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ORIGINAL ARTICLE

ALLELE FREQUENCY DISTRIBUTIONS ON POLYMORPHIC ESTERASE LOCI IN EXPERIENTAL POPULATIONS OF THREE *Macrobrachium* SPECIES

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ABSTRACT: Allele frequency distribution in three *Macrobrachium* species (*M. lamarrei,* n=100; *M. rosenbergii,* n=100 and *M. malcolmsonii,* n=150) was counted on different esterase isozyme loci on 7.5% Polyacrylamide Gel Electrophoresis (PAGE) stained with α- and β- naphthyl acetates from the eye tissues. Altogether three (Est-$1^{1.00\pm0.02}$, Est-$2^{0.60\pm0.06}$ and Est-$5^{0.10\pm0.01}$), five (Est-$1^{0.99\pm0.03}$, Est-$2^{0.59\pm0.04}$, Est-$3^{0.46\pm0.02}$, Est-$4^{0.33\pm0.03}$ and Est-$5^{0.10\pm0.02}$) and four (Est-$1^{0.99\pm0.03}$, Est-$2^{0.66\pm0.09}$, Est-$4^{0.27\pm0.03}$ and Est-$5^{0.11\pm0.03}$) esterase bands were detected in the above three species in order. Est-5 band of *M. lamarrei* (94%) and that of *M. rosenbergii* (99%) and Est-4 band of *M. malcolmsonii* (89%) was found to be prominent in the sampled populations. Individuals, containing different number of bands, also varied within each species and each band showed a remarkable extent of polymorphism. Calculated data indicated that the population of *M. lamarrei* (χ^2 = 31.12) and *M. rosenbergii* (χ^2 = 24.63) was out of Hardy-Weinberg equilibrium (HWE) whereas *M. malcolmsonii* (χ^2 = 7.13) was within equilibrium.
KEY WORDS: Allele frequency, Esterase, Polymorphism, *Macrobrachium* species

INTRODUCTION

Allele frequency is a measure of the relative frequency of an allele on a genetic locus in a population which shows the genetic diversity of a species or equivalently the richness of its gene pool. It also studies the different "forces" that might lead to changes in the distribution and frequencies of alleles - in other words, to evolution. In the absence of migration, mutation, natural selection, and assortative mating, genotype frequencies at any locus are a simple function of allele frequencies (Wigginton *et al.,* 2005). The original description of Hardy-Weinberg equilibrium (HWE) is an important landmark in the history of population genetics (Crow, 1988) and it is now common practice to check whether observed genotypes conform to HW expectations or not. These expectations appear to hold for most populations and deviations from HWE at particular markers may suggest problems with genotyping or population structure (Wigginton *et al.,* 2005). Populations can be scored with respect to loci scored with molecular probes. The use of allozyme variation has a long tradition in population genetics and more recent application in conservation biology (Gitzendanner and Soltis, 2000). At present, prawn grounds are getting contaminated with the increased use of agricultural pesticides and esterases are the enzymes that play an intermediary role in conferring or in contributing to insecticide resistance. This enzyme occurs in numerous isoforms expressed by distinct gene loci and each band can be considered to be an allele of a locus (Stordeur, 1976). Nonspecific esterases are usual markers in genetic studies of animals, plants and microorganisms because they are easy to detect and appear to be highly polymorphic. Hence, a simple trial was attempted to analyze the polymorphism on esterase loci of the experimental prawns that species play an important role in the national economy of Bangladesh.

