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カワヒバリガイとコウロエンカワヒバリガイ（イガイ科）の著しい遺伝的差異

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Large Genetic Differentiation of the Mussels *Limnoperna fortunei fortunei* (Dunker) and *Limnoperna fortunei kikuchii* Habe (Bivalvia: Mytilidae)

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Abstract: *Limnoperna fortunei fortunei* (Dunker, 1856) and *L. fortunei kikuchii* Habe, 1981 were introduced in Japan in the 1990's and 1970's, respectively. The latter has been recognized as a subspecies on the basis of features of shell morphology. However, validity of its subspecific status has been questioned, because these two mytilids are clearly distinguished also by several other characteristics. In the present study, genetic differences were examined between these two mytilids, which were collected from two sites in the Nagara River flowing into Ise Bay along the Pacific coast of central Japan. The sample of *L. fortunei fortunei* was from an upstream site while that of *L. fortunei kikuchii* was from a downstream site. Examination of these samples for 10 enzymes using gel electrophoresis showed genetic variation to be found at 14 loci, 13 of which indicated complete allelic substitution between the two samples. No hybrid was observed in samples containing the two mytilids. Nei's genetic distance (D) was very high (2.78) between the two. The observed average heterozygosity (Ho) was 0.0048 in *L. fortunei fortunei* and 0.0783 in *L. fortunei kikuchii*, the values being significantly different between the samples. These support that their genetic difference would be in the specific level.

Keywords: Mytilidae, *Limnoperna*, Genetic differentiation, Isozyme, Electrophoresis

Introduction

Different from other mytilid genera, the genus *Limnoperna* includes both of freshwater and brackish species. This genus, with four species and one subspecies, is distributed throughout the southeast Asia, e.g., Mainland China, Taiwan, Korea, Hong Kong and Japan (Kimura, 1994b). To our knowledge, only two mytilids of this genus, i.e., *L. fortunei fortunei* (Dunker, 1856) and *L. fortunei kikuchii* Habe, 1981* are found in Japan.

* At the 1996 annual meeting in the Malacological Society of Japan, Kimura, T. and Shikano, Y. read the paper that *Limnoperna fortunei kikuchii* Habe is *Xenostrobus securis* (Lamarck). They are preparing for publishing the paper.

Limnoperna fortunei fortunei was found in the Ibi River in 1990, the occurrence first being recorded in Japan (Kimura, 1994a). Since then, the species has been introduced into the southern part of the Lake Biwa, and also into the lower reaches and tidal areas of the Kiso Rivers (Kiso, Ibi, Nagara) (Kimura, 1994a) and the Yodo River (Nakai, 1995). The species has been described as a pest in the dam and water supply system in Mainland China, Taiwan, Hong Kong and Korea since the 1960's because it causes blockage of the water supply system or reduces the water quality (Morton, 1975; Liu *et al.*, 1979; Kojima, 1982; Tan *et al.*, 1987). On the other hand, *L. fortunei kikuchii* has been found in Japan since the 1970's (Kimura, 1994b). The subspecies is found in several bays and also in estuarine and/or brackish waters in Japan, e.g., bays from Tokyo Bay to Urado Bay (Kochi Pref.) along the Pacific coast, the Seto Inland Sea, and bays from Dokai Bay (Fukuoka Pref.) to Lake Shinji-ko (Shimane Pref.) along the Japan Sea coast (Kimura, 1994b). The subspecies is presumed to be an introduced species, but the provenance and routes of introduction have not yet been confirmed. The above mentioned two mytilids were found to occur together at a certain point of the Nagara River where a very few specimens of these mytilids were observed (pers. comm., Dr. K. Nakai).

