred at PGM locus between Sikka and Vizhinjam populations. The PGM allele 86 was represented only by the Sikka sample. It was absent in Vizhinjam. The reason for its absence in Vizhinjam could be due to its small sample size. Since PGM was not tested in Tuticorin its fate in Tuticorin is not known. The Sikka population also differed from that of Vizhinjam and Tuticorin in having its own rare allele, 68 at MDH-

Table 7. Allele frequencies at 17 loci examined in 3 wild populations and 3 batches of hatchery populations of *P. fucata*

Locus	Wild Populations			Hatchery Populations		
	Sikka	Tuticorin	Vizhinjam	Adults	Juveniles	Spats
AAT						
(N)	(20)	(9)	(24)	(54)	(15)	(15)
100	1.000	1.000	1.000	1.000	1.000	1.000
EST-1						
(N)	(20)	(20)	(21)	(37)	(6)	(11)
106		0.000		0.000	0.000	0.182
100	*	0.700	*	0.716	0.917	0.727
94		0.300		0.284	0.083	0.091
EST-2						
(N)	(17)	(28)	(21)	(37)	(21)	(26)
113	0.000	0.000	0.000	0.082	0.000	0.019
100	0.676	0.714	0.619	0.635	0.667	0.808
87	0.324	0.286	0.381	0.284	0.333	0.173
ESD-1						
(N)	(20)	(9)	(24)	(21)	(24)	(15)
100	1.000	1.000	1.000	1.000	1.000	1.000
ESD-2						
(N)	(20)	(9)	(24)	(21)	(24)	(15)
103	0.375	0.167	0.312	0.524	0.750	0.833
100	0.625	0.833	0.688	0.476	0.250	0.167

Locus	Wild Populations			Hatchery Populations		
	Sikka	Tuticorin	Vizhinjam	Adults	Juveniles	Spats
GPI						
(N)	(20)	(0)	(14)	(17)	(14)	(0)
100	0.850		0.821	0.824	0.786	
73	0.150	-	0.179	0.176	0.214	-
MDH-1						
(N)	(20)	(30)	(24)	(54)	(15)	(15)
100	1.000	1.000	1.000	1.000	0.000	0.000
MDH-2						
(N)	(20)	(30)	(24)	(54)	(25)	(53)
100	0.825	0.867	0.792	0.796	0.660	0.625
84	0.100	0.133	0.208	0.204	0.340	0.375
68	0.075	0.000	0.000	0.000	0.000	0.000
ME						
(N)	(20)	(30)	(24)	(54)	(0)	(0)
100	1.000	1.000	1.000	1.000	-	-
PGM						
(N)	(15)	(0)	(9)	(24)	(24)	(0)
100	0.467		0.611	0.179	0.146	
96	0.467	-	0.333	0.357	0.646	-
90	0.033		0.056	0.393	0.188	
86	0.033		0.000	0.071	0.020	

Locus	Wild Populations			Hatchery Populations		
	Sikka	Tuticorin	Vizhinjam	Adults	Juveniles	Spats
SOD-1						
(N)	(20)	(12)	(24)	(42)	(15)	(15)
114	0.000	0.000	0.000	0.071	0.000	0.000
100	1.000	1.000	1.000	0.929	0.000	0.000
SOD-2						
(N)	(20)	(15)	(21)	(42)	(15)	(15)
100	1.000	1.000	1.000	0.964	1.000	1.000
63	0.000	0.000	0.000	0.035	0.000	0.000
PROT-1						
(N)	(20)	(21)	(24)	(47)	(25)	(15)
100	1.000	1.000	1.000	1.000	1.000	D.S
PROT-2						
(N)	(20)	(21)	(24)	(47)	(25)	(15)
100	1.000	1.000	1.000	1.000	1.000	D.S
PROT-3						
(N)	(20)	(21)	(24)	(47)	(25)	(15)
100	0.925	0.786	0.542	0.660	0.480	D.S
86	0.000	0.190	0.438	0.298	0.520	
73	0.075	0.024	0.020	0.042	0.000	
PROT-4						
(N)	(20)	(21)	(24)	(47)	(25)	(15)
100	1.000	1.000	1.000	1.000	1.000	D.S
PROT-5						
(N)	(20)	(21)	(24)	(47)	(25)	(15)
100	1.000	1.000	1.000	1.000	1.000	D.S

*Feeble activity, did record. -Did not examine. D.S. Did not score.

2 locus and having 7% frequency. The sample size was fairly large and comparable in all three regions while the sample size was smaller at Sikka where the rare allele occurred (Table 7).

The significance of the allele frequency differences between paired populations was tested at all loci by Chi-square heterogeneity analyses (Table 9). Significant level of population heterogeneity occurred only at PROT-3 locus, especially between Sikka and Vizhinjam regions.

4. 2. 1b F- analyses

The index of genetic differentiation among the three populations was also measured by F_{ST} values (Wright 1951). It was significant only at the protein-3 locus (Table 10). Highly significant value was obtained between for Sikka and Vizhinjam populations. However, when Sikka was compared with Tuticorin the value was not as significant as that of Vizhinjam. None among the other loci (EST-1, EST-2, ESD-2, GPI, MDH-2 and PGM) showed significant F_{ST} values. Out of the 18 F_{ST} values, 11 were negative .(F_{ST} can assume negative values when finite sample sizes are used(Waples 1987)).

The level of heterozygote deficiency was measured by F_{IS} values. Significant heterozygote deficiency was produced only at EST-2 between Tuticorin/Vizhinjam and at GPI between Sikka/Vizhinjam (Table 11).

Table 8. Observed genotype frequencies at the variable loci and their values of Chi-Square test of deviation from Hardy-Weinberg equilibrium.

Locus	Wild Populations						Hatchery Populations					
	Sikka		Tuticorin		Vizhinjam		Adults		Juveniles		Spats	
	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value
EST-1												
(N)	(20)		(20)		(21)		(37)		(6)		(11)	
106/106			0(0)				0(0)		0 (0)		0(0.36)	
106/100	*	*	0(0)	1.6327	*	*	0(0)	23.7260$^+$	0(0)	0.0494	4(2.91)	1.4409
106/94			0(0)				0(0)		0 (0)		0 (0.36)	
100/100			11(9.8)				25(19)		5(5.05)		5(5.81)	
100/94			6(8.4)				3(15.1)		1 (0.91)		2 (1.46)	
94/94			3(1.8)				9(2.98)		0 (0.04)		0(0.09)	
EST-2												
(N)	(17)		(28)		(21)		(37)		(21)		(26)	
113/113	0 (0))		0(0)		0(0)		3 (0.25)		0 (0)		0(0.01)	
113/100	0 (0)	0.7406	0(0)	6.3162$^+$	0 (0)	3.2618	0(3.85)	42.1430$^+$	0 (0)	1.4305	1 (0.8)	1.4671
113/87	0 (0)		0(0)		0(0)		0(1.72)		0(0)		0 (0.17)	
100/100	7(7.7)		17(14.3)		10(8.05)		20(14.9)		10(9.34)		16(17)	
100/87	9 (7.45)		6 (11.44)		6 (9.91)		7(13.4)		8 (9.32)		9 (7.27)	
87/87	1 (1.78)		5 (2.29)		5 (3.05)		7 (2.98)		4 (2.33)		0(0.78)	

Contd....