MATERIALS AND METHODS

Experimental prawns were collected from three different places; *M. lamarrei* from Sirajgonj ($24^0$27´N, $89^0$44´E), *M. rosenbergii* from Khulna ($22^0$44´N, $89^0$33´E) and *M. malcolmsonii* from Kaptai Lake ($22^0$40´N, $92^0$10´E).The specimens were transported to the laboratory with ice cool pack and were identified according to classification proposed by Holthuis (1980), these were dissected to collect measured amount (~0.016 g) of the selected tissues in 'Genetics and Molecular Biology Laboratory', University of Dhaka during June to December, 2010. Each sample was separately squashed in TBE buffer (50µl), centrifuged at 12500 rpm for 15 min and 10µl aliquot from each sample was subjected to total protein

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estimation (Lowry *et al.*, 1951) and after equivalence of total protein of all the aliquots, 20µl of each sample was loaded on the gel slots for electrophoresis (Rashid *et al.*, 2012a). The entire technique for polyacrylamide gel electrophoresis (PAGE) was followed by Shahjahan *et al.*, (2008) and the electrophoretic bands of esterase resulting from stained gel with napthyl acetates were assigned to increasing numbers based on decreasing mobility following Richardson (1986). The possible epigenetic bands were prevented by the presence of EDTA in the gel buffer (Callaghan *et al.*, 1994). The reproducibility of the experiments was standardized and representative gels were subjected to analysis. Allele frequency was calculated using a reiterative technique developed by Cappellini *et al.* (1955) and slightly modified by Townson (1972). It was assumed that the F and S forms of the enzyme were controlled by two alleles Est-1^{f} and Est-1^{s} (abbreviated to f and s) and that a third allele Est-1^{x} (abbreviated to x), occurs at the same locus. Thus, theoretically, there could be six possible genotypic combinations: f/s and f/x producing the F_, s/s and s/x producing the S_, f/s producing the M_, and x/x producing the null_ phenotypes. Assuming equilibrium in the natural population and assuming that selection takes place at each locus, the gene frequencies were calculated from the phenotype frequencies (Falconer, 1960).

RESULTS AND DISCUSSION

Allozyme variation of nonspecific esterases was performed to analyze the population structure of *M. lamarrei* (n= 100), *M. rosenbergii* (n= 100) and *M. malcolmsonii* (n= 150). Altogether three (Est-1, Est-2 and Est-5), five (Est-1, Est-2, Est-3, Est-4 and Est-5) and four (Est-1, Est-2, Est-4 and Est-5) distinct esterase bands were detected in the population of *M. lamarrei, M. rosenbergii* and *M. malcolmsonii* in order (Figure 1-3, Table 1) that could be used to identify the prawn species (Shengming et al., 1988). Est-3 was not found in *M. lamarrei* and *M. malcolmsonii*. Similarly Est-4 could not be detected in *M. malcolmsonii* (Table 1). Number of esterase bands may vary from species to species. As for example, eight, seven, six and five esterase bands were found in *Oreochromis aureus* (Hongtudo *et al.*, 1993), in *Megalobrana mblycephala* (Sifa *et al.*, 1993), in *Ictalurus punctatus* (Knowles *et al.*, 1968) and in *Oreochromis niloticus* (Shahjahan *et al.*, 2008) respectively.

