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著者	SUZUKI Masae, YAMASHITA Masayori, KIJIMA Akihiro
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Estimation of Isozyme Marker Genes and Genetic Variability in Shijimi Clam, *Corbicula japonica* in Japan

*[1]Masae SUZUKI, *[2]Masayori YAMASHITA and *[2]Akihiro KIJIMA

*[1]Oyster Research Institute, Izumi, Sendai, Miyagi, 981-3217, Japan
*[2]Laboratory of Integrated Aquatic Biology, Graduate School of Agricultural Science, Tohoku University, Onagawa, Oshika, Miyagi, 986-2242, Japan

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Abstract

In order to estimate the isozyme marker genes in Shijimi clam, *Corbicula japonica*, starch gel electrophoresis was carried out about 27 enzymes for three tissues. As a result, twelve isozyme loci, *Aat-1, Aat-2, Ak-2, Fdp, Gpi, Idh-1, Mdh-1, Mdh-2, Mdh-3, 6Pgd, Sod-1* and *Sod-2* coding for eight enzymes were estimated as genetic markers. Tissue specific patterns were not observed, but foot tissue was better used for isozyme analysis based on clarity of bands.

Using the above marker genes, genetic variability was calculated as follows: the proportion of polymorphic loci ranged from 0.17 to 0.42 with a mean of 0.31, expected average heterozygosity was ranged from 0.041 to 0.103 with a mean of 0.071, and mean number of alleles per locus ranged from 1.8 to 2.0 with a mean of 1.9. The results revealed that genetic variability in *Corbicula japonica* was higher than fishes, crustacean and squids, but lower than the average of marine mollusks. These isozymes could be useful genetic markers for analyzing population structure of this species.

Introduction

The shijimi clam, *Corbicula japonica*, which has inhabited in brackish lakes and estuary around Japan, is one of the most commercially important species in inland fisheries (Nakamura 2000). Illegal ingressions of the other exotic *Corbicula* species from East Asian countries have been occurred. Ecological and genetic disturbance have been worried.

Isozymes are one of the most useful genetic markers for determination of species or subspecies in many aquatic animals. In *Corbicula* sp. of Japan, Sakai et al. (1994) and Hatsumi et al. (1995) reported the genetic difference among the three species using 12 isozyme loci. But, the genetic control of isozymes used in their reports was not necessarily the same. In fact, Hatsumi et al. (1995) estimated two loci controlling AAT and IDH, but one in Sakai et al. (1994), while Sakai et al. (1994) estimated two loci controlling SOD but one in Hatsumi et al. (1995). This means it is necessary to estimate the genetic control of isozymes before using as genetic markers.

The aims of the present study are to estimate the genetic control of isozymes and to evaluate the genetic variability in *C. japonica* using estimated isozyme marker genes.

Materials and Methods

Specimens of *Corbicula japonica* examined were collected from 3 localities, Lake Jusanko, Lake Ogawarako and Lake Shinjiko, from March to June 2003, as shown in Table 1. Samples were kept cool

Table 1. Sampling data of *Corbicula japonica* examined.

Location	Abbreviation	Date (d-m-y)	Sample No.	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)
Jusanko (Aomori)	JYU	3-Mar-03	128	19.5 ± 1.6	22.1 ± 2.0	13.7 ± 1.0	3.7 ± 0.9
Ogawarako (Aomori)	OGA	20-May-03	60	21.1 ± 3.1	22.8 ± 2.8	13.7 ± 2.0	4.5 ± 2.1
Shinjiko (Shimane)	SHI	11, 15-Jun-03	100	18.7 ± 1.4	20.2 ± 1.6	12.8 ± 0.9	3.1 ± 0.7

Shell height, Shell length, Shell width, Total weight : Mean ± S.D.

and transferred to laboratory where they were stored at -30°C until dissection.

Isozymes were detected by horizontal starch gel electrophoresis following the procedure of Fujio and Ikeda (1999). Adductor muscle (AM), digestive caecum (DC), and foot including siphon (FS), were used to analyze the tissue specific patterns. Approximately 200mg of tissue was minced in a 1.5 ml microtube with 30μl deionized water and frozen at -30°C until the electrophoretic run. After thawing the minced tissues at 4°C and centrifugation at 15,000rpm for 10min at 4°C, the tip of the filter paper (4×10mm) was dipped into the supernatant. These tips were used as electrophoretic samples.

Electrophoresis was carried out using 5mm thick 11% (W/V) starch gels under the constant voltage at 240V and 10mm thick gel at 300V. After electrophoretic run for 6 hours, the thick gel was cut into 1.5mm slices for staining. The electrode buffer systems were Tris-citrate buffer (135mM Tris, 43mM citrate, pH 7.0) for all the enzymes and citrate-aminopropylmorpholine buffer (40mM citrate, 0.96% aminopropyl -morpholine, pH 6.0) for AAT, GPI and IDH.

A total of 27 enzymes were examined, as shown in Table 2. The enzyme activity and convergence of bands were estimated by visual observation. The allele estimated from the most frequently appearing band in the sample of Lake Jusanko, was designated as allele *100* at each locus. Other alleles were named according to their relative mobility with respect to the band of allele *100*.

