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Genetic Evidence for the Presence of Distinct Fresh-water Prawn (*Macrobrachium nipponense*) Populations in a Single River System

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ABSTRACT—Genetic difference between two groups of individuals in the freshwater prawn *Macrobrachium nipponense,* which exhibit characteristically different egg sizes within a single river system, was examined electrophoretically and morphologically. Out of 16 prospective loci, *PGM* and *MPI-1* were polymorphic and allelic frequencies were significantly different between the two groups in both loci. In addition, rostral tooth count (number of spines on the dorsal margin of rostrum), which is regarded to be genetically controlled on the basis of crossing experiments, varied significantly between the two groups. These facts indicate that the two groups of individuals have genetically differentiated.

INTRODUCTION

A previous study on the freshwater prawn *Macrobrachium nipponense* (de Haan) [14] revealed that egg size remarkably varied from one local population to another, with an apparent relationship between egg size and the hydrogeographic feature of the habitats. Relatively large eggs (approximately 0.10 mm^3 per egg) were associated with freshwater lakes and rivers, small eggs (0.05 mm^3) with estuaries, and intermediate-sized eggs with brackish-water lagoons. Such populations with different-sized eggs were occasionally found even within a single water system, for instance, the Sagami River in central Japan where individuals with large and small eggs were found in the upper basin of the river and in the estuary, respectively [12]. Crossing experiments between these two groups of individuals, where the size of eggs produced by F_1 hybrids was intermediate between those of the parents, suggested that the different egg sizes among local populations of this species are controlled as a quantitative genetic trait [15]. The two groups were also different in larval physiological characteristics [13]. These facts all imply that *M. nipponense* is now differentiating into distinct local populations through modification of some life-history traits.

In the present study, the genetic difference between the two groups of *M. nipponense* in the Sagami River was further confirmed by means of both biochemical and morphological analyses. Electrophoretically different forms of enzyme with the same metabolic function (isozyme), especially at the same gene loci (allozyme), offer very useful information about population structures and systematics [e.g., 19, 23]. Chow and Fujio [4] have attempted this approach to *M. nipponense,* independently of our study without discriminating egg size, among a few local populations. Similarly, several authors [2, 6, 9, 10, 26] have made electrophoretic analyses among conspecific populations or different species in some taxonomic groups of freshwater prawns and shrimps. However, multiple conspecific populations coexisting in the same water system with different life-history traits was beyond the scope of those foregoing investigations, except the study by Chow *et al.* [5] on an inland-water prawn *Palaemon paucidens.* In the present study, in addition to the electrophoretic analysis, a morphological trait (rostral tooth count) was compared between the two groups of *M. nipponense* in the Sagami River with respect to

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its genetic bases.

MATERIALS AND METHODS

Adults were collected from the estuary (station Banyu) and the upper basin (stations Ogura and Lake Tsukui, approximately 35 and 40 km upstream, respectively, from the river mouth) of the Sagami River during the period 1985–1989. Topography of these collecting sites has been described previously in detail with a map [12].

Samples were kept frozen at below −50°C or kept alive in aquaria until electrophoresis. Approximately 0.4 g of abdominal muscle was minced and homogenized in 0.5 ml distilled water containing 25 μg NADP and centrifuged. The supernatant was absorbed into a filter paper wick and inserted into gel blocks. Horizontal starch-gel electrophoresis was run at the constant current of 4 to 5 mA/cm^2 (approximately 30 V/cm) in a cold chamber. During electrophoresis the gel was cooled with a glass-plate pan containing ice water, which was set on the gel. Usually electrophoreses were stopped when the marker of amid black 10B migrated 5 cm from the origin. The enzymes surveyed, along with their buffer systems, are summarized in Table 1. The MES/TEA buffer system (pH 7.0) was newly developed for separation of MPI by one of us (K.N.), where the gel contained 0.6 mg ATP/ml. After electrophoresis, each gel was sliced horizontally into 1 mm-thick sections and stained. The enzyme was detected according to Numachi [21], excepting ACP, HK, AO, MPI and GAPDH which were detected according to Redfield and Salini [22]. In the staining for PGM, MPI, HK, GPI and PEP, agarose solution (final concentration of 0.9%) containing reactants was overlaid on the gel. The stained starch gels were dried and preserved according to Numachi [20] for later analysis. The agarose gel was dried on a gel bond film.