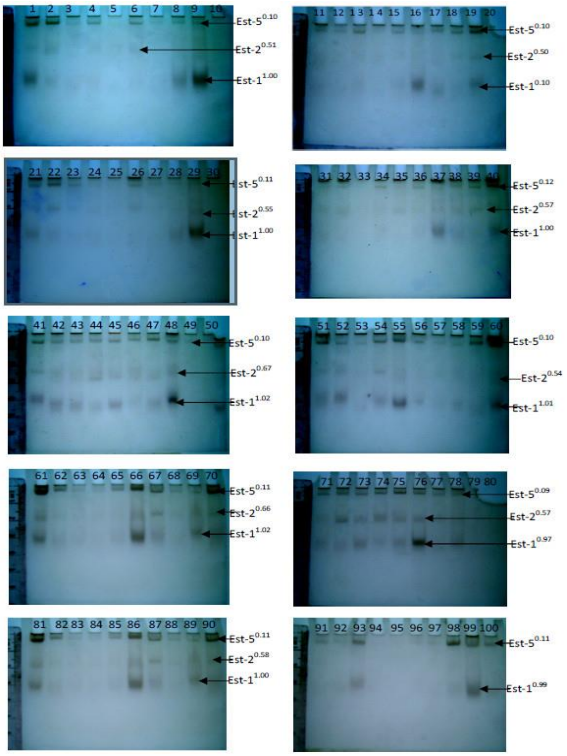

Figure 1: Esterase isozyme banding pattern in the eye tissues of M. lamarrei (sample 1-100) stained with both α and β naphthyl acetates on 7.5% PAGE where numerical number indicates the sample number, Est- represents the matched esterase isozyme and superscript delineates the relative mobility of corresponding band.

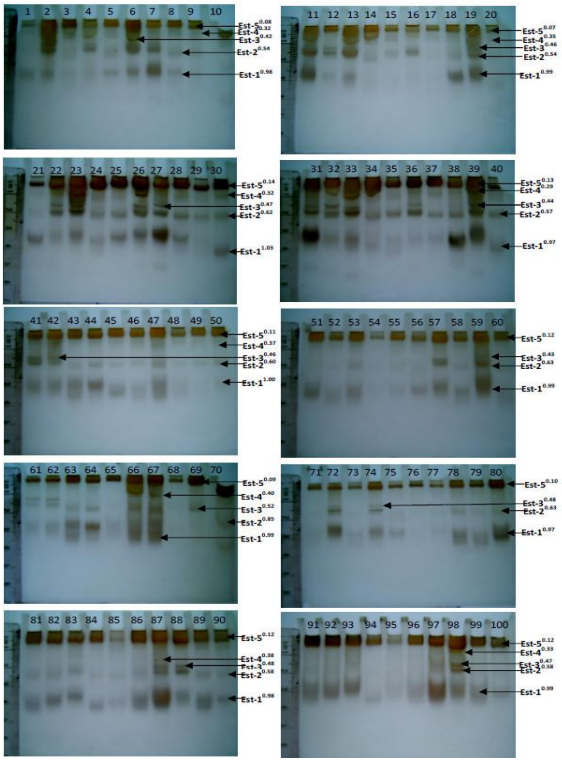

Figure 2: Esterase isozyme banding pattern in the eye tissues of M. rosenbergii (sample 1-100)

stained with both α and β naphthyl acetates on 7.5% PAGE where numerical number indicates the sample number, Est- represents the matched esterase isozyme and superscript delineates the relative mobility of corresponding band.

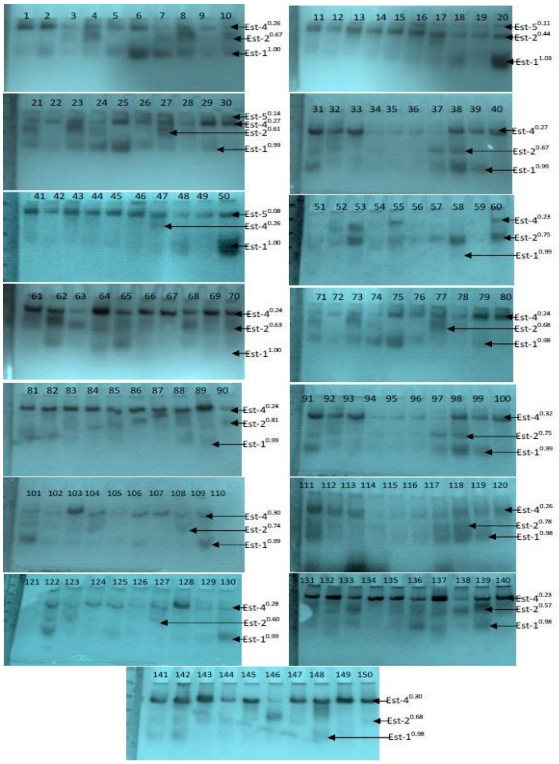

Figure 3: Esterase isozyme banding pattern in the eye tissues of M. malcolmsonii (sample 1-150) stained with both α and β naphthyl acetates on 7.5% PAGE where numerical number indicates the sample number, Est- represents the matched esterase isozyme and superscript delineates the relative mobility of corresponding band.