Chi-square tests were carried out to examine whether observed genotype number agreed with expected one under Hardy-Weinberg equilibrium (HWE). Genetic variability was estimated by the proportion of polymorphic loci (P^*), the proportion of variant loci (V^*) less than polymorphism, the average

Table 2. Enzymes surveyed (27enzymes).

Enzyme	Abbreviation	Enzyme Commission Number
Aspartate Aminotransferase	AAT	EC 2.6.1.1
Acid Phosphatase	ACP	EC 3.1.3.2
Alcohol Dehydrogenase	ADH	EC 1.1.1.1
Adenylate Kinase	AK	EC 2.7.4.3
Alkaline Phosphatase	ALP	EC 3.1.3.1
Creatine Kinase	CK	EC 2.7.3.2
Diaphorase	DIA	EC 1.6.*.*
Esterase	EST	EC 3.1.1.-
Fructose-1,6-Diphosphatase	FDP	EC 3.1.3.11
Fumarase	FH	EC 4.2.1.2
Galactose Dehydrogenase	GAD	EC 1.1.1.48
Glutamate Dehydrogenase	GDH	EC 1.4.1.-
Glucosephosphate Isomerase	GPI	EC 5.3.1.9
Glucose-6-phosphate Dehydrogenase	G6PDH	EC 1.1.1.49
Glycerol-3-phosphate Dehydrogenase	αGPD	EC 1.1.1.8
Hexokinase	HK	EC 2.7.1.1
Isocitric Dehydrogenase	IDH	EC 1.1.1.42
Laucine Aminopeptidase	LAP	EC 3.4.11.1
Lactate Dehydrogenase	LDH	EC 1.1.1.27
Malate Dehydrogenase	MDH	EC 1.1.1.37
Malic Enzyme	ME	EC 1.1.1.40
Mannose-6-phosphate Isomerase	MPI	EC 5.3.1.8
Octanol Dehydrogenase	ODH	EC 1.1.1.73
Phosphoglucomutase	PGM	EC 2.7.5.1
6-Phosphogluconate Dehydrogenase	6PGD	EC 1.1.1.44
Sorbitol Dehydrogenase	SDH	EC 1.1.1.14
Superoxide Dismutase	SOD	EC 1.15.1.1

number of alleles per locus (A/L), and observed and expected average heterozygosity (Ho and He). The observed heterozygosity was the direct count of heterozygous individuals in the sample, and the expected heterozygosity was calculated from the formula, $1-\sum\sum x_{ij}^2 / n$, where x_{ij} was the frequency of j-th allele of i-th locus and n was equal to number of examined loci. Genetic differentiation among localities was examined using the Genepop ver. 3.1 (Raymond and Rousset 1995a; 1995b; Goudet et al. 1996).

Results

Genetic control of isozymes

A total of 27 enzymes were examined in the adductor muscle (AM), digestive caecum (DC), and foot including siphon (FS). Electropherograms of the three tissue samples were shown in Fig. 1-1, 1-2, 1-3. Enzyme activity was observed in 23 enzymes out of 27. Tissue specific bands were not obtained though activity and clarity were not the same. Adequate activity was obtained in foot for the 23 enzymes. Enzyme activity and convergence of bands among the three tissue samples were shown in Table 3. As a result, converged bands were observed in 11 enzymes out of the 23. Finally, clear and stable expression bands were obtained in eight enzymes, that is, AAT, AK, FDP, GPI, IDH, MDH, 6PGD and SOD. In order to estimate the isozymes as a genetic marker, suitable electrophoretic condition was examined in the eight enzymes. Comparing the buffer system, clearer bands were observed by C-APM buffer system in AAT, GPI and IDH. In AK, FDP, MDH, 6PGD and SOD, T-C buffer system was suitable for electrophoresis.

In FDP, GPI and 6PGD, single zone was observed on the gel, indicating genetic control of single locus. In AAT, IDH and SOD, two zones always appeared on the gel, revealing two loci systems. Three active zones were observed in AK and MDH. Three loci coding for MDH were estimated, but only one locus was detected in AK because only one zone was always observed stably.

From the above results, twelve loci coding eight enzymes, namely *Aat-1, Aat-2, Ak-2, Fdp, Gpi, Idh-1, Mdh-1, Mdh-2, Mdh-3, 6Pgd, Sod-1* and *Sod-2*, were estimated (Table 4). These twelve loci were used as genetic markers for estimating genetic variability in the present study.

Typical electrophoretic patterns of allozymes of *C.*

Table 3. Tissue specific enzyme activity in the *Corbicula japonica* (n=5).

