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Morphometric Characteristics and Length of the 'Variable Region' in the Nonrepetitive Domain of the Adhesive Protein of *Mytilus* species in the Asamushi Area, Northern Japan

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ABSTRACT—Shell morphology and length of the nonrepetitive region of the foot protein 1 (adhesive protein) amplified by the polymerase chain reaction (PCR) using oligonucleotide primers, Me 15 and Me 16, were examined in mussels collected from Asamushi water, northern Japan, and compared to those of *M. galloprovincialis* Lamarck from Maruishi, Hiroshima, Japan. The Asamushi mussels were collected from a suspended rope substrate and *M. galloprovincialis* of Maruishi from a floating dock. The mussels of the Asamushi area have been referred to as *M. edulis* Linnaeus or *M. edulis galloprovincialis* Lamarck, and were recently supposed to be *M. galloprovincialis* and/or *M. trossulus* Gould based on the geographical distributions of these species. Although 14 of 19 variables of shell characters differed significantly between mussels of Asamushi and *M. galloprovincialis* from Maruishi, both canonical variates analysis using eighteen shell characters and PCR product analysis showed that the mussels from Asamushi were *M. galloprovincialis*. The result of PCR product analysis also indicated no hybridization between *M. galloprovincialis* and *M. trossulus* in the Asamushi area.

INTRODUCTION

Mussels of the genus *Mytilus* (family Mytilidae) are widely distributed throughout temperate coastal waters. Because they have impressive abilities to dominate a variety of habitats such as rocks, embankments, and floating docks, they are an ecologically important component of shallow water benthic communities as dominant space occupiers (Suchanek, 1985), habitat providers for associated organisms (Tsuchiya and Nishihira, 1985, 1986; Nishihira, 1992a, b; Matsumasa, 1994; Matsumasa and Nishihira, 1994), and/or ecosystem engineers (Jones *et al*., 1994; Crooks, 1998). They are used as a nutritious human food source. Moreover, their wide geographical distribution makes them suitable for biomonitoring organisms to assess coastal water quality (Widdows and Donkin, 1992; Tanabe, 1994). Many scientific studies have been conducted on numerous aspects of the biology of this interesting coastal marine species.

Among the five species of the genus *Mytilus*, three closely related species of so-called "*Mytilus edulis* complex" are morphologically similar and their shell shapes are often influenced by the local environment. Therefore, it has been difficult to identify these species (*M. edulis* Linnaeus, *M. galloprovincialis* Lamarck and *M. trossulus* Gould) based only on morphological characters (see, Gosling 1992a), and the confusing taxonomy has hampered the development of studies in biology and ecology of the *Mytilus edulis* complex. Recently, however, various powerful methods such as electrophoretic (Gosling, 1984, 1992a, b for reviews), mitochondrial DNA (e.g., Skibinsky, 1985; Koehn, 1991; Rawson and Hilbish, 1995), and PCR (polymerase chain reaction) product analyses (Inoue *et al*., 1995) have been proposed in addition to a fairly sophisticated morphological method, canonical variates analysis of eighteen morphometric characters of shells (McDonald *et al*., 1991).

In the course of our ecological study on the roles of mussel species in marine benthic community organization at Asamushi, northern Japan, we had doubts about the taxonomy of the mussel. The mussels of the Asamushi area had been referred to as *M. edulis* or *M. edulis galloprovincialis* (Matsumasa and Nishihira, 1994 and references therein; see also, Huq and Nishihira 1997) and have been recently supposed to be *M. galloprovincialis* based on its geographical distribution (McDonald *et al*., 1991; Gosling, 1992a; Nishikawa,