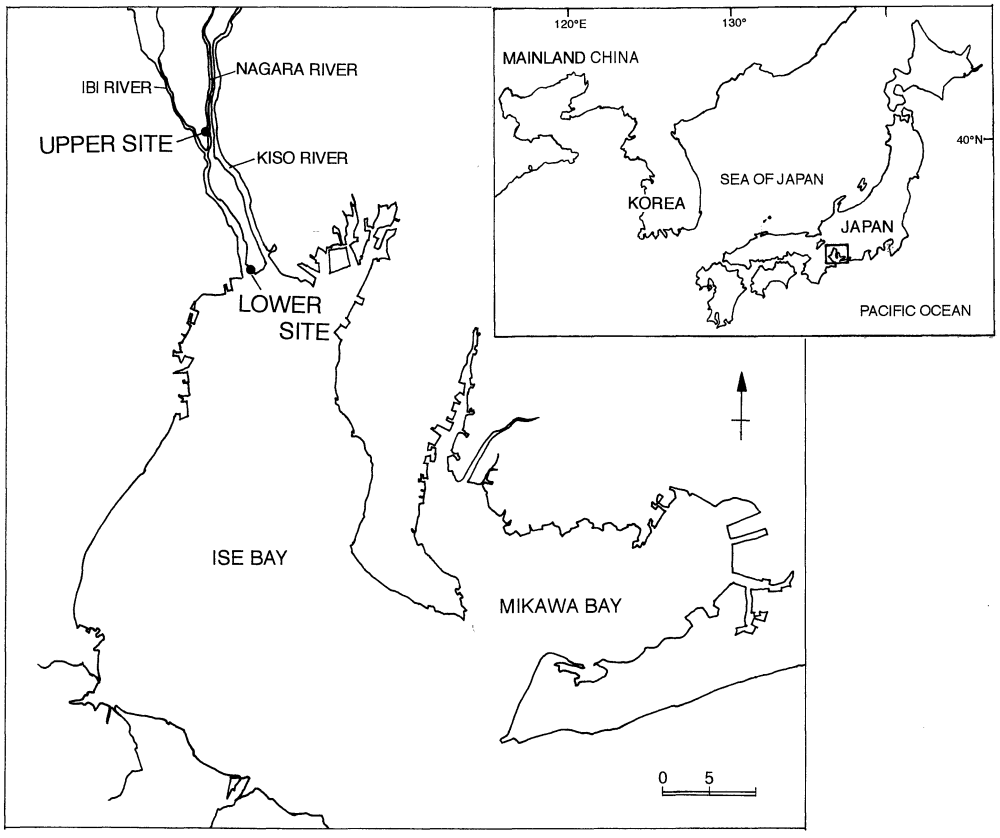

Fig. 1 Sampling sites of two *Limnoperna* populations in the Nagara River.
長良川におけるカワヒバリガイ属の採集地点

Limnoperna fortunei kikuchii has been treated as a subspecies, based on its shell morphology (Habe, 1981). However, some researchers clearly distinguished these mytilids from each other using morphological, physiological and genetic characteristics (shell color, posterior pedal retractor muscle scar, salinity tolerance, karyotype and nuclear DNA contents) (Kimura, 1994b; Kimura *et al.*, 1995; Ieyama, 1996). Furthermore, in Japan, *L. fortunei fortunei* mainly inhabits freshwater while *L. fortunei kikuchii* mainly inhabits brackish water (pers. obs.). Thus, validness of the subspecific status of *L. fortunei kikuchii* has been questioned. In this study using an electrophoretic method, we examined genetic differentiation between samples collected at two sites of the Nagara River.

Materials and Methods

Samples were collected from the two sites in the Nagara River in August 1994 (Fig. 1). The upstream site was in a tidal area from which *L. fortunei fortunei* specimens were collected. The downstream site was a river mouth where *L. fortunei kikuchii* specimens were collected. Identification was done based on morphological features according to Kimura (1994b). All live samples were transported to the laboratory and stored at −20°C or −80°C until an electrophoretic analysis. Mantle tissue extracts of 30 individuals in each sample were used for electrophoretic analysis using horizontal starchgel electrophoresis, procedures being basically done following May *et al.* (1979) and May (1992). Staining producers were according to Harris and Hopkinson (1976) and May (1992). The combinations of buffer systems, enzymes, enzyme abbreviations, enzyme numbers and loci are listed in Table 1. Identification of loci and alleles, genetic nomenclature, and inscriptions were