		T-C*1 pH7.0		
Enzyme	Isozyme	AM*2	DC*3	FS*4
AAT	AAT-1	++ ○	+ △	++ ○
	AAT-2	nd	nd	++ ○
ACP	(ACP-1)	+ ▲	+ ▲	+ ▲
	(ACP-2)	+ △	± ▲	+ △
ADH	—	-	-	-
AK	(AK-1)	+ △	+ △	+ △
	AK-2	++ ○	+ ○	++ ○
	(AK-3)	+ △	+ △	+ △
ALP	(ALP)	± △	± △	± △
CK	(CK)	++ △	+ △	++ △
DIA	(DIA-1)	+ △	± ▲	± ▲
	(DIA-2)	+ △	± ▲	± ▲
EST	(EST-1)	+ ▲	± ▲	± ▲
	(EST-2)	+++ ▲	+++ ▲	++ ▲
FDP	FDP	++ ○	+ △	++ ○
FH	(FH-1)	+ △	± ×	± ▲
	(FH-2)	+ △	± ×	± ×
GAD	—	-	-	-
GDH	—	-	-	-
GPI	GPI	++ ○	++ △	++ △
G6PDH	(G6PDH)	++ △	++ △	++ △
αGPD	(αGPD-1)	+ △	+ △	+ △
	(αGPD-2)	+ △	+ △	+ △
HK	(HK-1)	++ △	+ △	++ △
	(HK-2)	+ ×	± ×	+ ×
IDH	IDH-1	++ ○	++ ○	++ ○
	(IDH-2)	+ ×	+ ×	+ ×
LAP	(LAP-1)	++ ○	++ ○	++ ○
	(LAP-2)	++ ○	++ ○	++ ○
LDH	—	-	-	-
MDH	MDH-1	++ ○	++ ○	++ ○
	MDH-2	++ ○	++ ○	++ ○
	MDH-3	+ ○	+ ○	+ ○
ME	(ME-1)	+ △	+ △	+ △
	(ME-2)	++ ×	++ ×	++ ×
MPI	(MPI)	+ △	+ △	+ △
ODH	(ODH)	± ○	± ○	± ○
PGM	(PGM-1)	+ △	+ △	+ △
	(PGM-2)	+ ○	+ ○	+ ○
6PGD	6PGD	++ ○	++ ○	++ ○
SDH	(SDH)	+ ×	+ ×	+ ×
SOD	SOD-1	++ ○	++ ○	++ ○
	SOD-2	++ ○	++ ○	++ ○

* Activity	* Convergence
+++ : very high	○ : veryclear
++ : high	△ : unclear
+ : low	▲ : hard to converge
± : unstable	× : diffused
- : nothing	nd : no data

*1T-C : tris-citrate buffer
*2AM : adductor muscle
*3DC : digestive caecum
*4FS : foot (including siphon)

Table 4. Expected loci and available as genetic marker in the *Corbicula japonica* of Jusanko in Aomori prefecture.

Enzyme	Tissue	Buffer*1	pH	Expected loci	Available as genetic marker*2
AAT	Foot	C-APM	6	*Aat-1*	○
	Foot	C-APM	6	*Aat-2*	○
AK	Foot	T-C	7	*(Ak-1)*	×
	Foot	T-C	7	*Ak-2*	○
	Foot	T-C	7	*(Ak-3)*	×
FDP	Foot	T-C	7	*Fdp*	○
GPI	Foot	C-APM	6	*Gpi*	○
IDH	Foot	C-APM	6	*Idh-1*	○
	Foot	C-APM	6	*(Idh-2)*	(○)
MDH	Foot	T-C	7	*Mdh-1*	○
	Foot	T-C	7	*Mdh-2*	○
	Foot	T-C	7	*Mdh-3*	○
6PGD	Foot	T-C	7	*6Pgd*	○
SOD	Foot	T-C	7	*Sod-1*	○
	Foot	T-C	7	*Sod-2*	○
				15 loci controlling 8 enzymes	12 loci controlling 8 enzymes

*1C-APM : citrate-aminopropylmorpholine buffer

*1T-C : tris-citrate buffer

*2being able to be used as genetic marker

japonica are shown in Fig.2-1 and Fig.2-2. In AAT, one or three bands were observed both on the anodal and cathodal zones individually, estimating three alleles at *Aat-1* and two at *Aat-2*. In AK, one or two bands were observed individually, indicating a typical genetic variation of monomeric enzyme structure. Based on the banding positions, five alleles were estimated. The FDP showed only one band in all individuals, revealing monomorphic at the one locus, *Fdp*. In GPI and 6PGD, typical genetic variations of dimeric enzyme structure at one zone were observed. According to the banding positions, three alleles were estimated at *Gpi*, and four alleles at *6Pgd*. In IDH, two banding zones were observed separately on the anodal (IDH-1) and the cathodal areas (IDH-2), but IDH-2 bands were not always detected. In the IDH-1, one or three bands were observed, indicating a typical genetic variation of dimeric enzyme structure, and then three alleles were estimated at the *Idh-1*. At least three bands and maximally five bands were observed in MDH. The first and second bands from origin were always appeared but at the most anodal zone, one or two or three bands were observed individually. The third band from origin was always appeared. Therefore, three loci controlling MDH were estimated. The *Mdh-2* and *Mdh-3* loci were monomorphic while *Mdh-1* was polymorphic with three alleles. The band controlled allele *100* at *Mdh-1* appeared at the same position with that of *Mdh-2*. In SOD, two active zones were observed on the anodal area, indicating two different loci (*Sod-1* and *Sod-2*). Both two loci were monomorphic.

Genetic variability of isozymes

Allele frequencies at the 12 loci in the 3 localities of *C. japonica* are shown in Table 5. Significant deviation from HWE was not observed at all loci in all localities. Homogeneity tests for allele distribution at each locus between every pair of localities were done. Significant difference was observed in every pair of localities, indicating that an independent local population was constructed in each location.