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1997 and references therein). However, some characters of shells of Asamushi mussels are apparently distinct from *M. galloprovincialis* collected from other sites (see, Figs 1–3). Besides, since Asamushi is close to Hokkaido where both native *M. trossulus* and introduced *M. galloprovincialis* are distributed (Inoue *et al.*, 1995) and hybridization often occurs between species of the *M. edulis* complex (Skibinski *et al.*, 1983; McDonald and Koehn, 1988; Skibinski and Roderick, 1991; Inoue *et al.*, 1995), there is a possibility that the mussels of Asamushi were *M. trossulus* or represented a mixed population including hybrids between *M. galloprovincialis* and *M. trossulus*. To clarify this, we examined the mussels using (1) canonical variates analysis of 18 morphometric characters of shells (McDonald *et al.*, 1991), and (2) PCR product analysis which discriminates interspecific differences in length of the nonrepetitive 'variable region' of the adhesive protein gene using a set of primers (Me 15 and Me 16; Inoue *et al.*, 1995). In addition to the 18 morphometric characters used for canonical variates analysis, another shell character (distance between the anterior end of posterior retractor muscle scar and pallial line) was also examined, because it was reported that this distance was narrower in *M. trossulus* than in *M. galloprovincialis* (see, Nishikawa, 1997 and references therein). Using specimens of *M. galloprovincialis* from a 'pure' site (Maruishi, Hiroshima, Japan) for the sake of contrast, our results shed light on the geographical distributions of the *Mytilus* species and provide a test of the usefulness of morphological and PCR methods of identifying species at the boundary of geographical distributions of the *Mytilus edulis* complex.

MATERIALS AND METHODS

Morphometric analysis

Specimens were collected in the vicinities of the Asamushi Marine Biological Station of Tohoku University at Asamushi, Aomori Prefecture (sampling date, 8 Nov. 1996), and of the National Research Institute of Fisheries and Environment of Inland Sea, at Maruishi, Hiroshima Prefecture (sampling date, 25 Feb. 1997), Japan (Fig. 1). The Asamushi mussels were collected from a rope that had been artificially suspended as the substrate and the Maruishi ones from a floating dock (sampling depth, ca 50 cm). On these substrates, both Asamushi and Maruishi mussels have formed their crowding beds and were densely packed. Eighteen shell characters used by McDonald *et al.* (1991) were measured on mussels from Asamushi and Maruishi. We measured an additional character (i.e., the distance between the anterior end of posterior retractor muscle scar and pallial line: character 'e' in Fig. 2) but did not use this for the canonical variates analysis based on McDonald *et al.* (1991). Characters were measured in millimeters using either electronic digital calipers (Mitutoyo, DC-20) or an ocular micrometer fitted to a stereo microscope (Nikon, SMZ-U). To standardize the variables for size, all characters but number of teeth were log-transformed (using base 10 logs) and divided by the log-transformed shell length. The shells used for morphometrics ranged from 25.01 to 63.66 mm and from 27.25 to 71.37 mm in length for specimens from Asamushi and Maruishi, respectively. These ranges in shell length were within that of mussels used by McDonald *et al.* (1991) (i.e, 23–80 mm).

Differences in the distribution of each morphometric variable were examined between Asamushi and Maruishi mussels by the Kolmogorov-Smirnov two sample test using SYSTAT (Wilkinson, 1992). First and second canonical variates for each individual were calculated using the raw canonical coefficients given in McDonald *et al.* (1991). The canonical variate for each individual is the sum of each morphometric variable (log-transformed and length-standardized) multiplied by its raw canonical coefficient. See McDonald *et al.* (1991) for details in the canonical variate analysis.