Table 1 Enzymes examined, their loci and buffer systems used in electrophoresis
使用した酵素，遺伝子座，緩衝液

Enzyme	Enzyme number	Locus	Buffer
Aspartate aminotransferase	2.6.1.1	*AAT**	1
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3PDH**	2
Glucose-6-phosphate isomerase	5.3.1.9	*GPI**	2
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42	*IDHP-1**	2
		*IDHP-2**	2
Lactate dehydrogenase	1.1.1.27	*LDH**	1
Malate dehydrogenase	1.1.1.37	*MDH-1**	2
		*MDH-2**	2
Tripeptide aminopeptidase	3.4.-.-	*PEPB-1**	1
		*PEPB-2**	1
		*PEPB-3**	1
Proline dipeptidase	3.4.13.9	*PEPD**	1
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH**	2
Superoxide dismutase	1.15.1.1	*SOD**	1

Buffer: 1 = a Tris-boric acid-EDTA buffer (pH 8.5) described by Markert and Faulhazber (1965)

Buffer: 2 = an amin (n-(3-aminopropyl)-morpholine) citrate buffer (pH 6.5) described by Clayton and Tretiak (1972)

done following Fujio (1984) and Shaklee *et al.* (1990). Briefly, alleles were scored by arbitrarily designating the most common allele at each locus in samples from the upper site as the standard "**100*" allele. Other alleles were assigned as numerals on the basis of the mobility of their products relative to that of the standard allele.

Results

Fourteen loci were presumed for 10 enzymes examined in this study. Chi-square tests of allelic frequencies in each locus in the samples were applied to detect the deviation from the Hardy-Weinberg equilibrium. There were no significant differences between expected and observed frequencies (χ^2 = 0.008 − 2.290, $0.10 < P < 0.80$). Allele frequencies at the loci are given in Table 2. Examples of electropherograms of isozymes are given in Fig. 2. A brief description of the 10 enzymes is as follows:

A single locus was coded for AAT, G3PDH, GPI, LDH, PEPD, PGDH and SOD. Two loci were coded for IDHP and MDH. Three loci were coded for PEPB. Three-banded in heterozygotes were coded for AAT, G3PDH, GPI, IDHP, MDH and PGDH, which were dimeric enzymes. One-banded were coded for LDH, PEPB, PEPD and SOD. Only *AAT** had the same dominant allele in samples. Nei's (1972) genetic distance (D) between the two samples was 2.78. No hybrid was observed in samples containing of *L. fortunei fortunei* and *L. fortunei kikuchii*. Genetic polymorphism ($p < 0.95$) was not found in specimens of *L. fortunei fortunei*, while that was 0.214 confined to *AAT**, *IDHP-2** and *PGDH** in samples of *L. fortunei kikuchii*. Then, observed average heterozygosities (Ho) in specimens of *L. fortunei fortunei* and of *L. fortunei kikuchii* were 0.0048 and