Genetic variability of *C. japonica* was estimated based on the 12 loci, as shown in Table 6. Genetic variation was observed in 6 to 7 loci out of 12. The number of polymorphic loci, defined as the locus that had the maximum allele frequency of $\leqq 0.95$, was 4, 5 and 2, in Lake Jusanko, Lake Ogawarako and Lake Shinjiko, respectively. The proportion of polymorphic loci (P^*) and the proportion of variant loci (V^*) ranged from 0.17 to 0.42, with a mean of 0.31, and from 0.17 to 0.42 with a mean of 0.25, respectively. The

Table 5. Gene frequency at 12 loci in the 3 local lots for *Corbicula japonica*.

locus	*allele*	JYU	OGA	SHI
Aat-1	N*	90	55	98
	150	-	0.082	0.005
	100	0.950	0.918	0.990
	80	0.050	-	0.005
Aat-2	N	82	57	100
	-70	0.061	0.088	0.020
	-100	0.939	0.912	0.980
Ak-2	N	90	60	100
	120	-	-	0.005
	110	-	0.017	0.005
	100	1.000	0.950	0.970
	90	-	0.017	0.020
	80	-	0.017	-
Fdp	N	90	60	97
	100	1.000	1.000	1.000
Gpi	N	90	59	100
	100	0.900	0.856	0.925
	90	0.083	0.119	-
	80	0.017	0.025	0.075
Idh-1	N	90	44	100
	140	0.006	-	-
	100	0.894	0.875	0.995
	75	0.100	0.125	0.005
Mdh-1	N	90	60	100
	140	0.111	0.208	0.020
	110	-	-	0.005
	100	0.889	0.792	0.975
Mdh-2	N	90	60	100
	100	1.000	1.000	1.000
Mdh-3	N	90	60	100
	100	1.000	1.000	1.000
6Pgd	N	90	60	100
	140	0.011	-	0.090
	100	0.978	0.992	0.905
	90	0.006	-	-
	65	0.006	0.008	0.005
Sod-1	N	90	60	100
	100	1.000	1.000	1.000
Sod-2	N	89	60	100
	100	1.000	1.000	1.000

*N : sample size

proportion of total variant loci (P*+V*) from 0.50 to 0.58 with a mean of 0.56. Mean number of alleles per locus (A/L) was counted as 1.8 and 2.0 with a mean of 1.9. The observed average heterozygosity (Ho) and expected one (He) were distributed from 0.042 to 0.108 with a mean of 0.073, and 0.041 to 0.103 with a mean of 0.071, respectively. Ho/He values were 1.006 to 1.050 with a mean of 1.025. Genetic variability was roughly similar among localities.

Discussion

Detection of Isozymes

In *C. japonica*, Sakai et al. (1994) estimated isozyme loci used adductor muscle and mid-gut gland

(equal to digestive caecum) and Hatsumi et al. (1995) used mid-gut gland, respectively. In the present study, tissue specific pattern was not observed, and then any tissues can be used for isozyme analysis. Therefore, the present data could be compared with their reports on the view point of used tissues.

In the present study, 12 loci coding for 8 enzymes were estimated in *C. japonica*. In the same species, Sakai et al. (1994) reported 12 loci, *Aat, Acp, Cap, Gpi, Idh, Mdh-1, Mdh-2, Pgd, Pgm-1, Pgm-2, Sod-1* and *Sod-2* coding for 9 enzymes, and Hatsumi et al. (1995) reported 12 loci, *Aat-1, Aat-2, Cat, Idh-1, Idh-2, Lap, Mdh-1, Mdh-2, 6Pgd, Pgm-1, Pgm-2* and *Sod* coding for 8 enzymes, respectively. Different kinds of loci were estimated in these studies. This is probably caused by the difference of electrophoretic condition such as buffer systems, kinds of chemicals for staining, and condition of samples storage. Actually different buffer systems were used as shown in Table 7.

Table 6. Gene variability of isozymes in 3 localities for *Corbicula japonica*.

Locus	JYU	OGA	SHI
Aat-1	V(2)*	P(2)	V(3)
Aat-2	P(2)	P(2)	V(2)
Ak-2	M(1)	V(4)	V(4)
Fdp	M(1)	M(1)	M(1)
Gpi	P(3)	P(3)	P(2)
Idh-1	P(3)	P(2)	V(2)
Mdh-1	P(2)	P(2)	V(3)
Mdh-2	M(1)	M(1)	M(1)
Mdh-3	M(1)	M(1)	M(1)
6Pgd	V(4)	V(2)	P(3)
Sod-1	M(1)	M(1)	M(1)
Sod-2	M(1)	M(1)	M(1)
P*	0.33	0.42	0.17
V*	0.17	0.17	0.42
P*+V*	0.50	0.58	0.58
A/L	1.8	1.8	2.0
H_o	0.069	0.108	0.042
H_e	0.069	0.103	0.041
H_o/H_e	1.006	1.050	1.019

*( ) : No.of alleles
P : Polymorphic (maximum allele frequency $<$ 0.95)
V : Variant (maximum allele frequency $\geqq$ 0.95)
M : Monomorphic
P* : Proportion of polymorphic loci
V* : Proportion of variant loci
P*+V* : Proportion of P+V
A/L : Average Number of alleles per locus
Ho : Average heterozygosity (observed)
He : Average heterozygosity (expected)

Genetic variability

Genetic variability of *C. japonica* was reported using 12 loci by Sakai et al. (1994) and Hatsumi et al. (1995). The proportion of polymorphic loci, the average number of alleles per locus and expected average heterozygosity were reported as 0.33 to 0.42, 1.8 to 2.0 and 0.111 to 0.132 by Sakai et al. (1994) and 0.42 to 0.58, 1.7 to 2.1, 0.127 to 0.206 by Hatsumi et al. (1995), respectively. Compared with the present result, genetic variability was almost the same, suggesting that obvious change in genetic variability has not been occurred during recent decade.