The number of teeth on the dorsal margin of rostrum (excluding the top spine of the rostrum itself) was counted for the two groups of individuals collected from the estuary (Banyu) and the upper basin (Ogura) of the Sagami River. Besides the field-collected individuals, this trait was examined for F_1 individuals by reciprocal crossings between and within the two groups (hybrid and control, respectively). Those F_1 individuals were raised for about 6 months after hatching under nearly identical laboratory conditions. The procedures to obtain the hybrids and rear them have been described previously in detail [15].

RESULTS

In electrophoresis, a total of 17 genic loci were routinely assayed (Table 1). Among them, *PGM* and *MPI-1* showed polymorphic banding patterns according to the definition of polymorphism that frequency of the most common allele was no greater than 0.95 in at least one locality. *HK-1* also appeared to be polymorphic, but its banding pattern was not always definitely scorable. All other loci were judged to be monomorphic, and the proportion of polymorphic loci was 0.13 (excluding *HK-1*).

Phenotypes and interpreted genotypes in *PGM* and *MPI-1* are shown in Figure 1, where one or two bands appeared in each individual, showing monomeric protein structure in both loci. The allelic variants were alphabetically designated in the order of faster anodal migration. Relative mobility of each allele in comparison to the most common one (1.00) was: *a*, 1.84; *b*, 1.47; *c*, 1.00 in *PGM*, and *a*, 1.09; *b*, 1.04; *c*, 1.00; *d*, 0.95; *e*, 0.91 in *MPI-1*. Allelic frequency was not affected by the sex in either locus.

Table 2 shows the observed and expected genotypic frequencies and allelic frequencies of *PGM* and *MPI-1* in individuals collected from the three stations of the Sagami River. All the genotypes of *PGM* and *MPI-1* were at Hardy-Weinberg equilibrium in each station ($P>0.05$ in all, chi-square test). However, allelic frequencies was significantly different between the small-egg (station Banyu) and the large-egg (stations Ogura and Tsukui) groups of individuals for the alleles *b* and *c* in *PGM* and the allele *a* in *MPI-1* ($P<0.05$ in all of them, chi-square test). This means that the two groups of individuals, one with large-sized and the other with small-sized eggs, consist of distinct gene pools. Between the two groups of individuals laying large eggs at Ogura ad Tsukui, on the other hand, no difference in allelic frequency was

TABLE 1. The enzymes and loci surveyed, and corresponding buffer systems. Numerals suffixed to abbreviated enzyme name represent one of multiple banding zones (loci) assigned in the order of faster anodal migration. The abbreviation of enzyme name is based on Shaklee *et al.* [25]

Enzyme (E.C.Number)	Locus	Buffer
Phosphogluconate dehydrogenase (1.1.1.44)	*PGDH*	TBE*
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	*G3PDH*	ibid
Acid phosphatase (3.1.3.2)	*ACP*	ibid
Valyl-leucine peptidase (3.4.—.—.)	*PEPA*	ibid
Esterase (3.1.1.—)	*EST-3*	ibid
Hexokinase (2.7.1.1)	*HK-1*	TME**
Lactate dehydrogenase (1.1.2.7)	*LDH*	ibid
Isocitrate dehydrogenase (1.1.1.42)	*IDHP-1*	ibid
Aldehyde oxydase (1.2.3.1)	*AO*	ibid
Phosphoglucomutase (5.4.2.2)	*PGM*	CAPM***
Asparate aminotransferase (2.6.1.1)	*AAT-1*	ibid
Malate dehydrogenase (1.1.1.37)	*MDH*	ibid
Malic enzyme (1.1.1.40)	*MEP*	ibid
Superoxide dismutase (1.15.1.1)	*SOD-2*	ibid
Mannose-6-phosphate isomerase (5.3.1.8)	*MPI-1*	MES/TEA****
Glucose-6-phosphate isomerase (5.3.1.9)	*GPI-1*	ibid
Glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12)	*GAPDH*	ibid

* Tris-borate-EDTA, pH 8.7 [11]
** Tris-maleate-EDTA, pH 7.4 [8] modified by Numachi [21]
*** Citrate-N-(3-Aminopropyl) morpholine, pH 6.0 [7] modified by Numachi [21]
**** 2-(N-Morpholino)ethanesulfonic acid (MES) 21.32 g, Triethanolamine 16.96 g/1000 ml D.W. for electrode, and its 1/10 dilution for gel, pH 7.0