Locus	Wild Populations						Hatchery Populations					
	Sikka		Tuticorin		Vizhinjam		Adults		Juveniles		Spats	
	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value
ESD-2												
(N)	(20)		(9)		(24)		(21)		(24)		(15)	
103/103	4 (2.81)		0 (1.27)		0 (2.34)		6 (5.77)		13 (13.5)		10(10)	
103/100	7 (9.38)	1.2891	3 (2.5)	1.3800	15(10.3)	4.9749^{+}	10(10.5)	0.0433	10 (9)	0.2963	5 (4.17)	0.6014
100/100	9 (7.81)		6 (6.25)		9 (11.4)		5 (4.76)		1 (1.5)		0 (0.42)	
GPI												
(N)	(20)		(0)		(14)		(17)		(14)		(0)	
100/100	16(14.5)		*	*	11(9.44)		11(11.5)		11(8.65)			
100/73	2 (5.1)	7.3895^{+}			1 (4.11)	7.9500^{+}	6 (4.93)	0.7875	0(4.71)	14.051^{+}	*	*
73/73	2 (0.45)				2 (0.45)		0 (0.53)		3 (0.64)			
MDH-2												
(N)	(20)		(30)		(24)		(54)		(25)		(53)	
100/100	13(13.6)		22 (22.5)		14 (15)		36(34.2)		9 (10.9)		!7(20.7)	
100/84	4 (3.3)		8 (6.92)		10 (7.9)		12(17.5)		15 (11.22)		32(25)	
100/68	3 (2.48)	0.8946	0 (0)	0.7120	0 (0)	1.6655	0 (0)	5.2035^{+}	0(0)	2.8375	0(0)	4.299^{+}
84/84	0 (0.2)		0(0.53)		0 (1.04)		5 (2.25)		1 (2.89)		4 (7.45)	
84/68	0(0.3)		0(0)		0(0)		0(0)		0(0)		0(0)	
68/68	0(0.11)		0(0)		0(0)		0(0)		0(0)		0(0)	

Contd.....

Locus	Wild Populations						Hatchery Populations					
	Sikka		Tuticorin		Vizhinjam		Adults		Juveniles		Spats	
	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value
PGM												
(N)	(15)		(0)		(9)		(14)		(24)		(0)	
100/100	3 (3.27)				4 (3.36)		0 (0.45)		2 (0.51)			
100/96	6 (6.54)				2(3.66)		1 (1.79)		2 (4.53)			
100/90	1 (0.46)				1(0.62)		4 (2.0)		0 (1.32)			
100/86	1 (0.46)	2.4877	*	*	0 (0)	2.4777	0 (0.36)	12.691^{+}	1 (0.14)	15.753^{+}	*	*
96/96	4 (3.27)				2 (1)		0 (1.78)		10 (10.02)			
96/90	0 (0.46)				0 (0.34)		7 (3.93)		9 (5.83)			
96/86	0 (0.46)				0 (0)		2 (0.71)		0(0.62)			
90/90	0(0.02)				0(0.03)		0(2.16)		0(0.85)			
90/86	0(0.03)				0(0)		0(0.78)		0(0.18)			
86/86	0(0.02)				0(0)		0(0.07)		0(0.01)			
SOD-1												
(N)	(20)		(12)		(24)		(42)		(0)		(0)	
63/63							2 (0.21)					
63/100	M	M	M	M	M	M	2 (5.54)	17.6041^{+}	-	-	-	-
100/100							38(36.3)					
SOD-2												
(N)	(20)		(12)		(24)		(42)		(15)		(15)	
120/120							0(0.05)	0.0102				
120/100	M	M	M	M	M	M	3(2.83)		M	M	M	M
100/100							39(39)					

Contd.....

Locus	Wild Populations						Hatchery Populations					
	Sikka		Tuticorin		Vizhinjam		Adults		Juveniles		Spats	
	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value	Observed genotype frequency	χ^2 value
PROT – 3												
(N)	(20)		(21)		(24)		(47)		(25)			
100/100	17(17.1)		12(13)		4 (7.05)		15(20.5)		4(5.76)			
100/86	0 (0)		8 (6.27)		17(11.4)		28(18.5)		16 (12.48)			
100/73	3 (2.78)	0.1281	1 (0.79)	2.1657	1 (0.52)	6.4130$^+$	4 (2.61)	12.523$^+$	0 (0)	2.0000	*	*
86/86	0 (0)		0 (0.76)		2 (4.6)		0(4.17)		5 (6.76)			
86/73	0(0)		0(0.19)		0(0.42)		0(1.18)		0(0)			
73/73	0(0.11)		0(0.01)		0(0.01)		0(0.08)		0(0)			

* Did not record the locus.

+ $P<0.05$ Significant deviation from Hardy-Weinberg equilibrium.

M Monomorphic locus.

- Locus not present.

Expected genotype frequency values in parenthesis.

Table 9. χ^2 heterogenity analysis for the samples examined for P. *fucata*

Comparisons	EST-1	EST-2	ESD-2	GPI	MDH-2	PGM	PROT-3
Wild Samples							
All Sites	-	0.987	2.506	-	10.159^{+}	-	25.642^{+}
Sikka/ Tuticorin	-	0.145	2.509	-	4.768	-	9.153^{+}
Sikka/Vizhinjam	-	0.266	0.386	0.102	5.231	1.669	23.456^{+}
Tuticorin/Vizhinjam	-	0.985	1.389	-	1.082	-	6.325^{+}
Hatchery Samples							
Adult Oysters/Wild Tuticorin	0.032	4.889	6.606^{+}	-	1.325	-	2.197
Adult Oysters and Juveniles	2.190	3.700	4.994^{+}	0.142	3.398	9.002^{+}	8.158^{+}
Adult Oysters/Spats	16.101^{+}	5.037	7.732^{+}	-	7.781^{+}	-	-
Juveniles/Spats	2.543	5.986	3.851	-	0.201	-	-

$^{+}P < 0.05$ Significant Variation.
– Did not record.

Table 10. Values of F_{st} for the pair wise comparison of the samples of *P. fucata*

Comparisons	EST-1	EST-2	ESD-2	GPI	MDH-2	PGM	PROT-3
Wild Samples							
All Sites	-	-0.01915	0.01044	-	0.00433	-	0.19241^{+}
Sikka/ Tuticorin	-	-0.02811	-0.00070	-	-0.01024	-	0.03411
Sikka/Vizhinjam	-	-0.02829	-0.01673	-0.05247	-0.00461	-0.03747	0.16626^{+}
Tuticorin/Vizhinjam	-	-0.02077	0.00197	-	-0.00517	-	0.04834^{+}
Hatchery Samples							
Adult Oysters/Wild Tuticorin	-.03202	-0.00184	0.08287^{+}	-	-0.00706	-	0.00485
Adult Oysters and Juveniles	-.0350^{+}	-0.02586	0.02966^{+}	-0.06281	0.00884	0.03405	0.03094^{+}
Adult Oysters/Spats	.00545	0.00276	0.07188^{+}	-	0.02669^{+}	-	-
Juveniles/Spats	-.00626	-.002168	0.07091	-	-0.00908	-	-

$^{+}$ P<0.025 Significant Variation.
– Did not record.

Table 11. Values of F_{IS} for the pairwise comparison of the samples of *P. fucata*

Comparisons	EST-1	EST-2	ESD-2	GPI	MDH-2	PGM	PROT-3
Wild Samples							
All Sites	-	0.29194*	-0.09825	-	-0.17614	-	-0.32022*
Sikka/Tuticorin	-	0.22790	0.21117	-	-0.12990	-	-0.13741
Sikka/Vizhinjam	-	0.16350	-0.09251	0.6909*	-0.18480	0.20564	-0.21512
Tuticorin/Vizhinjam	-	0.45717*	-0.36367*	-	-0.18986	-	-0.31165
Hatchery Samples							
Adult Oysters/Wild Tuticorin	0.6272*	0.58310*	0.09110	-	0.19706	-	-0.3663*
Adult Oysters and Juveniles	0.7628*	0.62191*	-0.21008	0.8818*	0.08407	-0.217	-0.3424*
Adult Oysters/Spats	0.5664*	0.41294*	0.05982	-	-0.00827	-	-
Juveniles/Spats	-0.1514	0.26483*	0.05105	-	-0.2886*	-	-

* $P < 0.025$ Significant Variation.
– Did not record.

4. 2. 1c Genetic Identity of Wild Populations

Nei's genetic identity values obtained for the three populations are presented in table 12. The values produced on pairwise comparisons were high and ranged from 0.981 to 0.992. These values indicate that the three wild populations from Sikka, Tuticorin and Vizhinjam are genetically identical.