Genetic variability, especially expected average heterozygosity was reported in many fishes and shellfishes, including crustacean. In fish species, average heterozygosity is reported as 0.059±0.007 (Fujio and Kato 1979), 0.043±0.028 in decapod crustacea (Chow and Fujio 1987), 0.033±0.010 in squids (Fujio and Kawada 1989) and 0.147±0.011 in marine mollusks (Fujio et al. 1983). Compared with *C. japonica* including the data from Sakai et al. (1994), Hatsumi et al. (1995) and our result (He = 0.073±0.033), average heterozygosity of *C. japonica* is higher than fishes, crustacean and squids, but lower than an average of the other marine mollusks.

In the present study, observation of genetic differentiation among localities would mean the existence of local populations. Therefore, 12 allozyme loci estimated in the present study will be useful genetic markers for analyzing population structure of the species.

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Table 7. Comparison of isozyme loci detected among the three independent papers

Enzyme	Sakai et.al (1994)		Hatsumi et.al (1995)		Present study	
	Locus	buffer* (pH)	Locus	buffer (pH)	Locus	buffer (pH)
AAT	*Aat*	T-C (8)	*Aat-1*	T-C (7)	*Aat-1*	C-APM (6)
			Aat-2	T-C (7)	*Aat-2*	C-APM (6)
ACP	*Acp*	AC (6)	nd		*(Acp)*	T-C (7)
AK	nd		nd		*Ak-2*	T-C (7)
CAT	nd		*Cat*	others (8)	nd	
CAP	*Cap*	RW (8.5)	nd		nd	
FDP	nd		nd		*Fdp*	T-C (7)
GPI	*Gpi*	T-C (8)	nd		*Gpi*	C-APM (6)
IDH	*Idh*	T-C (8)	*Idh-1*	C-APM (6.2)	*Idh-1*	C-APM (6)
			Idh-2	C-APM (6.2)	*(Idh-2)*	C-APM (6)
LAP	nd		*Lap*	T-C (7)	*(Lap-1)*	T-C (7)
					(Lap-2)	T-C (7)
MDH	*Mdh-1*	T-C (8)	*Mdh-1*	C-APM (6.2)	*Mdh-1*	T-C (7)
	Mdh-2	T-C (8)	*Mdh-2*	C-APM (6.2)	*Mdh-2*	T-C (7)
					Mdh-3	T-C (7)
PGM	*Pgm-1*	RW (8.5)	*Pgm-1*	C-APM (6.2)	*(Pgm-1)*	T-C (7)
	Pgm-2	RW (8.5)	*Pgm-2*	C-APM (6.2)	*(Pgm-2)*	T-C (7)
6PGD	*6Pgd*	T-C (8)	*6Pgd*	T-C (7)	*6Pgd*	T-C (7)
SOD	*Sod-1*	RW (8.5)	*Sod*	T-C (7)	*Sod-1*	T-C (7)
	Sod-2	RW (8.5)			*Sod-2*	T-C (7)
No. loci	12		12		12	

*T-C : tris-citrate buffer
*AC : amine citrate buffer
*RW : tris-citric acid, lithium hydroxide-boric acid buffer
*C-APM : citrate-aminopropylmorpholine buffer
*others : tris-EDTA-borate buffer
nd : no data