PCR products analysis

The lengths of the 'variable region' in the nonrepetitive domain of

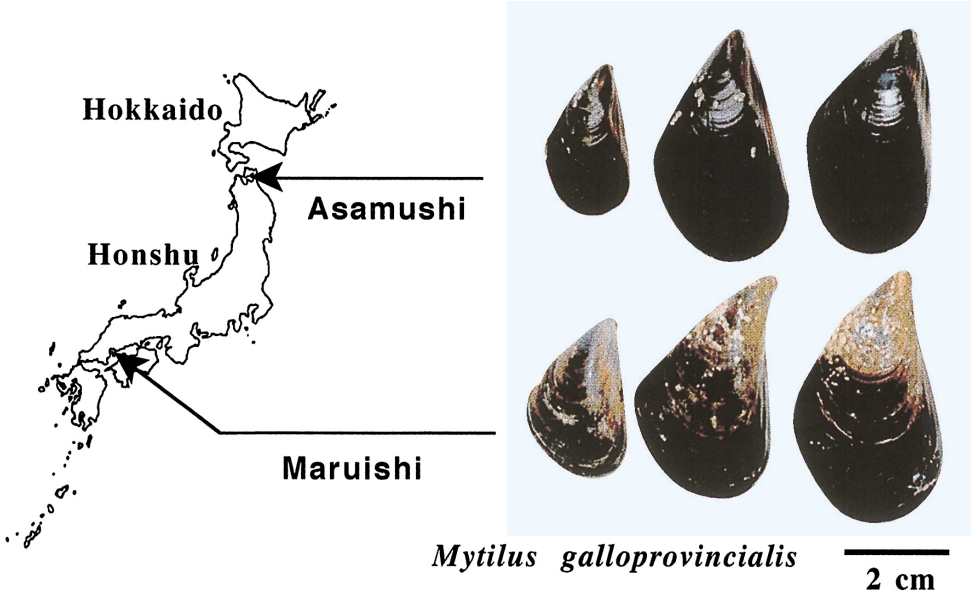

Fig. 1. *Mytilus* collected from Asamushi and Maruishi sites. Based on the PCR analysis for the nonrepetitive region of the adhesive protein gene (Inoue *et al.*, 1995), it has been considered that the only *M. galloprovincialis* occurs among species of the *M. edulis* complex at the Maruishi site (Hamaguchi, unpublished data). See 'RESULTS' for differences in shell morphology between mussels from Asamushi and Maruishi.

the adhesive protein gene (Inoue *et al*., 1995) were examined for 28 individuals from the Asamushi site (sampling date, 8 Nov. 1996) as well as *M. galloprovincialis* (n=30) from the Maruishi site (sampling date, 25 Feb. 1997) and *M. trossulus* (n=32) from Akkeshi, Hokkaido (sampling dates, 10 Jan. and 25 Apr. 1997) for the sake of contrast. The shell length of individuals from Asamushi ranged from 12.80 to 62.30 mm. A piece of the gill from each mussel, about 0.5 cm^2, was incubated in 500 μl lysis buffer containing 50 mM Tris-HCL (pH 7.5), 10 mM EDTA, 0.5% SDS, 500 μg/ml Proteinase K at 55°C for 2–4 hr. Samples were then extracted twice with equal volumes of saturated phenol and twice with phenol chloroform: isoamyl alcohol (25:24:1). The aqueous phase was precipitated with ethanol and dissolved in 50 μl TE (10 mM Tris, 1 mM EDTA). About 100 ng of DNA was dissolved in 100 μl 1×Tth buffer (TOYOBO, Japan) containing 6 μg sense primer, 6 μg antisense primer, and 200 μM dNTP. After preheating to 95°C, 1 unit of Tth DNA polymerase (TOYOBO, Japan) was added and 35 cycles of amplification were performed. Each cycle consisted of 30 sec at 94°C, 30 sec at 56°C, and 90 sec at 72°C, using a set of oligonucleotide primers, Me 15 and Me 16 (Inoue *et al*., 1995). Ten microliters of PCR product was mixed with the loading dye solution containing bromophenol blue (BPB) and xylene cyanol and subjected to agarose gel electrophoresis [4.8% NuSieve GTG agarose gel (FMC)]. Electrophoresis was continued until BPB reached the end of the gel. The shell morphology was also examined for all individuals from the Asamushi site, in addition to the above PCR products analysis.

RESULTS

Shell morphology

The shells of mussels from Maruishi (*Mytilus gallprovincials*), particularly of large individuals, exhibited the typical morphological characteristics of *M. gallprovincials* (see Gosling, 1984, 1992), but individuals from Asamushi only vaguely exhibited these characteristics (Fig. 1). The anterior ends of the shells of the Maruishi mussels were distinctly beaked or incurved, while those from Asamushi were not incurved. The shells of the Maruishi mussels tended to be higher and flatter than those from Asamushi.