Nonspecific esterase isozymes after electrophoresis were also used to identify different species of *Anabas* (Ramaseshaiah and Dutt, 1984), *Pangasius* (Amin *et al.*, 2005), *Notopterus* (Begum *et al.*, 2012a), *Clarias* (Begum *et al.*, 2012b) and *Bactrocera* (Rashid *et al.*, 2012b) based on relative mobility and presence or absence of certain band. Thus, isozyme patterns showed pronounced differentiation in many organisms and have been decisive in determining the taxonomic and population status (Ferguson *et al.*, 1995).

Maximum sampled individuals of *M. lamarrei* and *M. malcolmsonii* contained three esterase bands (60% and 53% respectively) whereas *M. rosenbergii* possessed four bands (40%) (Table 1). No esterase band could be detected in some individuals from each species (1% in *M. rosenbergii* & *M. malcolmsonii* and 6% in *M. lamarrei*) which might be due to either sub-standard sample quality or the presence of null allele at these loci and were ignored during analysis (Raymond *et al.*, 1996). Two bands containing individuals were also abundant in the sampled population of *M. lamarrei* (30%) and *M. malcolmsonii* (32%) whereas *M. rosenbergii* showed only 6%. Comparatively higher number of esterase bands was found in *M. rosenbergii* (Table 1). Variation in the number of bands was also observed in *Culex pipiens* where 0-, 1-, 2- and 3-banded individuals were found to be frequent (Raymond *et al.*, 1996).

Table 1: Allele frequency distribution of esterase isozymes* in the eye tissues of three Macrobrachium species

Species↓	Frequency (%) and total number of phenotypes (N = F + FS + S + Null)													Individuals containing different number of isozymic bands (%)					
Alleles→	Est-1		Est-2		Est-3		Est-4		Est-5		Average		Corel						
	%	N	%	N	%	N	%	N	%	N	%	N		0b	1b	2b	3b	4b	5b
M. lamarrei	82	9	67	15	0	0	0	0	94	9	81.0	10.33	-0.064	6	4	30	60	0	0
M. rosenbergii	89	11	88	12	36	11	46	13	99	11	71.6	11.60	-0.341	1	2	6	36	40	15
M. malcolmsonii	75	11	65	21	0	0	89	15	17	6	82.0	13.25	+0.618	1	11	32	53	3	0

* Scored from α_ and β_ naphthyl acetates stained gels.
Est-1, Est-2, Est-3, Est-4 and Est-5 represent the corresponding phenotype of each locus.
0b, 1b, 2b, 3b, 4b and 5b stand for corresponding number of bands.
F, FS and S represent the fast, medium and slow moving bands of the same locus.

Differences in the number of bands within a population may have underlying mechanisms regulating the esterase-related processes (Lima-Catelani *et al.*, 2004). Moreover, these differences in the expression of esterase isozymes could be due to presence of selective forces in the natural habitat. Variation in the number of esterase bands within population might be due to variability of the age and size of the individuals (Holmes and Whitt, 1970), as all the selected prawns were not equal in age and size. Age specific esterase isozyme variation was also observed in several insects and fish species viz. *Bactrocera dorsalis* and *B. tau* (Rashid *et al.*, 2012a), *B. cucurbitae* (Rashid *et al.*, 2012b), *Gambusia affinis* (Rashid, 2012), *Poecilia reticulate* (Ahmed *et al.*, 2011) and *Heteropneustes fossilis* (Begum *et al.*, 2011)

where it was shown that expression of isozyme pattern may or may not vary with developmental stages. However, variation might be resulted from the biological need of specific individual that was hard to explain from the current experimentation. As in the experiments with different specimens of *Hypophthalmicthyes molitrix* from the same locality exhibited different pattern of expression and regulation of esterase isozyme which indicated that genes encoding certain isozymes were active but exhibited different patterns of expression (Rashid and Habib, 2012; Lizhao *et al.*, 1993).