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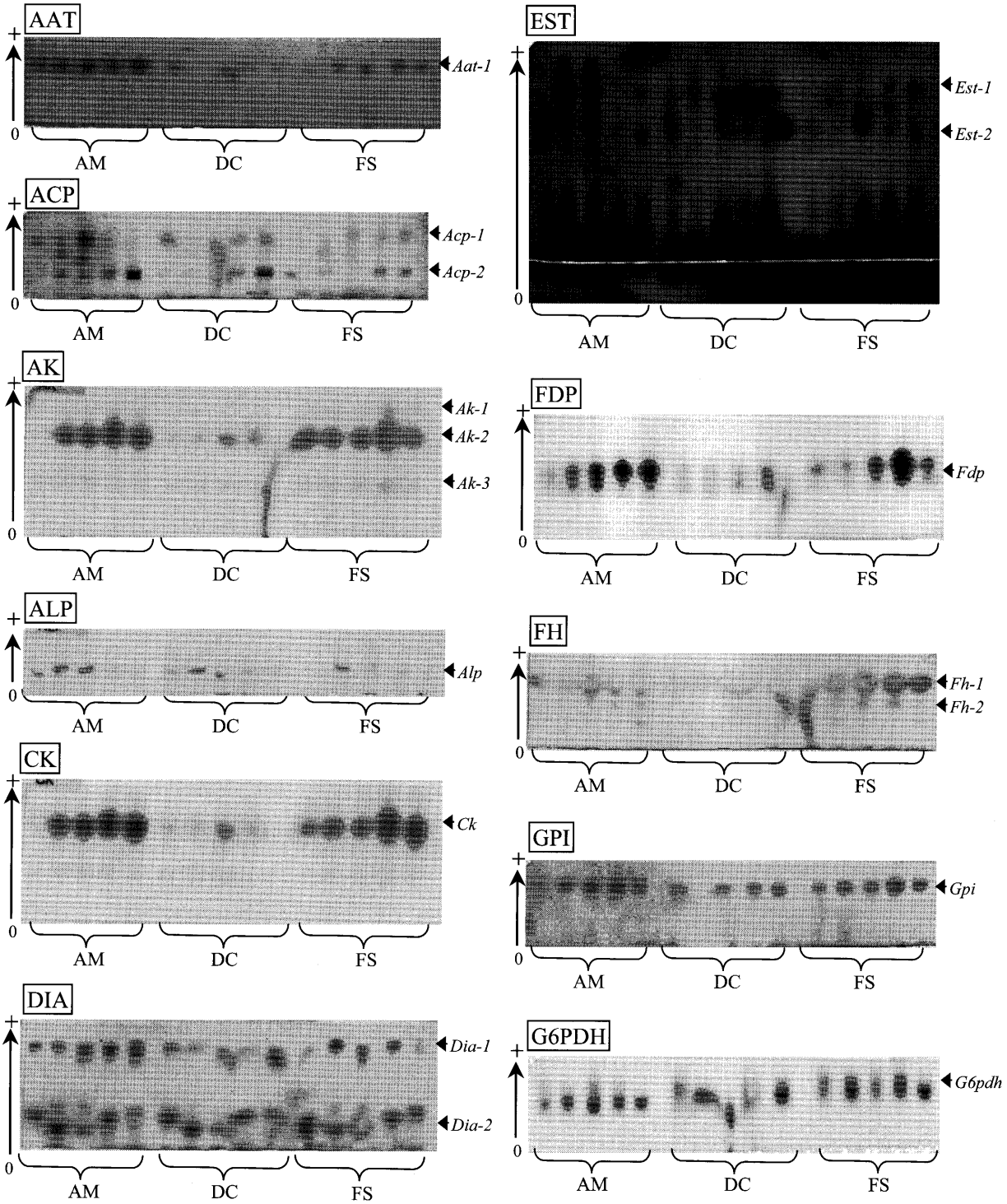

Fig. 1-1. Tissue specific isozyme patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