Fourteen of 19 characters examined showed the significant differences in length-standardized size distribution between Asamushi and Maruishi specimens (Fig. 2). In particular, the largest level of significance (Kolmogorov-Smirnov two sample tests; $p<0.0001$) in the four characters (Fig. 3a–d) quantitatively indicated that the mussels from Maruishi were higher and grew longer anterio-dorsally than those from Asamushi. The distance between the anterior end of posterior retractor muscle scar and pallial line (character e in Fig. 2) was significantly narrower in Asamushi than in Maruishi mussels ($p<0.0001$; Fig. 3e).

All 46 mussels from Maruishi were certified as *M. galloprovincialis* by canonical variates analysis for morphometric data (Fig. 3f). Of the 59 individuals from Asamushi, 51 mussels (86.4%) were identified as *M. galloprovincialis*, 6 (10.2%) as *M. trossulus*, and 2 (3.4%) as *M. edulis*.

PCR products

The sizes of amplified fragments of a part of the non-repetitive region including the primer sequences (primers, Me 15 and Me 16) are 180, 168, and 126 bp for *M. edulis*, *M.*

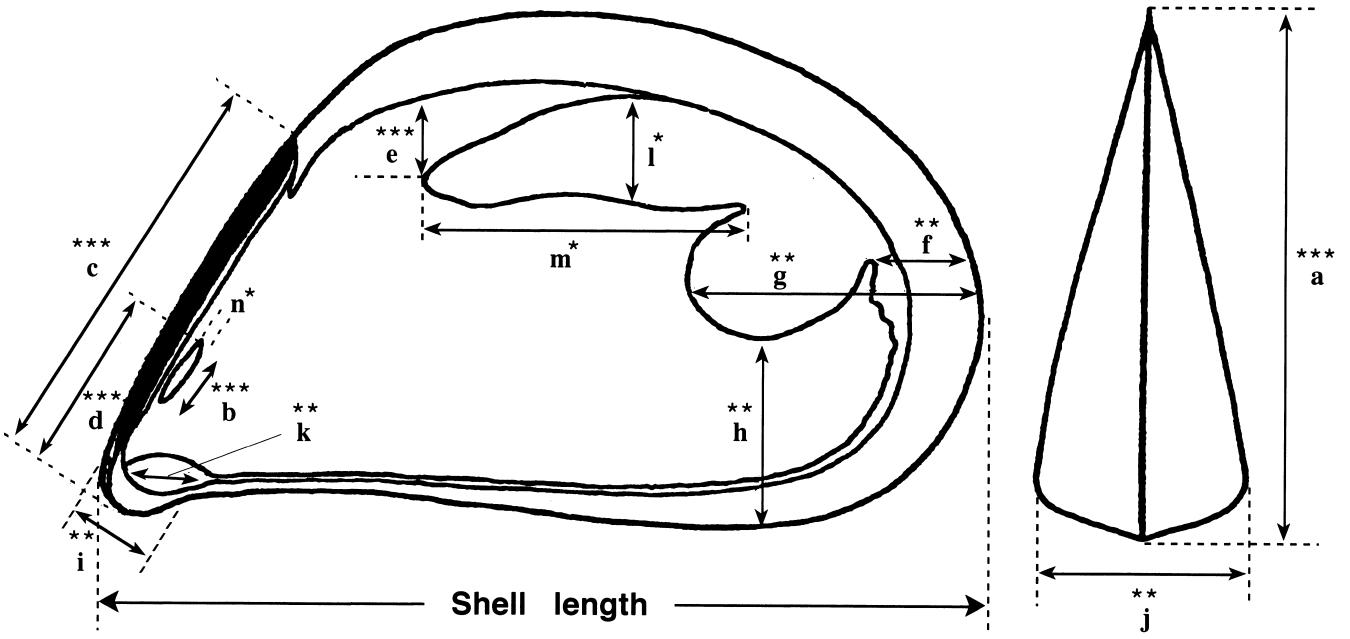

Fig. 2. Fourteen shell characters that significantly differ between Asamushi and Maruishi mussels. The Kolmogorov-Smirnov two sample tests were used for the log-transformed and length-standardized variables. Significance; (*) $0.01\leq p<0.05$, (**) $0.0001\leq p<0.001$, (***) $p<0.0001$. (a) shell height. (b) length of anterior retractor muscle scar. (c) distance between umbo and posterior end of the ligament. (d) distance between umbo and posterior end of anterior retractor scar. (e) distance between the anterior end of posterior retractor muscle scar and pallial line. (f) distance between posterior edge of posterior adductor muscle scar and posterior shell margin. (g) distance between anterior edge of posterior adductor muscle scar and posterior shell margin. (h) distance between ventral edge of posterior adductor muscle scar and ventral shell margin. (i) length of hinge plate. (j) shell width. (k) length of anterior adductor muscle scar. (l) width of posterior retractor muscle scar. (m) length of posterior retractor muscle scar. (n) width of anterior retractor muscle scar.