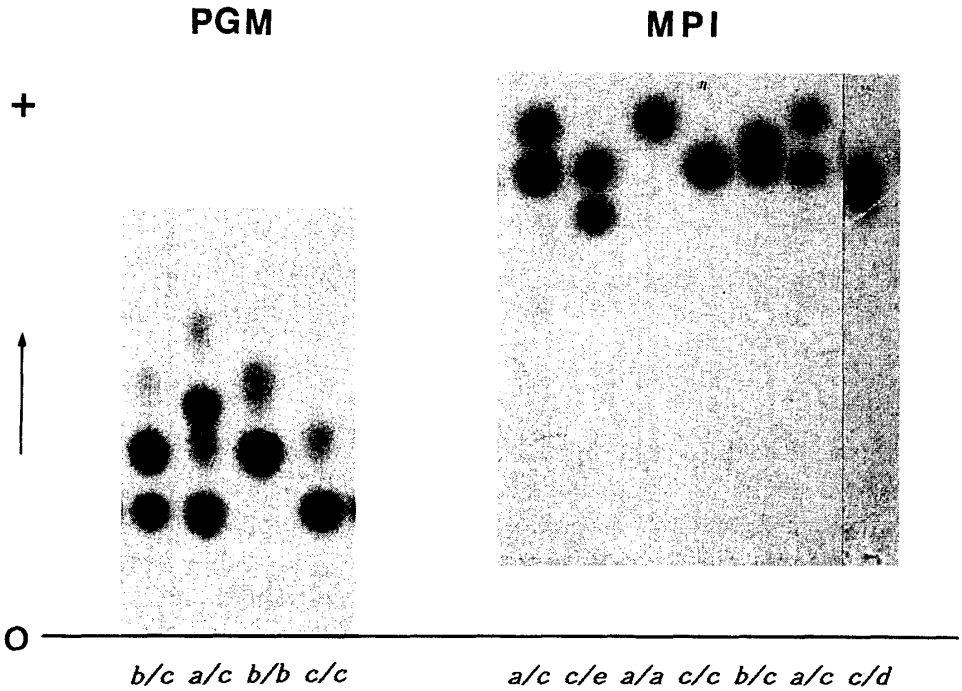

FIG. 1. Banding patterns of Phosphoglucomutase (PGM) and Mannose-6-phosphate isomerase (MPI), with interpreted genotypes for the loci of *PGM* and *MPI-1*. Gels for the reaction of MPI were usually cut off 10 mm toward the anode from the origin.

TABLE 2. Observed *vs.* expected genotypic frequencies and allelic frequencies at the loci of *PGM* and *MPI-1* for small-egg (station Banyu) and the large-egg (stations Ogura and Tsukui) populations of *M. nipponense* in the Sagami River. The expected genotypic frequency is from the Hardy-Weinberg equilibrium. The total number of specimens in each locality is indicated by the symbol n.

Locus	Station		Genotype				Allelic frequency		
			a/c	*b/b*	*b/c*	*c/c*	*a*	*b*	*c*
	Banyu	Obs.	3	19	22	13	0.03	0.53	0.45
	n=57	Exp.	1.3	15.8	26.8	11.4			
PGM	Ogura	Obs.	0	54	16	0	0.0	0.89	0.11
	n=70	Exp.	0.0	54.9	14.1	0.9			
	Tsukui	Obs.	0	32	6	1	0.0	0.90	0.10
	n=39	Exp.	0.0	31.3	7.1	0.4			

			a/c	*b/b*	*b/c*	*c/c*	*c/d*	*c/e*	*a*	*b*	*c*	*d*	*e*
	Banyu	Obs.	14	1	8	33	1	1	0.12	0.09	0.78	0.01	0.01
	n=58	Exp.	10.9	0.5	8.1	35.2	0.9	0.9					
MPI-1	Ogura	Obs.	2	0	13	55	0	5	0.01	0.09	0.87	0.0	0.03
	n=75	Exp.	1.3	0.6	11.6	56.0	0.0	4.0					
	Tsukui	Obs.	1	0	6	32	0	1	0.01	0.08	0.90	0.0	0.01
	n=40	Exp.	0.7	0.5	5.8	32.4	0.0	0.0					