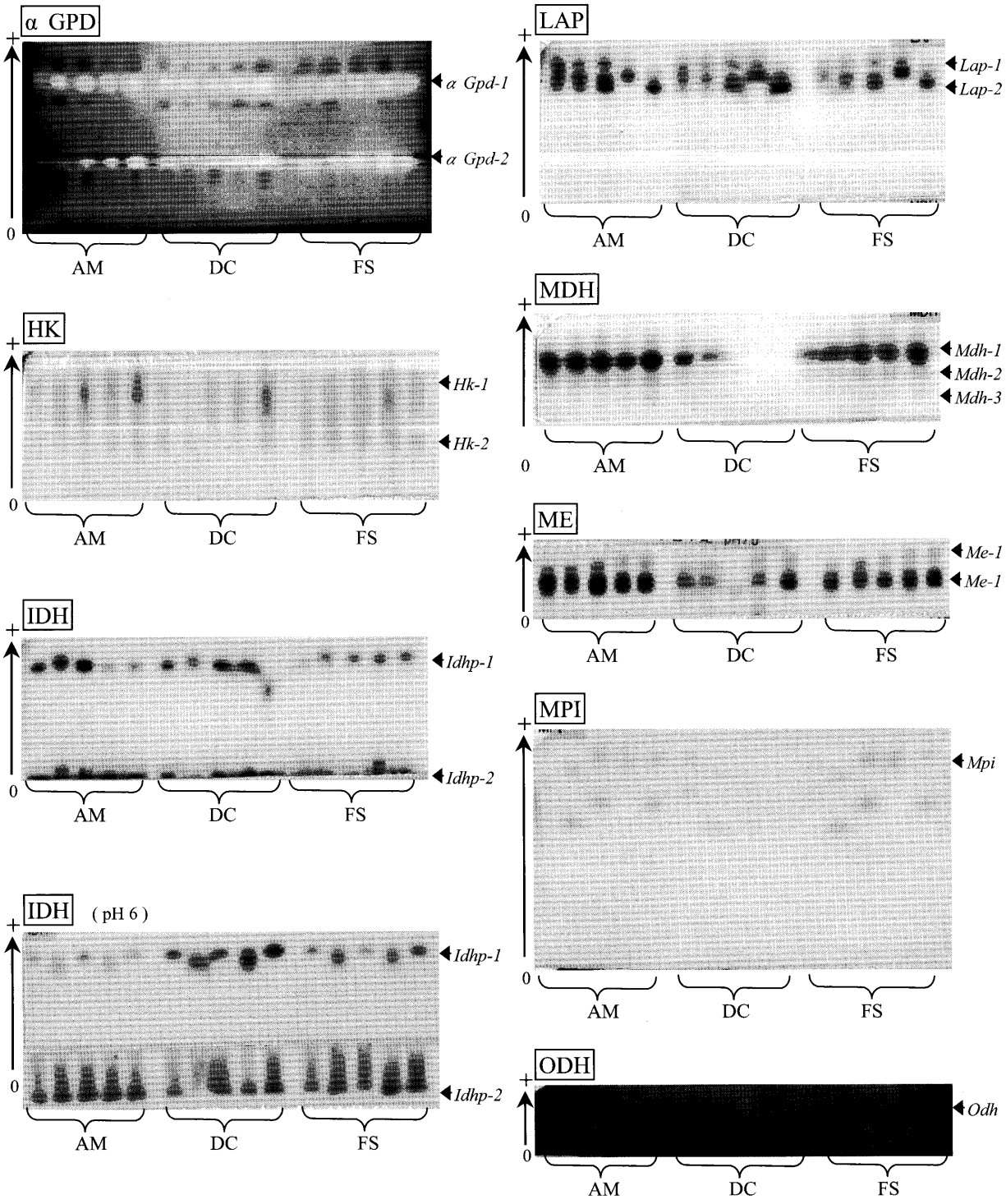

Fig. 1-2. Tissue specific isozyme Patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

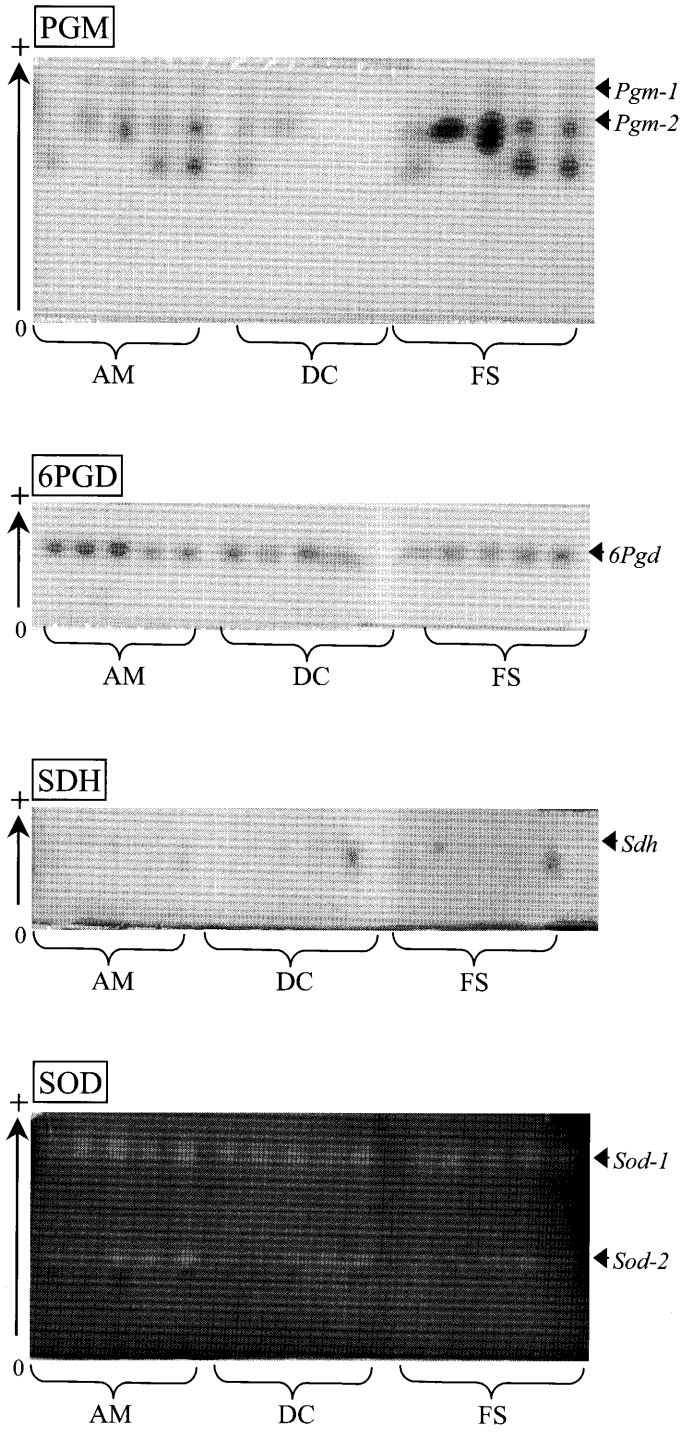

Fig. 1-3. Tissue specific isozyme Patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