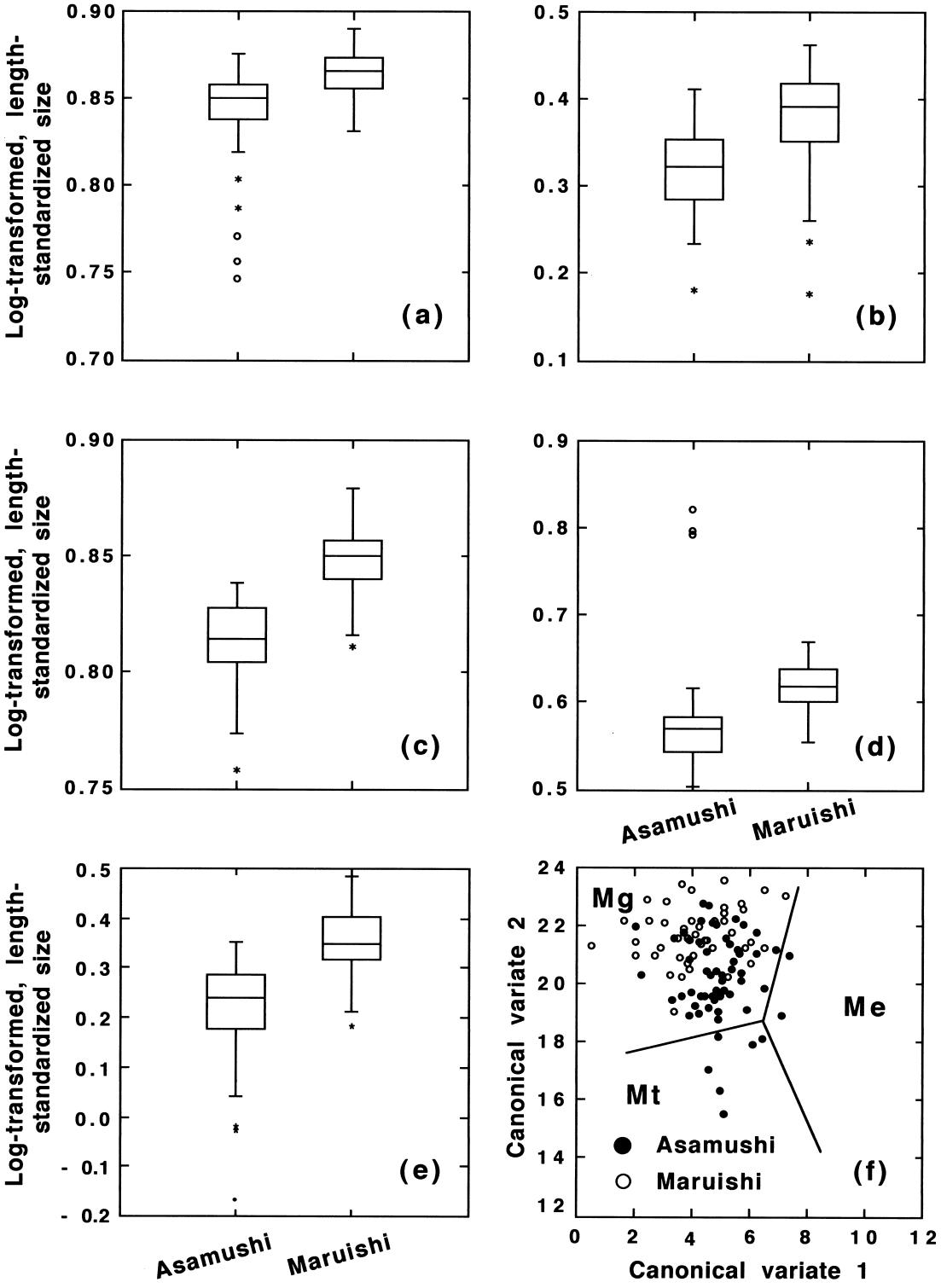

Fig. 3. Size distribution for the most significantly different shell characters (Kolmogorov-Smirnov two sample tests; $p<0.0001$) between Asamushi and Maruishi mussels [Fig. (a)–(e)] and first and second canonical variates from morphometric data for individuals from the two sites (f). (a) shell height. (b) length of anterior retractor muscle scar. (c) distance between umbo and posterior end of the ligament. (d) distance between umbo and posterior end of anterior retractor scar. (e) distance between the anterior end of posterior retractor muscle scar and pallial line. The median is marked by the center horizontal line. Lower and upper hinges comprise the edges of the central box. The hinges split the halves, into which the median split the ordered batch of numbers, in half again. Values outside the inner fences are plotted with asterisks, and those outside the outer fences are plotted with empty circles. The inner fences are defined as follows: lower fence=lower hinge–(1.5Hs), upper fence=upper hinge+(1.5Hs). The outer fences: lower fence=lower hinge–(3Hs), upper fence=upper hinge+(3Hs). Hs is the absolute value of the difference between the values of the two hinges (Wilkinson, 1992). (f) Lines separating the Northern Hemisphere species drawn by McDonald *et al.* (1991) were shown. Me, *Mytilus edulis*. Mg, *M. galloprovicialis*. Mt, *M. trossulus*.