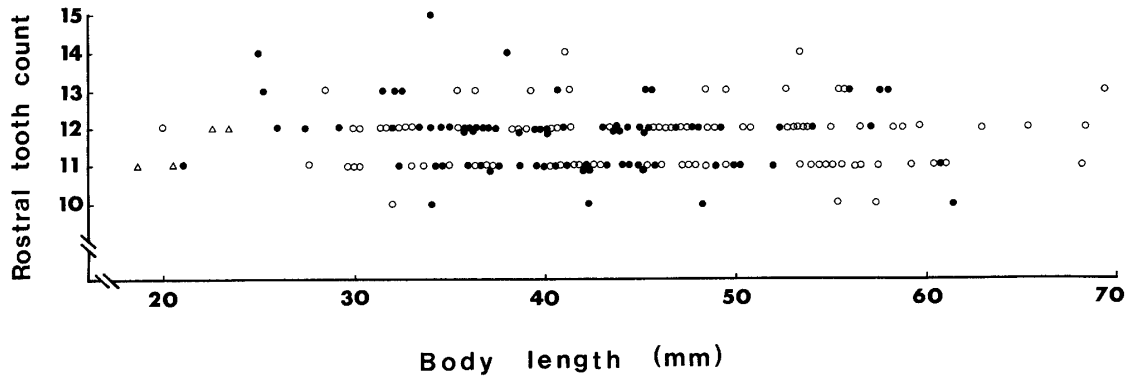

FIG. 2. Rostral tooth counts plotted against body length in individuals collected from station Ogura in the Sagami River. Solid circles, open circles and open triangles indicate males, females and sexually unidentified juveniles, respectively.

observed in both loci. Genetic identity and distance [17] between the large and the small-egg populations of *M. nipponense* in the Sagami River were 0.989 and 0.011, respectively, which were calculated from the results on 16 loci (*HK-1* excluded). The values of average heterozygosity, calculated from actual allelic frequencies, in the large-egg and the small-egg populations were 0.055 and 0.025, respectively.

Prior to analyzing the rostral tooth count, ontogenetic change of this trait was examined using specimens collected from the station of Ogura (Fig. 2). This count fluctuated from 10 to 15 among individuals larger than 20 mm in body length, but was almost independent of the body size of individuals. In the regression equation for this relationship, $Y=12.1-0.0063X$, $r=-0.08$, where X and Y mean the body length (mm) and