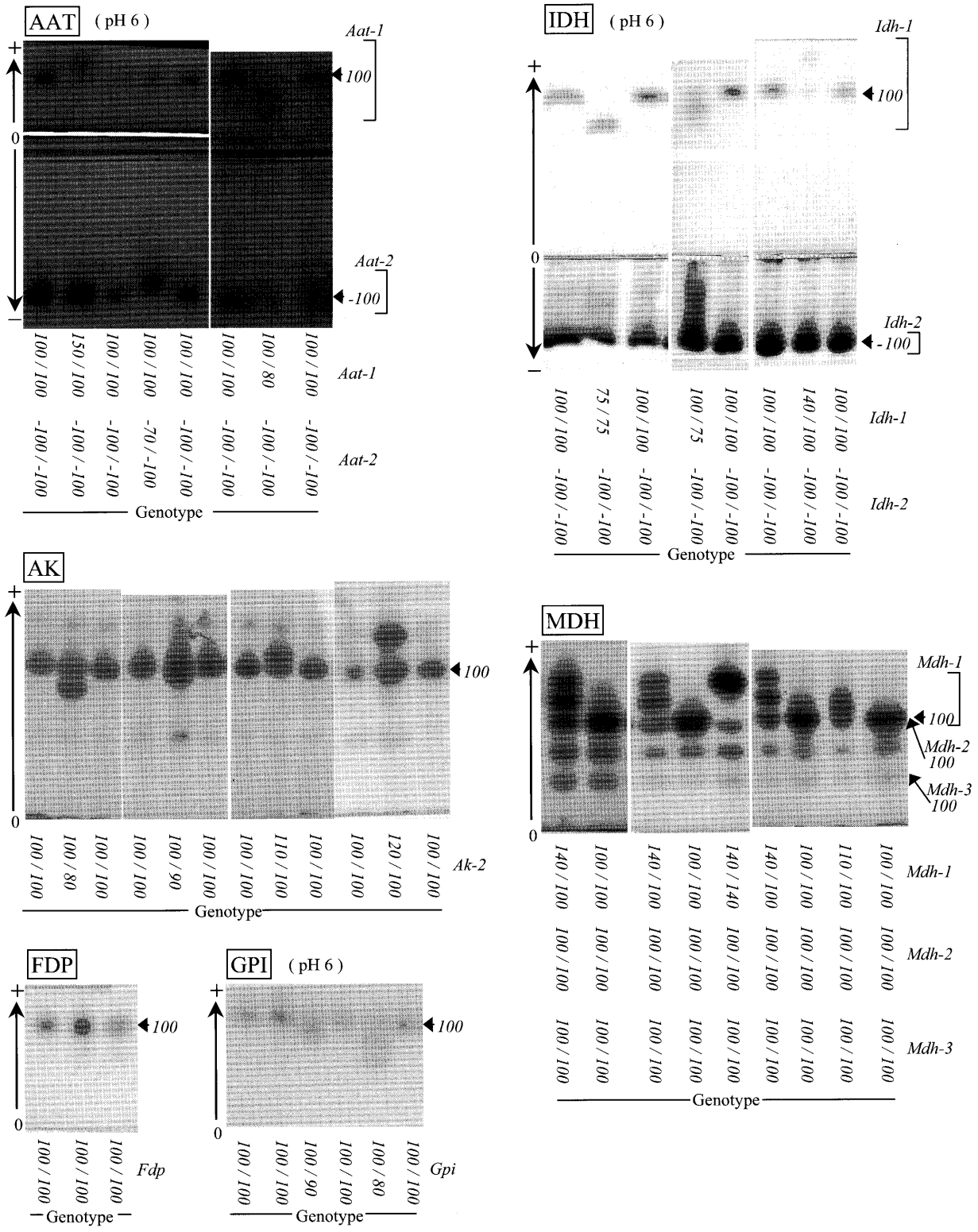

Fig.2-1. Electropherograms of 12 isozymes in *Corbicula japonica*.

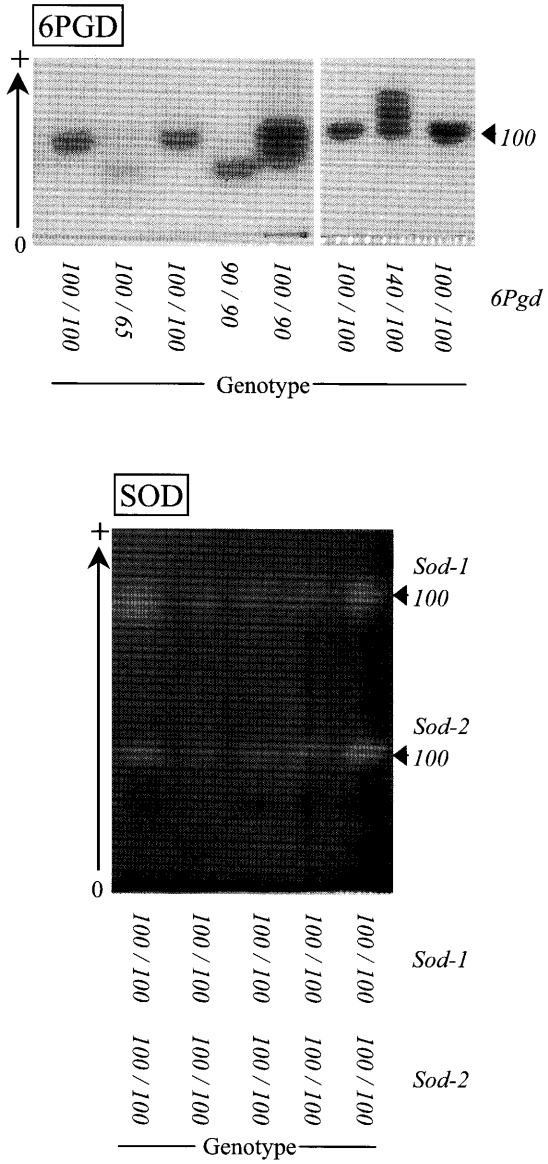

Fig.2-2. Electropherograms of 12 isozymes in *Corbicula japonica*.