Est-1 and Est-2 were abundant in the sampled population of all three species of *Macrobrachium* (Table 1). Est-3 was not detected in *M. lamarrei* and *M. malcolmsonii,* but detected in nearly one third (36%) samples of *M. rosenbergii.* Est-4 band was mostly found in the population of *M. malcolmsonii* (89%), and in about half of the population of *M. rosenbergii* (46%) but not in *M. lamarrei* (Table 1). Est-5 band was prominent in the population of *M. lamarrei* (94%) and *M. rosenbergii* (99%) whereas *M. malcolmsonii* showed only 17%. It was difficult to explain, why certain bands were profoundly expressed in the sampled population, but their presence conformed the essentiality of those particular alleles to be expressed. However, similar trends was also observed in other species viz. Est-2 in Chironomids (Rashid and Rozy, 2013), Est-3 in *B. cucurbitae* (Rashid *et al.*, 2012b), Est-4 in *H. molitrix* (Rashid and Habib, 2012), Est-5 in *B. dorsalis* (Rashid *et al.*, 2012a) and Est-6 in *B. tau* (Rashid *et al.*, 2012a). The finding of such a complex distribution of allozymes would seem difficult to explain on any basis other than selection (Strickberger, 1996). One of the examples of such condition was Milkman's (1973) finding that, in *E. coli* clones for each of five different enzymes, one particular electrophoretic band was frequent in almost all samples.

PAGE enabled to determine the degree to which individual gene was polymorphic in the natural population (Raven and Johnson, 1986). All the studied isozyme loci found in the natural populations of prawns were polymorphic. However, there was no information on the extent to which components of rich polymorphisms were stable. Adults were capable of genetically effective dispersal; environmental heterogeneity within each locality was thought to be the prime factor maintaining the polymorphism (Burns and Johnson, 1971).

Maximum 15, 12 and 21 polymorphs were found in the Est-2 locus of *M. lamarrei, M. rosenbergii* and *M. malcolmsonii* respectively based on their relative nobilities. On the other hand, 9, 11 and 6 polymorphs were identified in the Est-5 locus of above mentioned species in order. Although 11 polymorphs were detected in the Est-3 locus of *M. rosenbergii* but it was not detected in the sampled population of *M. lamarrei* and *M. malcolmsonii.* Unlike *M. lamarrei* (regarding Est-4 non-detection), *M. rosenbergii* and *M. malcolmsonii* showed 13 and 15 polymorphs of the Est-4 band. Moreover, polymorphs of *M. rosenbergii* and *M. malcolmsonii* at the Est-1 locus were same (11 polymorphs) where as it was less (9) in case of *M. lamarrei.* This higher extent of polymorphism might serve the advantages to be adapted with the differential environmental conditions. Most allozymic variants have a minimal effect on fitness and were in population because of a combination of mutation, finite population size and migration (Hedrick *et al.*, 1976). We think the rich polymorphism reflects heterozygous advantage and an adaptation to undetected environmental heterogeneity. Stordeur (1976) found nine alleles of an esterase locus in a southern France population of *Culex pipiens*. Polymorphism of esterase isozyme was also studied in rainbow trout (Kingsbury and Masters, 1972), in shrimp (Harris *et al.*, 1990) and in Russian sturgeon (Kuz'min, 2002) where remarkable polymorphism in certain locus was found.