4. 3. Genetic Structure of Hatchery Produced Adult Oyster Population

Five loci (EST-1, EST-2, MDH-2, SOD-1 and PROT-3) among the nine variable loci (EST-1, EST-2, ESD-2, GPI, MDH-2, PGM, SOD-1, SOD-2 and PROT-3) showed significant deviations from Hardy-Weinberg equilibrium (Table 8). The mean observed heterozygosity was 0.18(±0.07) and mean expected heterozygosity was 0.20(±0.06) which is higher than that of the wild populations (0.13). Mean number of alleles per locus and the mean effective number of alleles per locus were 1.76 and 1.41 respectively, which were also more than that of the wild populations.

4. 3. 1. Allelic Comparison with Wild Stock

Allelic frequencies in the wild and hatchery adult population were closely similar at all loci except at ESD-2. The loci SOD-1 and SOD-2 were polymorphic in the hatchery adult population while the wild populations were

Table 12. Nei's genetic similarity values for *P. fucata*

Comparison	Sikka	Tuticorin	Vizhinjam
Sikka	-	0.99186	0.98183
Tuticorin	0.0082	-	0.98891
Vizhinjam	0.0183	0.0112	-

Values above the diagonal are genetic identities and below the diagonal are genetic distances.

not polymorphic at these loci (Table 7). The Chi-square heterogeneity test between the hatchery adults and wild populations was significant only at ESD-2 locus (Table 10).

4. 3. 2. F-analyses

F_{ST}values were insignificant between the hatchery adult and wild samples except for ESD-2 (Table 10). F_{IS} values were significant for EST-1 EST-2 and PROT-3 (Table 11) indicating highly significant heterozygote deficiency at EST-1 and EST-2 whereas an excess of heterozygotes at protein locus .

4. 3. 3. Genetic Identity of Wild and Hatchery Populations

Genetic identity values between the two populations for all loci were high. The loci EST-1, EST-2, ESD-2, MDH-2, SOD-1, SOD-2 and protein-3 had 0.9962, 0.9992, 0.8048, 0.9951, 0.9971, 0.9997 and 0.9822 identity values respectively. The mean value was 0.983 indicating that the hatchery adults and wild adults are genetically identical.

4. 4. Ontogenic Studies

All the loci except ME in juveniles and spats, GPI and PGM in spats were examined for ontogenic variations. Among all the loci examined (AAT, EST, ESD, MDH, PGM, SOD and PROT) MDH-1 and SOD-1 were not detected in the juveniles and spats. All other loci had comparable expressions in the

juveniles and spats. Though five protein loci expressed their phenotypes in the adults and the juveniles none of these loci was sufficiently active for typing in the spats. The rare allele PROT-3/73 was not detected in the juveniles. The spats showed protein band patterns significantly different from the adults and juveniles (Fig. 22, Plate 22). The protein bands expressed by adults/juveniles at third locus were absent in the spats (Fig. 23, Plate 23). Besides, the number of minor bands, their positions and staining intensities in the spats differed from that of juveniles/adults. However, protein bands expressed by the juveniles were not very different from that of the adults. Slight differences in the band position of minor bands in the area of third to fifth loci were noticeable between the two groups.

4. 4. 1. Allelic Frequencies

The allelic frequencies at all the loci except ESD-2, PGM and PROT-3 were closely similar in adults, juveniles and spats. Significant frequency differences occurred between the adults and the juveniles at PGM locus where the frequency of allele PGM-96 was much higher in the juveniles than in the adults. On the otherhand, the frequencies of ESD-2/100 and PROT-3/100 were much less in the juveniles/spats than that of the adults.

4. 4. 2. F-analyses

F- statistic analyses showed significant differences between adult oysters and juveniles at EST-1, ESD-2 and PROT-3 (Table 10). ESD-2 and MDH-2

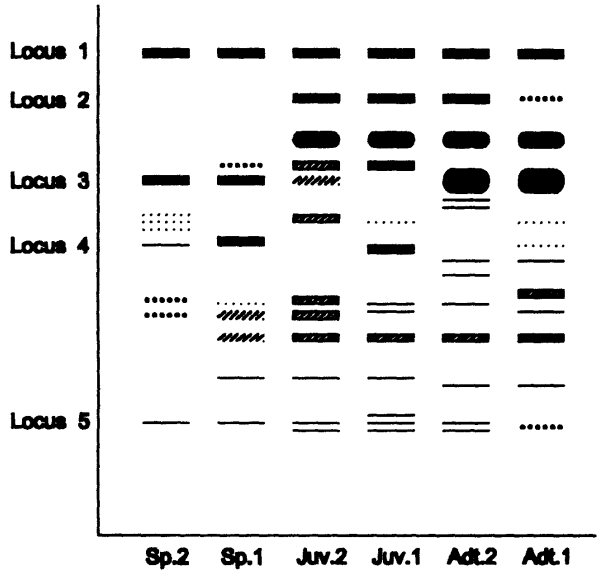

Fig. 22. Zymogram for PROT showing ontogenic variation in *P. fucata* (Adt: Adult; Juv: Juvenile; Sp: Spat)

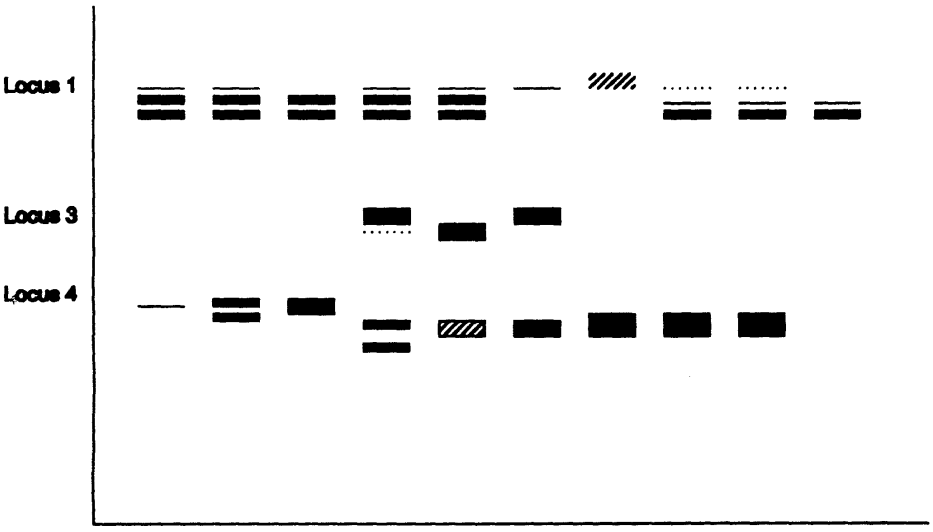

Fig. 23. Zymogram for PROT in spats of *P. fucata*

Faint band

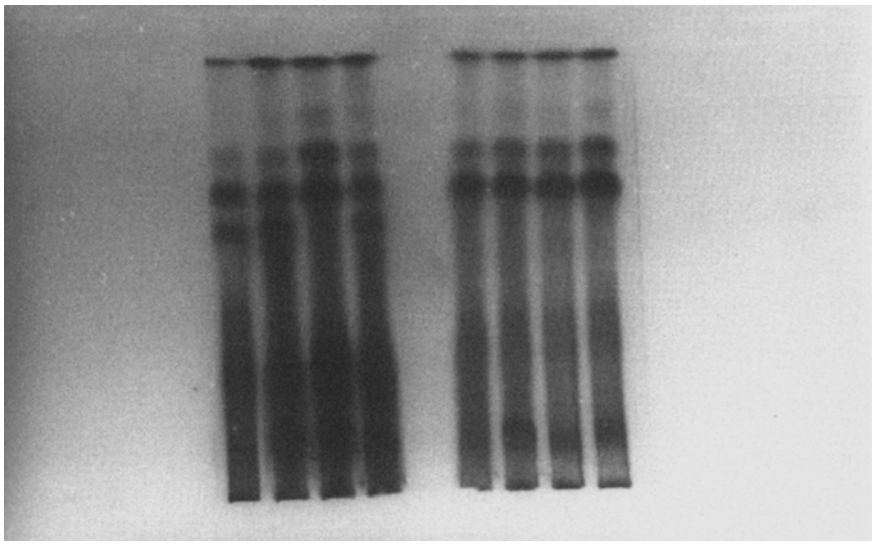

Plate 21. Pattern observed for general proteins in adductor muscle of *P. fucata* from Vizhinjam (L to R: Lanes 1 to 4) and Sikka (L to R: Lane 6 to 9)