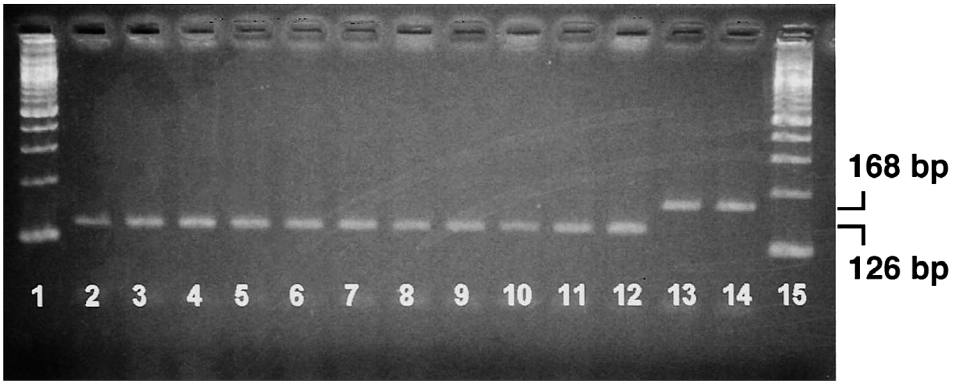

Fig.4. Amplification of the 'variable region' of the adhesive protein gene of Asamushi, Maruishi and Akkeshi mussels. Lines 2–10, mussels from Asamushi site. Lines 11 and 12, *Mytilus galloprovincials* from Maruishi, Hiroshima. Lines 13 and 14, *Mytilus trossulus* from Akkeshi, Hokkaido. All of the tested mussels from the Asamushi site showed a single band of 126bp. Lines 1 and 15, molecular markers (100bp DNA ladder; TOYOBO, Japan).

trossulus, and *M. galloprovincialis*, respectively (Inoue *et al*., 1995; see also Filpula *et al*., 1990). In addition, it is considered that the hybrid between *M. galloprovincialis* and *M. trossulus* exhibits two bands, 126 and 168 bp (Inoue *et al*., 1995). All 28 individuals of Asamushi mussels, including 5 individuals which were not morphologically considered as *M. galloprovincialis*, showed a single band of 126 bp, which was consistent with that of *M. galloprovincialis* from the Maruishi site (Fig. 4). This indicated that mussels from Asamushi were *M. galloprovincialis*, and that hybrids between *M. galloprovincialis* and *M. trossulus* were not included in the tested samples.