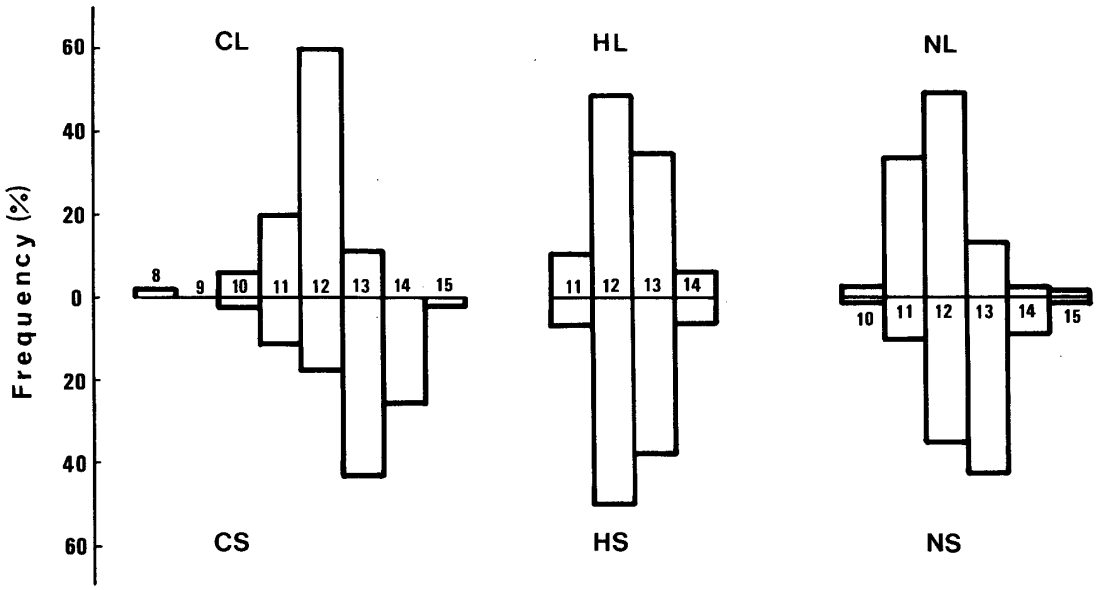

FIG. 3. Frequency distributions of the rostral tooth count in six groups of individuals, NL: natural large-egg population at station Ogura (mean 11.8±SD 0.8, n=93), NS: natural small-egg population at station Banyu (12.6 ±0.9, n=87), HL: F_1 hybrids between females of the large-egg population and males of the small-egg population (12.4±0.8, n=66, HS: F_1 hybrids between females of the small-egg population and males of the large-egg population (12.4±0.7, n=105), CL: control of F_1 individuals by crosses within the large-egg population (11.7± 0.9, n=45), and CS: control of F_1 individuals by crosses within the small-egg population (12.7±1.0, n=46). Numbers on the horizontal axes of histogram represent the ranks of the rostral tooth count.

rostral tooth count, respectively, the slope of −0.0063 did not significantly differ from zero ($P>$ 0.3, ANCOVA). Moreover, the tooth count did not significantly differ between the two sexes ($P>$ 0.5, student's t-test). Thus, comparison of the mean value of this morphological trait among populations is justified even when the composition of individual body size and sex differ among them.

Figure 3 shows the rostral tooth count in the six groups of individuals, NL: natural large-egg population at Ogura; NS, natural small-egg population at Banyu, HL: F_1 hybrids between females of the large-egg population and males of the small-egg population, HS: F_1 hybrids between females of the small-egg population and males of the large-egg population, and CL and CS: controls of F_1 individuals obtained by crosses within the large-egg and the small-egg populations, respectively. The histogram for F_1 individuals in each type of cross was drawn from the results accumulated for more than three parental females. All individuals examined here were larger than 20 mm in body length. The mean rostral tooth counts for NL and NS, 11.8 and 12.6 (12 and 13 in mode), respectively, were significantly different from each other ($P<0.001$, student's t-test). This difference persisted between CL (11.7 in mean and 12 in mode) and CS (12.7 and 13; significant difference in the mean value, $P<0.001$). On the other hand, the mean tooth count was 12.4 (12 in mode) in both hybrids (HL and HS), which was nearly, though not precisely, intermediate between the values of CL and CS, as expected from quantitative inheritance. The differences between HL and CL and between HS and CS were significant ($P<$ 0.001 and $P<0.05$, respectively). Thus the different rostral tooth counts in the two groups of field-collected individuals (NL and NS) are considered to have a genetic basis as a quantitative trait, like different egg sizes as mentioned at the beginning.

DISCUSSION

Both allozymic and morphological data indicate that the two groups of *M. nipponense* occurring in the Sagami River are genetically different, and these two groups of individuals appear to be more

or less reproductively isolated within the single river system. The reproductive isolation between them has been also suggested from another fact that, while experimentally crossed F_1 hybrids lay intermediate-sized eggs between parental populations, no female carrying such intermediate-sized eggs has been found in the field [15]. Since the two groups of individuals easily intercrossed in the laboratory, and since viability and fertility do not decline significantly in F_1 hybrids [15], any post-mating isolation factor does not seem to work effectively between them. It is likely that, while individuals of this species are apt to move downstream especially during planktonic larval stage, spatial separation of habitats at the estuary and at the upper basin of river plays an important role of the reproductive barrier between the two groups of *M. nipponense* in the Sagami River. However, the detailed mechanism of this reproductive isolation remains to be solved in the future.

It is generally recognized that in natural heterogenous environments a single species consists of genetically diversified local populations or races, fundamentally due to the depression of free gene flow among them [e.g., 1, 24]. For such local populations, Nei's genetic distance is calculated to be, very roughly speaking, no greater than 0.05 in many organisms [18]. This holds also in inland-water prawns and shrimps [e.g., 3, 6, 26]. According to this criterion, the degree of differentiation between the two groups of *M. nipponense* in the Sagami River (genetic distance 0.011) is considered to be within the level of local populations. This suggests that, though the two groups of *M. nipponense* remarkably differ in some life-historical and morphological traits, they have differentiated rather recently. It was presumed previously that the divergence of this species from the presumptive original population with small eggs to those with large and intermediate-sized eggs took place within recent, at most, several thousand years [16]. This consideration comes from the established geological recognition that most of the coastal lagoons in Japan (sea-relict lakes), where the populations with large and intermediate-sized eggs were specifically found [14], were originally formed by alluvial actions after the most recent (Jōmon-period) marine transgression of the Holocene. The electrophoretic data in the present study well coincide with the previous view.

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