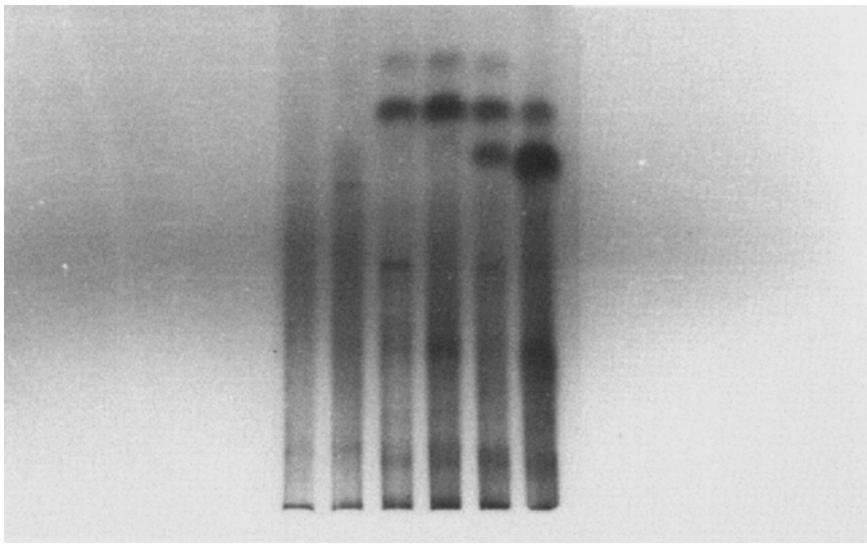

Plate 22. Pattern observed for general proteins in in spats (L to R : Lanes 1 & 2), juveniles(L to R: Lanes 3 & 4) and adults (L to R : Lanes 5 & 6 of *P. fucata* from hatchery.

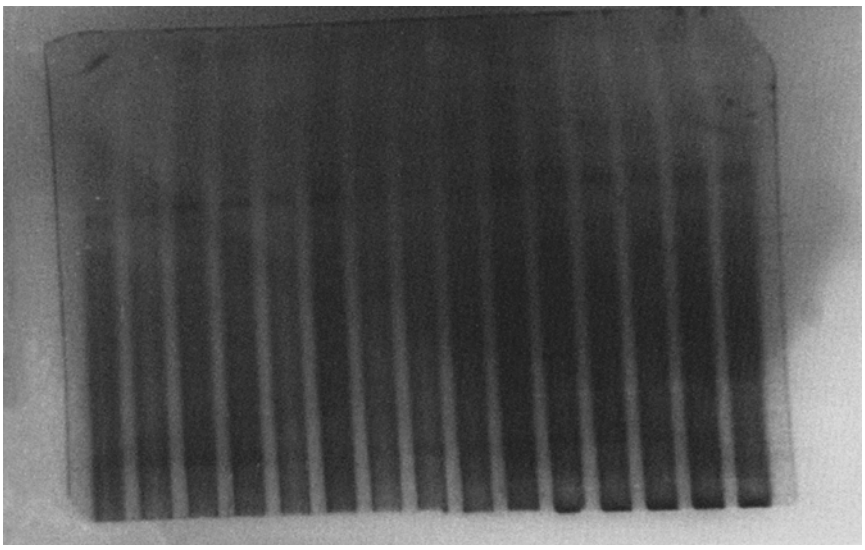

Plate 23. Pattern observed for general proteins in spats of *P. fucata*

also exhibited significant F_{ST} values for adult/spat comparison. None of the polymorphic loci examined for spats and juveniles (EST-2, ESD-2 and MDH-2) exhibited significant F_{ST} values. Significant heterozygote deficiencies were observed at EST-2 and MDH-2, as indicated from the positive F_{IS} values (Table 11). F_{IS} value at PROT-3 locus was highly significant between adults and juveniles (F_{IS} = -0.34, $P<0.025$). The level of heterozygosities at MDH-2 for adults, juveniles and spats were 0.22, 0.60 and 0.60 respectively. For adults and juveniles, these at protein locus were 0.68 and 0.64 respectively.

Thus the comparison of zymogram patterns of eight enzymes and general proteins between adults and juveniles between adults and spats and between juveniles and spats revealed that some of the phenotype variations noticed were of ontogenetic nature. Such ontogenetic differences were sharper in the spats than in the juveniles. A statistical comparison of F_{ST} values at the polymorphic loci between the adults and the juveniles showed significant differences at three (EST-1, ESD-2 and PROT-3) out of seven polymorphic loci. The F_{ST} values between adults and spats were significant at two loci (ESD-2 and MDH-2). The F_{IS} values between adults and juveniles were significant at EST-1, EST- 2 and GPI and PROT-3 loci. The values between adults and spats were also significant at EST-1 and EST-2 loci. The values between juveniles and spats were significant at EST-2 and MDH-2 loci.

4. 5. Morphometric Analyses

The average values of hinge length, dorsoventral length, width, shell weight and convexity of the shell for the samples tested are shown in Table 1. Tuticorin population was smaller in size as compared to Sikka and Vizhinjam populations. The convexity of the shells of Sikka sample was significantly different ($P<0.05$) from Tuticorin and Vizhinjam samples. Sikka population had heavy valves, but less deeper as compared to Tuticorin or Vizhinjam populations. The colour of nacre on the selected area of the shell for Sikka population was predominantly cream - yellow, and for Vizhinjam and Tuticorin it was more towards white with tinges of pink, green and blue (Plate 4). The prismatic layer of Sikka population was cream without any radial brown bands or flecks, which were characteristic of Tuticorin and Vizhinjam populations (Plate 3). However, towards the margin of the shell on the inner side, some dark spots were observed in the sample from Sikka. Thus the examined shell morphology of the Sikka sample appears to be different from that of the Vizhinjam and Tuticorin regions.

5. *Discussion*

The application of biochemical genetic techniques (Smithies 1955; Hunter and Markert 1957; Smith 1968; Brewer 1970) in the analysis of tissue proteins and enzymes has established a phenomenon of intraspecies genetic polymorphism in all kinds of organsims tested, including the laboratory populations of drosophila (Lewontin and Hubby 1966), aquatic organisms like the finfish (Sick 1961; de Ligny 1969; Allendorf and Utter 1979) and shellfishes like, shrimps (Lester 1979; Sbordoni et al. 1986; Li et al. 1993) and a range of bivalves like pearl oysters (Wada 1982; Durand et at. 1993; Benzie and Ballment 1994). The implications of the natural biochemical genetic variations in the management of the commercially important fish and shellfish resources of the world have been thoroughly discussed, especially with reference to the scientific exploitation and conservation of the fisheries resources (de Ligny 1971; Ryman and Utter 1986). Meanwhile, studies to correlate the observed genetic variability with economically important traits like growth rate, fecundity, disease resistance, etc. have also been attempted by others to augment the production through modern aquaculture practices (Newkirk 1980; Wilkins 1975; Bye and Ponniah 1983).

The general objectives of the electrophoretic analysis of proteins and enzymes in different commercially important fish and shellfishes are to answer the basic fisheries management related questions. Such as (1) What is the level of the genetic variation in the species and its different populations? (2) Whether the allelic frequencies in the sample populations are similar or different? (3) Whether the observed and expected genotype frequencies are

in Hardy – Weinberg equilibrium? and (4) If the populations are genetically homogeneous or heterogeneous, then what are the implications of the findings with reference to their exploitation and conservation ?