Polymorphs of the Est-2 locus found in *M. lamarrei* and *M. malcolmsonii* overlapped with the polymorphs of Est-3 locus of *M. rosenbergii* when the relative mobility of each phenotype was considered (Figure 4) which was probably coincidental due to the large number of electromorphs (Raymond *et al.*, 1996). It could be assumed that there might be present another independent locus, but it was difficult to firmly establish since four-banded (*M. lamarrei*) and five-banded (*M. malcolmsonii*) individuals in the sampled populations were not detected. Rather, it was better to think that the molecular weights of these polymorphs covered a wide range of variation, probably due to biological need of the species. However, polymorphism was concentrated on the average molecular weights in most of the locus. Byrne and Devonshire (1993) found certain polymorphism of esterases predominated in susceptible and resistant populations of the Tobacco Whitefly from the southern United States, Middle East, Central America and Northern Europe.

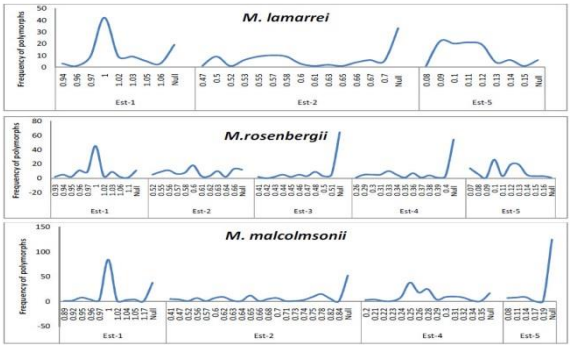

Figure 4: Frequencies of the polymorphic forms of esterase isozymes in the eye tissues of three Macrobrachium species (M. lamarrei, M. rosenbergii and M. malcolmsonii) scored from both α and β naphthyl acetates on 7.5% PAGE.

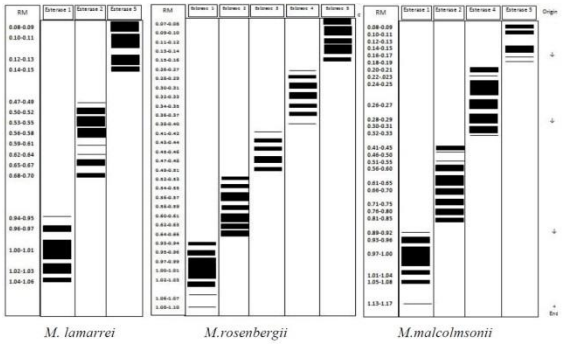

Figure 5: Zymograms prepared from the allele frequency distribution of polymorphic esterase loci in three Macrobrachium species.

Null alleles at some locus were frequent, the cause of which was not clearly understood. Similar results were also observed in the insecticide susceptible populations of *Culex pipiens* (Raymond *et al.*, 1996), Greek population of *Dacus oleae* (Krimbas and Tsakas, 1971) and *Colias eurytheme* (Burns and Johnson, 1967).

Relationship between frequency distribution and number of polymorphs was found to be insignificant and negatively correlated in *M. lamarrei* (r = -0.064) and *M. rosenbergii* (r = -0.341), but positive and significant in *M. malcolmsonii* (r = 0.618). However, considering frequency distribution at each locus of all three species, the correlation was found to be highly positive (r = 0.71).

Polymorphism of most frequent band of each species (Est-5 locus of *M. lamarrei* and of *M. rosenbergii* and Est-4 locus of *M. malcolmsonii*) was tested for the Hardy-Weinberg equilibrium. Other bands were assumed to be controlled by other independent loci and were not considered for this purpose. It was found that the population of *M. lamarrei* and *M. rosenbergii* were out of Hardy-Weinberg equilibrium (Table 2). Natural populations with whole genotypes in Hardy-Weinberg equilibrium were rarely found; one or more of the assumptions must be violated in most situations. If nothing else, most populations were under the influence of natural selection. Certainly no population could be infinite, but many populations were not even large enough to be functionally infinite. Oftentimes, populations were not completely isolated from one another, and migration of individuals into or out of one population could change its genetic makeup. Mutations could potentially alter the gene pool significantly, although the majorities were thought to have little or no effect (neutral mutations). Finally, individuals often mate selectively rather than randomly. Deviations from Hardy-Weinberg equilibrium (HWE) indicated inbreeding, population stratification, and even problems in genotyping (Wigginton *et al.*, 2005). Selection might also be occurred due to differences in survival during the winter which were believed to contribute to the polymorphisms, although other factors were not excluded (Semeonoff and Robertson, 1968).