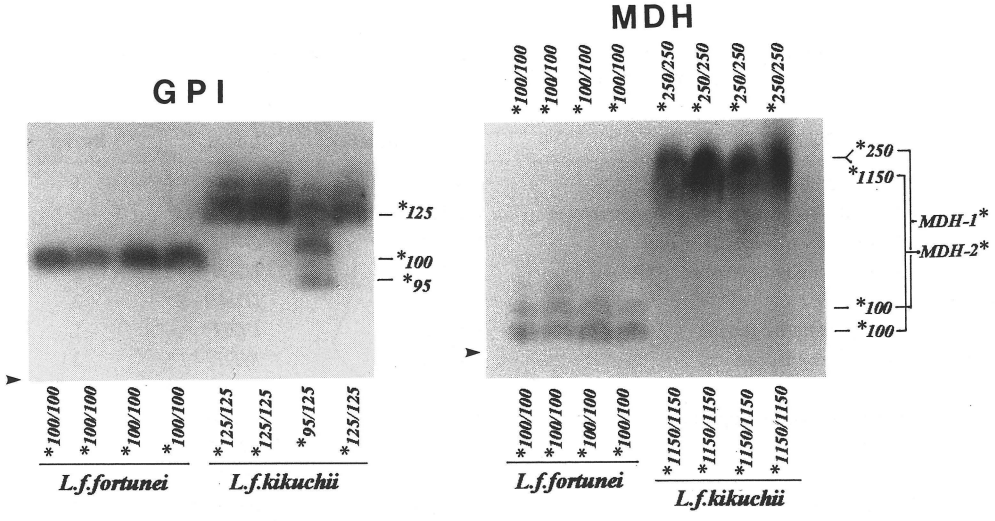

Fig. 2 Glucose-6-phosphate isomerase (GPI) and Malate dehydrogenase (MDH) of isozyme phenotypes of *Limnoperna* specimens. Corresponding loci and alleles are indicated at the right of each gel. Genotypes assined are indicated at top and bottom of each gel. Triangles indicate the sample origin. Anode is toward the top. カワヒバリガイとコウロエンカワヒバリガイの泳動像　対応する遺伝子座および対立遺伝子名はゲルの右側に，遺伝子型はゲルの上下に示す。三角形は原点を示し，上側が陽極.

Table 2 Allele frequency at 14 loci for two populations of *Limnoperna*
カワヒバリガイとコウロエンカワヒバリガイの遺伝子頻度

Locus		*L. fortunei fortunei*	*L. fortunei kikuchii*
*AAT**	*70	0.000	0.033
	*90	0.000	0.017
	*100	1.000	0.833
	*125	0.000	0.117
*G3PDH**	*70	0.000	0.033
	*95	0.000	0.967
	*100	1.000	0.000
*GPI**	*95	0.000	0.017
	*100	1.000	0.000
	*125	0.000	0.983
*IDHP-1**	*40	0.000	0.050
	*50	0.000	0.950
	*100	1.000	0.000
*IDHP-2**	* – 100	1.000	0.000
	*65	0.000	0.017
	*100	0.000	0.933
	*135	0.000	0.050
*LDH**	*100	1.000	0.000
	*130	0.000	1.000
*MDH-1**	*70	0.017	0.000
	*75	0.017	0.000
	*100	0.950	0.000
	*110	0.017	0.000
	*250	0.000	1.000
*MDH-2**	*100	1.000	0.000
	*1150	0.000	1.000
*PEPB-1**	*80	0.000	1.000
	*100	1.000	0.000
*PEPB-2**	*95	0.000	1.000
	*100	1.000	0.000
*PEPB-3**	*100	1.000	0.000
	*110	0.000	1.000
*PEPD**	*100	1.000	0.000
	*105	0.000	1.000
*PGDH**	* – 30	0.000	0.083
	*80	0.000	0.783
	*100	1.000	0.000
	*140	0.000	0.067
	*150	0.000	0.017
	*170	0.000	0.033
	*200	0.000	0.017
*SOD**	*100	1.000	0.000
	*460	0.000	1.000

0,0783, respectively, being significantly higher in specimens of *L. fortunei kikuchii* than in those of *L. fortunei fortunei* (Wilcoxon signed rank test, $p<0.05$).

Discussion

As indicated in the present study, 13 (excluding *AAT**) of 14 loci were fixed for different alleles between the samples from the two sites of the Nagara River. No hybrid was observed in collected samples containing specimens of *L. fortunei fortunei* and *L. fortunei kikuchii*. The two sampling sites were located within the tidal area of the Nagara River (The Ministry of Construction, 1995). Deducing from that the two mytilids were found to occur together at a certain point of the Nagara River (pers. comm., Dr. K. Nakai) and that the Nagara River Estuary Dam located between the two sites was not yet operated when the present samples were collected, the planktonic larvae of the two mytilids would have been able to move easily between the two sites. This suggests that reproductive isolation between the two mytilids would be established.