5. 1. Genetic Structure of the Wild Populations

The basic question tried to be answered through the present biochemical genetic studies on the pearl oyster *Pinctada fucata* of India were the same as listed above. Hence, the original results obtained from the present investigation have been discussed here in detail with a view to evaluating the significance of the research findings and their application in the management of the Indian pearl oyster, *P. fucata* which is considered as a vulnerable species(James 1994).

It is important to ascertain that the aspects of materials collected and the methods followed have not affected the results. As all the specimen samples used here were live, alterations in the quality of tissues used for test have not occurred and not affected the results. To avoid any age related effect on the pattern of results, adult animals in the size group of 46mm (DVL) to 63mm (DVL) were tested. Juveniles and spats were separately tested and analysed.

It is known that the qualitative and quantitative nature of enzymes may vary considerably in different tissues of an organism and usually these may

express tissue specific patterns. Many popular buffer systems are known to produce satisfactory separation and resolution of enzymes/proteins present in different species (Shaw and Prasad 1970; Schaal and Anderson 1974; Harris and Hopkinson 1976; Gordon 1980; Richardson *et al.* 1986). However, to obtain the most satisfactory results, it was necessary to compare enzyme patterns in different tissues and different buffer systems. In the present investigation, the visual quality and quantity of seventeen enzymes were first evaluated in different tissues, like, the adductor muscle, digestive diverticula and mantle, and in different buffer systems, like, TC, TEC, TBC and TG to produce the best possible results. In consequence the adductor muscle and digestive diverticula, and the buffer systems TBC and TG, were found to produce better results. Even slight modifications like, change in the strength and pH of the buffer or gel percentage composition have benefited in obtaining better results in the local species *P. fucata*. In nutshell, the materials and methods adopted here have not adversely affected the results thus obtained.

It is interesting to record that eight of the 17 enzymes tested were found unsuitable for the population genetic analyses in *P. fucata*. Some of these enzymes, like, A0, LAP, LDH and ODH apparently did not show any detectable level of enzyme activity or others like AAT, ADH, DIAPH, G6PD and IDH were either insufficiently active or poorly resolved. A comparable pattern of results have also been reported in other species of molluscs (Fujio et al. 1983; Benzie et al. 1993; Benzie and Ballment 1994). However, an

exception from the above statement was reported in three species of Japanese pearl oysters, including *P. fucata* (Wada 1975c, 1982, 1986c) where a strong activity for LAP locus with high level of genetic variation was observed. A possible explanation for its non - activity in the Indian species is that the variation in the electrophoretic methodologies adopted in the two investigations may have caused the difference. Wada (1975c) followed starch gel electrophoresis for 17 hours, while PAGE for about 2 hours was adopted in the present investigation. These two opposing patterns of results in the case of LAP enzyme suggest that some negative results may be indeed due to certain aspects of the methodology followed, and these negative results therefore may become positive, if a suitable method is tried. The above problem in the Indian species can be solved by adopting exactly the same methods followed for the Japanese species by Wada (1975c).

Another interesting difference noticeable between the Indian and Japanese pearl oyster, *P. fucata*, is that the major zone of superoxide dismutase was found monomorphic in Indian species, while it was reported as highly polymorphic in the Japanese species *P. fucata* (Wada 1982) and the subspecies *P. fucata martensii* (Wada 1986c). Once again, the major reason for the difference in the results compared may not be attributed to the methodological differences alone. Because what was observed as monomorphic SOD bands in the middle part of the polyacrylamide gel was a major zone of strong enzyme activity with additional two minor zones in the Indian species. Adductor muscle was used in both the cases to study SOD.

Thus the basic patterns of SOD enzyme in the Indian and Japanese *P. fucata* appeared to be very distinct.

The basic banding patterns of adductor muscle proteins in three Japanese species, *P. fucata*, *P. maculata* and *P. albina* were determined by three alleles, A, B and C in the decreasing order of mobility. The A type was observed only in *P. albina* whereas B and C alleles and their combinations as heterozygotes were present in the other two species. Quite interestingly, the three alleles typed as A, B and C, in the foreign species of *P. fucata* were present in Indian species (Fig. 13, Plate 16). A significant difference between Indian and Japanese *P. fucata* is that the A allele which was absent in the Japanese species apparently dominated in the Indian species. Besides, the protein allelic frequencies appeared to be very different in Indian and Japanese pearl oyster populations. Another parameter that showed significant differences between the Indian and the Japanese *P. fucata* is the zymogram pattern of the malate dehydrogenase enzyme. In Indian species, the MDH enzyme showed identical polymorphic patterns in the tissues, adductor muscle, digestive diverticula and mantle, at MDH-2 locus, and a single banded monomorphic pattern at MDH-1 locus. The MDH banding patterns in the Japanese *P. fucata* varied from 2 to 7 bands in individuals tested and produced twelve types of combinations (Wada 1975b). It appears that these twelve types in the Japanese species were merely phenotypic variants and not genotypic variants as expected in a genetic polymorphism. Thus the MDH patterns in both the Indian and the Japanese *P. fucata* are

very different. On the other hand, comparable morphometric traits studied in the species from India and Japan do not appear to be significantly different.

The above discussion on the significant differences in the zymogram patterns of superoxide dismutase, malate dehydrogenase and general muscle protein in the Indian and Japanese species of *P. fucata* suggests that the species in the two geographical regions may be genetically very different. Unfortunately, many enzyme loci studied now in the Indian species were not examined in the Japanese species. Otherwise, a comprehensive evaluation of the two results could have been attempted. Hence, it is necessary to conduct a comparative study of as many enzymes as possible and of the general proteins in identical electrophoretic conditions so as to test the above hypothesis that populations of *P. fucata* in India and Japan are geographically distinct.

Now, the results of the present investigation on the Indian *P. fucata* and its populations should be discussed with a view to evaluating the genetic variability within the species, and the stock structure of the three populations. The values of heterozygosity, average alleles per locus and percentage polymorphic loci can indicate the level of genetic polymorphism in a species while the mean heterozygosity alone can indicate the degree of genetic variations (Ayala and Kiger 1980). The estimated mean heterozygosity in the Indian *P. fucata* is 0.13. This is closely comparable to that of marine

invertebrates (0.124). The value is much higher than that of fishes (0.082)(Tab.18.11, Ayala and Kiger 1980). As the heterozygosity value was not reported in the Japanese *P. fucata* (Wada 1975b, 1982) an evaluation of genetic variability between the two regions is not possible. However, the fact that the two enzymes SOD and LAP , and muscle protein examined in Japanese *P. fucata* were polymorphic suggests that the Japanese species also has high genetic variations (Wada 1982). Besides, the average heterozygosities, like, 0.57 in *P.margaritifera* (Benzie and Ballment 1994), 0.60 in *P. fucata martensii* (Wada 1986) indicate higher genetic variability in the other pearl oyster species of *Pinctada*. The polymorphism indicated by the average number of about seven alleles at 17 loci and a maximum number of 22 alleles at GPI locus in *P. margaritifera* (Benzie and Ballment 1994) suggest that the incidence of mutation at enzyme loci in this pearl oyster species was more than in that of *P. fucata*. A probable reason for such very high average heterozygosity in *P. margaritifera* is that monomorphic loci were not considered in finding the average heterozygosities as done in the same species by Durand et al. (1993) where the heterozygosity reported was only 0.237 on the basis of eleven polymorphic and seven monomorphic loci. Comparatively high genetic variations were also reported in other bivalves, like , *Crassostrea gigas* (Buroker et al. 1975) and *Saccostrea commercialis* (Buroker et al. 1979a, 1979b). The higher genetic variability present in the bivalves, like, the pearl oysters, compared to the vertebrates, like, fish (Fujio et al. 1983; Ayala and Kiger 1980) suggests that the built in genetic strategy may differ not only between species but also between higher levels of species

organisation. The advantages of possessing high genetic variability at isozyme loci are not clearly known. The apparent correlations of variant genotypes or alleles to any biological traits, like, growth, fecundity or resistance to diseases, unfavourable situations etc. have to be assessed through breeding experiments. Positive or negative or lack of correlations between genotypes and growth rate or survival rate or fecundity have been reported (Adamkewicz et al. 1984; Beaumont et al. 1983; Rodhouse and Gaffney 1984; Diehl and Koehn 1985; Mallet et al. 1985; Hoare and Beaumont 1995). The results of the attempts of these authors to measure detectable level of correlations between enzyme genotype variants and specific biological traits suggest that the phenomenon of correlation or lack of it is too complex to evaluate in many cases. The details of genetic variability or genotype characteristics gathered in the present investigation on Indian *P. fucata* could not be tested against any correlation factors, because, such a testing was impractical and was beyond the scope of the present investigation.