Table 2: Observed and expected frequencies of the esterase bands* in natural populations** of three Macrobrachium species.

Species	Fob (f/f & f/x)	Fcal (p2 + 2pr)	FSob (f/s)	FScal (r2)	Sob (s/s & s/x)	Scal (q2 + 2qr)	Nullob (x/x)	Nullcal (2pq)	***X2	HWE
M. lamarrei	13.00	25.90	57.00	35.70	24.00	36.00	6.00	2.20	31.12	Rejected
M. rosenbergii	11.00	22.08	67.00	45.99	21.00	31.79	1.00	0.13	24.63	Rejected
M. malcolmsonii	19.33	25.91	32.67	22.29	36.67	22.29	11.30	10.37	7.13	Accepted

* Scored from α_ and β_ naphthyl acetates stained gels (Est-5 locus of M. lamarrei and M. rosenbergii and Est-4 locus of M. malcolmsonii).
**On the basis of an equilibrium of the allele f, s and x.
***at 5% level of significance with 3 degree of freedom (Xtab =7.815).
p, q and r stands for the frequency of the allele f, s and x in order.
ob and cal represents observed and calculated frequencies respectively.

Frequency analysis of the Est-4 phenotypes in *M. malcolmsonii* provided negative evidence of selection by showing Hardy-Weinberg equilibrium (Table 2). However negative evidence did not disprove selection of any kind, since it was impossible to test experimentally all possible environmental variables (Krimbas and Tsakas, 1971). Sugama *et al.* (2002) studied

genetic variation and population structure of the giant tiger prawn in Indonesia and found six polymorphic loci in Hardy–Weinberg equilibrium at all localities.

Samples were collected from Kaptai Lake of Rangamati where there was no direct migration channel to salt water and the population was generally consistent with the region that could be one of the causes of Hardy-Weinberg equilibrium (Sugama *et al.,* 2002). There are evidences of selection for esterase alleles in other species, although no general pattern emerges for how selection operates (Raymond *et al.,* 1996). The genotypes of individuals and frequencies of alleles in small populations, geographic patterns of variation were characterized by uniformity of allelic frequency in major physiographic or climatic regions, as would be expected if selection was determining the frequencies (Robert, 1970). Osakabe and Sakagami (1993) stated that the selection by pesticides influenced the allele frequency of α-*Estl* in Japanese populations of *Panonychus citri*. That might be a cause of selection as various pesticides used in agricultural field were washed out into this lake.

Allele frequency test in natural population depend critically on the accurate enumeration of alleles. However, present data indicated that the assumption of one locus controlling the three allele f, s and x might be true, but insufficient to be firmly established.

Presence of more than two morphs where all the bands had more or less similar relative mobility as one of the bands detected in the other individuals, suggested that they were not artifactual (Raymond *et al.,* 1996). Polymorphism could be explained as a result of hetrozygosity in which additional intermediate bands appeared (Riva and Robinson, 1986). However, Genetic control of these polymorphic systems was unknown, but, high enzymatic activity of these esterases, sufficient for quantitative electrophretic detection, permits utilization of these polymorphisms for phenotypic monitoring of prawn populations.

Esterase bands in some individuals were highly stained, indicating that an esterase overproduction mechanism was probably present in these populations (Raymond *et al.,* 1996). Gene duplications were not unusual and could easily arise from an unequal crossing over event that produces a recombinant product processing increased chromosome material (Strickberger, 1996).

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