According to several studies done by Skibinski *et al.* (1980), Grant and Cherry (1985) and Väinölä and Hvilsom (1991) that dealt with three mussel species (*Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*), the Nei's (1972) distance (D) among these three mussels was in the range of 0.16–0.28, suggesting that hybrid between populations of any pair species would occur (McDonald and Koehn, 1988; Koehn, 1991; Väinölä and Hvilsom, 1991). Then, Gosling (1992) regarded the above three mussels as a subspecies status. Based on morphological differences, the swan mussel *Anodonta woodiana* has been known to show two types that living in the same pond in Japan, though Tabe *et al.* (1994) made clear that the D distance between these two types was 0.707 and no hybrid was observed. They suggested that these two types should be regarded as distinct species.

In the present study, no hybrid was observed between *L. fortunei fortunei* and *L. fortunei kikuchii*, and the D distance was higher than the values observed for other mussels (e.g., Skibinski *et al.*, 1980; Grant and Cherry, 1985; Väinölä and Hvilsom, 1991; Tabe *et al.*, 1994). This suggests that each of the above two mytilids, which shows different characteristics of morphological, physiological and ecological aspects (Kimura, 1994b, Kimura *et al.*, 1995), would occupy a species status.

Genetic polymorphism ($p<0.95$) and observed average heterozygosities (Ho) were higher in *L. fortunei kikuchii* than in *L. fortunei fortunei*. These evidences may be interpreted as indicating that genetic variability of *L. fortunei kikuchii* is relatively high as compared to that of *L. fortunei fortunei*. This result may be due to through one of following two alternative processes: First, the present heterozygosities of the two mytilids would reflect those of their provenance. Further investigation is needed to confirm the provenance of the two mytilids and to examine their genetic variations. Secondly, if heterozygosities in their provenance are similar between the two mytilids, and then if the introduced number of *L. fortunei kikuchii* is larger than that of *L. fortunei fortunei*, heterozygosity of the former would be low through an artificial bottleneck effect. *L. fortunei fortunei* in the Kiso Rivers comes from Asia probably through contamination with the edible freshwater shell *Corbicula* sp. imported from Asian countries such as Mainland of China (Nakai, 1995), while *L. fortunei kikuchii*, euryhaline to be tolerant in 0–30‰, is presumed to be introduced

by ballast waters of commercial vessels or by fouling of vessel bottoms (Kimura *et al.*, 1995). The introduced number of the two mytilids would differ depending on these different routes of introduction into Japan. However, it is impossible to estimate the introduced number of the two mytilids. Therefore, it is difficult to decide that this result may be due to different introduced number or different mortality after introduction, if heterozygosities in their provenance are similar between the two mytilids.

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要　　約

付着性二枚貝のカワヒバリガイ *Limnoperna fortunei fortunei* (Dunker, 1856) とその亜種コウロエンカワヒバリガイ *L. fortunei kikuchii* Habe, 1981 はいずれもイガイ科カワヒバリガイ属の移入種である。前者は1990年代初めに日本の淡水域に移入し，後者は1970年代に日本の内湾汽水域に移入した。

コウロエンカワヒバリガイは，殻の形態の違いをもとにカワヒバリガイの亜種とされた。しかし，これらは殻の色彩，閉殻筋痕，塩分濃度耐性が明らかに異なり，別種の可能性が高いと指摘されてきた。今回，同一河川内のこれら2種類についてアイソザイム分析を行い，遺伝的分化程度を推定した。試料は岐阜県長良川流域の2地点において採集した。10酵素を用いて分析した結果，14遺伝子座においてその遺伝様式を推定することができた。遺伝様式の推定できた14遺伝子座のうち*AAT* *を除く全ての遺伝子座において2種類間に対立遺伝子の置換がみられた。遺伝的距離はD=2.78と大きく，いずれの遺伝子座においても交雑個体と考えられる泳動像は観察されなかった。これらのことから2種類は別種と考えられる。集団内の遺伝的変異の程度を示す遺伝子多様度は，カワヒバリガイではHo = 0.0048なのに対し，コウロエンカワヒバリガイではHo = 0.0783とカワヒバリガイの方が有意に小さかった。この理由について考察した。

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