The range of the mean heterozygosities in the species varied from 0.10 in Tuticorin to 0.16 in Vizhinjam. The mean heterozygosites in the populations of *P.fucata* from Sikka(0.13) and Tuticorin (0.10) do not appear to be significantly different. The fact that the sites of the lowest heterozygosity (Tuticorin) and the highest (Vizhinjam) are geographically closer is intriguing. Does it indicate that the Vizhinjam population is genetically more heterogeneous than that of Tuticorin? A detailed discussion on the allelic

frequencies and other relevant facts in these three populations should answer this question.

The allele frequencies at six polymorphic loci (EST-1, EST-2, ESD-2, GPI, MDH-2 and PGM) were not significantly different in the three populations tested from Sikka (Gulf of Kutch), Tuticorin (Gulf of Mannar) and Vizhinjam. However, at the muscle protein, the allele frequencies in the three populations differed significantly. The allele 86 was totally absent in Sikka population while it was highest (0.436) in Vizhinjam and lowest (0.19) in Tuticorin. Besides, the allele 100 was significantly higher in Sikka(0.95) and Tuticorin (0.78) as compared to Vizhinjam (0.542). These allele frequency differences were found statistically significant by Chi-square heterogeneity and F_{ST} tests for paired samples between Sikka and Vizhinjam and Sikka and Tuticorin populations (Table 9, 10). Do these values at protein locus indicate that Sikka population is genetically heterogeneous? As sample size from all three regions were closely comparable (20 to 24), the size of the sample has not affected the results at this locus. The electrophoretic and genotype scoring methods were uniform and the specimens used were adults, in all the three regions. The fact that the allele frequencies were closely comparable at all other loci suggests that non-genetic parameters have not affected the results at the PROT locus in the present case. The next question to be answered is whether the protein phenotypes scored are of genetic nature or its frequencies were as expected in Hardy-Weinberg equilibrium? The observed frequencies at PROT locus were not significantly different from the expected values (Table

8). Hence, the difference at PROT locus is of genetic nature. However, a significant difference at a single locus out of seven loci compared does not statistically qualify to differentiate the Sikka or any other population as genetically different stock. Furthermore, the population structure cannot he determined on the basis of single locus when some loci in the same set of populations show little differentiation (Burton 1983). Moreover, Nei's genetic identity/distance for the population from Sikka, Vizhinjam and Tuticorin showed these three populations are genetically similar (Table 12). However, the question remaining is that whether the significant heterogeneity at the protein locus is a reflection of a hidden heterogeneity between Sikka population and Vizhinjam/Tuticorin populations, i.e., a heterogeneity not revealed by enzyme loci? There are instances when protein electrophoresis have failed to identify genetically discrete stocks (Utter 1981). An analysis of mitochondrial (mt) DNA in these three populations may throw more light on the population genetic difference of these three populations of *P. fucata*. A hidden genetic stock difference in the populations of the American oyster, *Crassostrea virginica* with continuous distribution was detected by mt DNA and nuclear DNA analysis (Reeb and Avise 1990), which by allozyme studies (Buroker 1983) exhibited homogeneity. Since, Sikka, Tuticorin and Vizhinjam population of the Indian pearl oyster, *P. fucata* have a discontinuous distribution, the chances of hidden genetic stock difference in Indian *P. fucata* could be more than that of the American oyster.

On the otherhand, the biological reasons for the present observation of genetic similarity at almost all the six polymorphic enzyme loci in the three populations of Indian *P. fucata* occurring at a distance of more than 3500 km. from Sikka to Tuticorin may be suggested. In this respect, the first favourable condition for generating genetic homogeneity among the distant populations of a species is the nature of long larval phase and easy larval dispersal through coastal currents. The larval phase of Indian pearl oyster is 22 days (Alagarswami et al. 1983) under normal circumstances and it provides the potential for dispersal over several hundred kilometers. The circulation of surface waters in Indian Ocean is influenced by the monsoons. During monsoon, the average speed of the current along the Indian coast is one knot, and with winds this can measure upto two knots. So, at a current speed of one knot, a distance of 950 km. can only be covered in 22 days.

A similar case of larval drift has been observed in the Western Pacific populations of *P. margaritifera* where high levels of gene flow exists (Benzie and Ballment 1994). Currents have been suggested as the causative factor for the low level of genetic divergence seen among some invertebrate populations in the Great Barrier Reef (Benzie 1995). In transoceanic populations of *Linckia laevigata* separated by more than 6500 km., no significant genetic differentiation was observed (Williams and Benzie 1993). For the maintenance of genetic uniformity in several widespread marine species in the absence of selection and geographic or hydrographic restraints, the high level of larval dispersal has been suggested as the probable cause (Silberman et al.

1994). If, the genetic homogeneity between the three populations of *P. fucata* is indeed due to larval dispersal, then at the mentioned current rate, it may take 80 days for the larvae to each Vizhinjam from Sikka or vice versa. It appears that under unfavourable conditions (the absence of particular physical or chemical cues), the metamorphosis of marine invertebrate larvae can be delayed to a considerable extent under laboratory conditions, like 280 days in gastropods (Kemf 1981) and echinoderms (Birkeland et al. 1971), and 45 days in *M. edulis* (Pechnik et al. 1990). So, in the present case, either the pearl oyster larvae delays its metamorphosis until it finds a suitable substratum or, there must be some region along the west coast of India where unexploited pearl oyster beds, with a continuous mixing of the other stocks of *P. fucata* exist. Deep water natural resources are thought to exist in case of certain pearl oyster species ensuring recruitment into the fishable, shallower stocks (Penn and Dybdahl 1988). Without any published reports on the occurrence of pearl oyster spat settlement along the west coast, except at Vizhinjam, we are left with the first assumption, i.e. delayed metamorphosis. This is once again strengthened by the fact that the coastal topographic profile of the west coast is mainly unsuitable (oozy substratum chiefly (Sewell 1994)) for the spat settlement of pearl oysters.

On the contrary, if the hypothesis of delayed metamorphosis and easy larval dispersal of *P. fucata* from Sikka to Tuticorin or vice versa is taken as a base for the genetic similarity of the three populations, the presence of allele 86 at a frequency of 0.432 in Vizhinjam and at 0.19 in Tuticorin and its total

absence in Sikka sample cannot be explained. Vizhinjam and Tuticorin cannot have a supply of allele 86 from the larvae of Sikka since it is devoid of allele 86 and likewise if the larvae of Vizhinjam or Tuticorin are the source of Sikka population, the absence of allele 86 in Sikka becomes unexplainable through larval distribution hypothesis.

An alternative hypothesis that may explain the similarity of allele frequencies at the six polymorphic loci in Sikka, Vizhinjam and Tuticorin, while total absence of protein allele 86 in Sikka and its considerable presence in Vizhinjam/Tuticorin regions, is the existence of a closely comparable selection pressure in all three populations at six enzyme loci, but, a non-comparable selection pressure at protein locus, especially in the Sikka population. Such similar or dissimilar selection pressure has been suggested for some geographically distant populations of marine molluscs (Koehn et al. 1980; Lassen and Turano 1978; Gosling 1979). The hypothesis of selection on the protein locus in the Indian *P. fucata* may be experimentally tested by planting the Vizhinjam stock in Sikka region or vice versa and keep monitoring the frequencies of alleles, 100, 86 and 73 for a few generations.

Thus the above discussion on the genetic variability in the Indian pearl oyster, *P. fucata* may be concluded as follows. The average alleles per locus (1.49), percentage polymorphic loci (41%) and the mean heterozygosity (0.13) estimated for the species in the present study suggests that the species has comparatively high genetic variability. A comparison of the biochemical

genetic characteristics discovered in the Indian *P. fucata* with those of the Japanese *P. fucata* (Wada 1975 b; 1982) clearly suggests that the populations of *P. fucata* from India and Japan are genetically two distinct geographical stocks, a hypothesis to be tested by a further electrophoretic study having identical experimental conditions. On the otherhand, the observed genetic characteristics of the three populations of Indian *P. fucata* tested from Sikka (Gulf of Kutch) , Vizhinjam and Tuticorin (Gulf of Mannar) are statistically homogeneous, inspite of the fact, that Sikka populations is isolated by a distance of more than 3500 km from the latter two populations. The hypothesis of delayed larval metamorphosis and larval dispersal through coastal currents may accomplish the phenomenon of genetic homogeneity in the three populations isolated by distance. However, the total absence of the allele 86 at protein locus in the Sikka population, while its occurrence at frequencies of 0.438 in Vizhinjam and 0.19 in Tuticorin, is a bottleneck in the above larval distribution hypothesis. Hence, an alternative technique of genetic analysis, like, mt DNA analysis in the populations of Indian pearl oyster, *P. fucata*, is recommended for obtaining an insight on the population genetic structure of the species.

5. 2. Genetic Structure of the Hatchery Adult Population

The value of allozyme analysis for monitoring the genetic structure of hatchery population in aquaculture has been stressed by authors like Wilkins (1975) and Hedgecock (1977). Genetic variation is required in a population if the attributes of that population are going to be changed via

selection. The average genetic variation in a population can be estimated by allozyme studies. It is generally accepted fact that the artificial production of marine organisms can lead to a reduction of genetic variation in the cultured stock as compared with that observed for wild populations. When hatchery stocks are compared with the natural populations of the same species, one might expect to see significant changes in allele frequencies and a reduction in the mean number of alleles per locus , and in the overall levels of heterozygosity and polymorphism.

In the present study, the genetic structure of the wild and hatchery adult populations of *P. fucata* did not differ significantly. The allele frequencies at all loci except at PGM and ESD-2 were closely similar. However, the F_{ST} and genetic identity values were not significantly different in the two populations. A similar situation was reported in *C. gigas* (Gosling 1982). Few more instances in marine molluscs exist where no significant overall genetic differences were observed between the natural and hatchery stocks (Dillon and Manzi 1987; Durand et al. 1993).

The SOD enzyme loci 1 and 2 were polymorphic only in the hatchery population. A comparable phenomenon was reported at XDH locus in *C. gigas* (Gosling 1982). It is true that sample size of wild populations were fifty percent less than the hatchery stock. Screening of larger wild populations may show up the polymorphic nature of SOD loci. On the contrary, some species have shown reduced levels of genetic variation in their hatchery stocks

(Allendorf and Utter 1979; Agnese et al. 1995; Sbordoni et al. 1986; Wada 1986).

Hatchery stock of *P. fucata* showed significant deviations from the expected genotype frequencies at many loci. Mainly these deviations arose because of the observed heterozygote deficiencies at the loci. Studies on the genetic variability in marine molluscs have shown that there is a general tendency for the hatchery population to show deficiency of heterozygotes (vide Singh and Green 1984). In the hatchery population of *C. gigas* the heterozygote deficiency was seen at three out of ten loci (Gosling 1982). She has suggested differential selection acting on these loci, or the differential mortality due to selection at other loci, as the reason.

The heterozygosity in the hatchery population of *P. fucata* was higher than that of wild populations. Reduced heterozygosity and reduction in the number of alleles were reported in hatchery populations of fish and shellfishes(Allendorf and Phelps 1980; Ryman and Stahl 1980, Sbordoni et al. 1986; Wada 1986). In case of *P. fucata* no such reduction in the number of alleles for hatchery stock in comparison to the natural stock was observed. But, heterozygote deficiency was observed at all loci, except protein. Why a reduction in the heterozygote level, when actually the mean number of alleles and heterozygosity observed in hatchery is more than of the wild is not understood. Reduction in the number of alleles and heterozygote deficiency usually occur when limited number of parents are used as brood stocks. In

case of Tuticorin hatchery, the practice of random mixing of the wild population with hatchery brood stock may be suggested to have a positive effect in maintaining the wild population structure in the hatchery stock. Similarly, Allendorf and Utter (1979) and Busack et al. (1979) have reported no reduction of heterozygosity or number of alleles in the hatchery reared population of finfish. In *P. maragaritifera* a reduction in the number of alleles was observed in the hatchery population (Durand et al. 1993). But there was no reduction in the level of heterozygosity. Heterozygosity is less sensitive than the total number of alleles to genetic changes in cultivated stocks (Hedgecock and Sly 1990). Besides, many facts inherent in the hatchery system may have influenced to express the observed differences in the hatchery adult population.

5. 3. Ontogenic Variations

The phenomenon of ontogenic variations occurring particularly in organisms with larval and juvenile developmental stages is well known. The ontogenic variations are due to differences in the gene expressions during early developmental stages through adulthood where a stabilised pattern emerges. Such differences easily detected by gel electrophoresis of enzymatic and non enzymatic proteins have been reported in many species of Crustacea and Decapoda (Lester and Cook 1987). The ontogenic expressions are easily detected in the form of differences in the number of electrophoretic bands, their electrophoretic positions and staining intensities (Lester and Cook 1987;

Samuel 1987). In the present comparative study of seven isozymes and general proteins in the adults, juveniles and spats of the Indian pearl oyster *P. fucata* has also revealed ontogenic variations, especially at specific enzyme loci. Thus the absence of MDH-1 and SOD-1 enzyme acticvity in juveniles and spats, the insufficient acticvity of about five protein loci in the spats, the absence of rare allele PROT-3/73 in juveniles, were some evidences of ontogenic variations in *P. fucata* tested here. The absence of ontogenic variations at other loci is interesting. It suggests that all loci are not equally involved in the developmental changes of the species. It is also interesting to note that allelic frequencies, and heterozygosites also differed at certain loci between adults and juveniles or between adults and spats. These differences may be due to differences in the selection pressure (Flowerdew and Crisp 1976) or mortality at different developmental stages (Mallet et al 1985).

5. 4. Morphological Variations in Wild Samples

Morphological variations is common in molluscs like mussels, gastropods, littorinids etc. A ubiquitous shell colour polymorphism has been reported in *M.edulis* (Newkirk 1980). The colour pattern of blue – black or brown in *M. edulis* was found to be controlled by a gene (Innes and Haley 1977 vide Berger 1983). Shell colour is also supposed to be influenced by the environment. The external colour of the bivalve shell is due to the presence of certain pigments associated with the prismatic layer of the shells. In *P. fucata* the most common colour morph is cream with dark brown radial

bands or spots. Rarely, green colour is also observed. Wada (1994) reports even red, yellow and white specimens. He has discussed that the white colour is under the control of a recessive gene. In the present morphological study of *P. fucata*, the difference observed in the shell colour of Sikka sample from that of others, may be due to the environmental effect. Absence of the radial bands or any dark spot in Sikka sample can be due to the regular and prolonged exposure of the animal to light during ebb tides. May be the lighter shell in Sikka population is just an adaptive phenomenon to avoid excessive heat when the animals are exposed during low tides, because less radiant energy is absorbed by light surfaces. A comparable trend was observed in some species of mussels occurring in colder waters, where darker shells are common to reduce the risk of freezing (Mitton 1977). Breeding experiments have to be conducted to determine the causes of the light shell colour of the Sikka population and shell colour/shape inheritance. The shell of Sikka population was less convex than that of the other two wild populations.

A uniform predominantly cream nacre colour was observed in Sikka alone. It has been proved in *P. fucata martensii that* the colour of pearl will depend upon the nacre colour (Wada 1969). *P. fucata* chiefly produces pearls of golden yellow or ivory white colours (Algarswami and Qasim 1973), some are pink. The colour of the pearls mainly results from the reflection and interference of light falling upon the granular/laminar structure of the nacre. The environmental conditions like depth, light, presence of certain metal ions, etc. will also influence the colour. It has been seen that golden or cream

coloured pearls will have more copper and gold content than the skin or pink colour pearls which have more of sodium and zinc (Matsui 1960). An iron bond peptide in the nacre has been shown to favour the formation of the yellow colour (Sawada 1961 vide Wada 1970). For the nacre colour polymorphism observed in *P. fucata* stocks, an extensive water quality as well as breeding data analyses have to be conducted to understand that which among the many factors is evoking the nacre colour differentiation. The importance of such studies should be highlighted since the yellow pearls fetch more price than white pearls in India where an ever growing domestic market for pearls exists.

6. Conclusions

1. The estimated indices of genetic variations, like, 1.49 alleles per locus, 41% polymorphic loci and a mean heterozygosity of 1.3 at the 17 loci examined suggest that the Indian pearl oyster, *Pinctada fucata* has a high genetic variability(Table 7).
2. The observed genotype frequencies deviated significantly from that of the expected only at five out of 17 occasions due to heterozygote deficiencies (Table 8) . The observed variations were of genetic nature.
3. The heterogeneity tests of allele frequencies in the three populations were statistically insignificant (Table 7, 9, 10 & 12). Therefore, the populations of *P. fucata* screened from Sikka, Vizhinjam and Tuticorin are genetically homogeneous and identical.
4. The heterogeneity in the allele frequencies / heterozygosities and the lack of allelic expressions at MDH-1 and SOD-1 loci in the juveniles and spats, as well as inconsistent banding patterns at five protein loci in the spats suggest that the phenomenon of ontogenic variations is present in the species (Table 7).

7. Suggestions

1. The present findings of the genetic homogeneity of *Pinctada fucata* populations in Sikka, Vizhinjam and Tuticorin may be evaluated by more sensitive methods, like, mitochondrial DNA (mt DNA) analysis.
2. The peptidase enzyme loci known to be highly polymorphic in other *Pinctada* species are to be screened in the future genetic analysis of *P. fucata* .
3. The hypothesis of selection at protein locus (PROT-3) in the Sikka population may be tested by suitable experiments, like, planting the Vizhinjam stock in the Sikka region or vice versa and keep monitoring the protein patterns through generations.
4. Breeding experiments may be necessary to determine the role of environmental factors evoking the cream nacre colour in the Sikka population.
5. The hypothesis that the Indian and the Japanese [illegible] be two distinct genetic stocks is to be tested by a comparative biochemical genetic analysis of the two geographical populations.

8. Summary

1. The thesis contains the results of " An investigation on the biochemical genetics of the Indian pearl oyster, *Pinctada fucata* (Gould)". The results have been described and discussed on the basis of data presented in 23 figures and plates and 12 tables.
2. Polyacrylamide gel electrophoresis methods were standardised to detect and analyse the biochemical genetic characteristics present in the form of tissue isozymes and general proteins. Sixteen enzyme systems were tested for activity. But only eight (AAT, EST, ESD, GPI, MDH, ME, PGM and SOD) were found good for screening the population. Detailed analyses of the zymograms of these eight isozymes enabled to discover twelve gene loci composed of eight polymorphic and four non-polymorphic loci. A total of five loci, one polymorphic and four monomorphic, were detected from the zymograms of general proteins.
3. To study the genetic structure of the wild populations of the species, the enzyme and protein loci were screened in the population samples taken from Sikka, Tuticorin and Vizhinjam oyster beds. To study the genetic structure of the hatchery population samples taken from the Tuticorin hatchery, the same loci screened in the wild populations were screened.
4. The data collected, loci and population wise, were separately analysed by standard statistical methods to determine the genetic nature of the observed electrophoretic phenotypes and to measure the level of genetic variability within the species and the genetic heterogeneity among its three populations.
5. The frequencies of the observed phenotypes did not deviate significantly

from that of the expected at any of the loci examined except at EST-2 in Tuticorin and GPI in Sikka and Vizhinjam and at ESD-2 and PROT-3 in Vizinjam populations.

6. The heterozygosities varied from 0.10 (Tuticorin) to 0.16 (Vizhinjam) giving 0.13 heterozygosity for the species. The level of heterozygote deficiency measured by F_{IS} analyses showed significant values at EST-2 between Tuticorin and Vizhinjam and at GPI between Sikka and Vizhinjam (Table 11).
7. The estimated allele frequencies were closely similar at all the loci in all the three populations except at PROT-3 locus. Its allele 86 was totally absent in the Sikka population whereas it had a frequency of 0.19 in Tuticorin and 0.43 in Vizhinjam. In the case of PGM locus, its rare allele 86 present in Sikka was not detected in Vizhinjam. Similarly the rare MDH – 68 was present only in the Sikka population.
8. The Chi-square heterogeneity analysis of the three populations showed significant values at PROT-3 locus where the highest heterogeneity occurred between Sikka and Vizhinjam (Table 9). The F_{ST} heterogeneity test values were not significant among three populations except at PROT-3 locus, which revealed highly significant differences between Sikka and Vizhinjam populations (Table 10).
9. Nei ' s genetic identity test carried out for the three populations, produced values ranging from 0.981 to 0.992 indicating the three wild populations are genetically identical (Table 12).
10. Genetic structure of the hatchery populations composed of adults,

juveniles and the spats was also analysed and studied separately. The hatchery adult populations also showed polymorphism at all the loci which were polymorphic in the wild populations and additionally, the loci SOD-1, SOD-2 that were monomorphic in the wild populations were polymorphic in the hatchery produced adults. The genetic variability in the hatchery adults was as high as in the wild populations. The observed-expected genetic frequencies deviated significantly at six- (EST-1, EST-2, MDH-2, PGM, SOD-1 and PROT-3) out of nine polymorphic loci examined. The mean heterozygosity 0.18 was higher than that of the wild population's (0.13). However, allelic frequencies at almost all loci were closely similar to that of wild populations. SOD-1 and SOD-2, which were monomorphic in the wild populations, were polymorphic in the hatchery adults at low frequencies. The Chi-square heterogeneity and F_{ST} tests were not significant at any loci except at ESD-2 (Table 9, 10). Heterozygote deficiency was noticed at EST-1 and EST-2 loci. The genetic identity test between the hatchery adults and wild populations was not significant, the mean value being 0.983.

11. All the loci examined for ontogenic variations, except MDH-1 and SOD-1, were equally expressed in juveniles and spats. The loci MDH-1 and SOD-1 were not expressed in adults, juveniles and spats. Though all the five protein loci expressed their alleles in the juveniles, protein phenotypes expressed in the spats were inconsistent and could not be scored as genotypes (Fig. 22, 23). The Chi-square and F_{ST} heterogeneity tests revealed significant differences between adults and juveniles/spats at

certain loci like EST-1, ESD-2, MDH-2, PGM and PROT-3. F_{IS} test for heterozygote deficiency also produced significant values at certain loci, like, EST-1, EST-2 and GPI between adults/juveniles and at EST-1 and EST-2 between adults/spats. The F_{IS} values were significant between juveniles and spats indicating heterozygote deficiency at EST-2 while an excess at MDH-2 loci. Excess of hetrozygotes also occurred at PROT-3 locus. These significant heterogeneities are indications of ontogenic variations in the species.

12. The shell morphology of the Sikka population appeared to be different from that of the Vizhinjam and Tuticorin populations.
13. A comparison of the available and comparable genetic data reported on the Japanese pearl oyster *P. fucata* with that of the Indian pearl oyster collected in the present investigation suggests that the Indian and Japanese pearl oyster species, *P. fucata*, may be geographically two distinct genetic groups.
14. In the light of the present investigation, suggestions for future research on the Indian pearl oyster *P. fucata* have also been listed in the thesis.

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