DISCUSSION

The results of both the PCR product and the canonical variates analyses indicated that the mussels from Asamushi were *M. galloprovincialis* (Figs. 3f and 4). Therefore, among the ecological and biological studies in the Asamushi area, the species name given to the mussel in Matsumasa and Nishihira (1994) must be changed from '*Mytilus edulis* L.' to '*M. gallprovincialis* Lamarck' at the very least. Both mussels in Matsumasa and Nishihira (1994) and in this study were collected from the same substrate at a similar depth from the same site. However, re-examination is needed for mussels dealt with in the other works of the Asamushi area (e.g., Hoshiai, 1958, 1960; Tsuchiya, 1979; Tsuchiya and Nishihira, 1985, 1986), because those mussels were from other habitats (i.e., intertidal natural rocks and embankments). It has been reported that beaches exposed to wave action are dominated by *M. galloprovincialis*, while more protected areas a short distance away are dominated by *M. edulis* in the British Isles (Gosling and Wilkins, 1981; Skibinski *et al*., 1983; Skibinski and Roderick, 1991). Moreover, mussels collected from an intertidal beach at Posjet Bay, USSR, contained only *M. trossulus*, while those from a floating dock a few meters away were all *M. galloprovincialis* (see, McDonald *et al*., 1991). Since the closely related mussels would appear to segregate in microhabitats within an area where their geographical distributions overlap, samples from a variety of habitats should be examined in northern Japan, including the Asamushi area, in the future.

As stated in previous studies (Gosling, 1984, 1992a; Koehn, 1991; McDonald *et al*., 1991), no single morphological character is reliable for identifying species within the mussel group. Fourteen of 19 shell characters of Asamushi mussels differed significantly from individuals of Maruishi which exhibited the typical characteristics of *M. galloprovincialis* (Fig. 2). This led to the erroneous presumption that the mussels of Asamushi were not *M. galloprovincialis*. The distance between the anterior end of posterior retractor muscle scar and pallial line is believed to be narrower in *M. trossulus* than in *M. galloprovincialis*, but the results of this study revealed that *M. galloprovincialis* had a great deal of intraspecific variation in this character (Fig. 3e). On the other hand, the canonical variates analysis based on 18 shell characters (McDonald *et al*., 1991) would be a good morphological method for field ecologists and biologists studying the '*M. edulis* complex.' The results of this study from the canonical variates analysis indicated that 86.4% of Asamushi mussels were the same species as those from Maruishi (i. e., *M. galloprovincialis*), even though 13 of 18 characters differed statistically in their standardized sizes between individuals from the two sites. However, when this method indicates a mixed population, other methods such as electrophoretic (Gosling, 1984, 1992a, b for reviews), mitochondrial DNA (e.g., Skibinsky, 1985; Koehn, 1991; Rawson and Hilbish, 1995), and PCR product analyses are indispensable.

Although the recently proposed method of PCR product analysis by Inoue *et al*. (1995), has rarely been tested for wild mussels, its reliability was supported by our result which was consistent with that of canonical variates analysis method that had been made in conjunction with the careful examination in allozyme characters (McDonald *et al*., 1991). Inoue *et al*. (1995) have pointed out two advantages of their method: (1) PCR requires only a small amount of DNA as a template, so that the method may be used for larvae or young individuals.

(2) The hybrids within the '*M. edulis* complex' can be identified by detecting haplotypes of different species. The first point is very important for understanding a variety of phenomena in the field. Patterns of larval dispersion and recruitment are essential in determining distributions and population dynamics of benthic organisms (e.g., Hall *et al.*, 1994), but taxonomic difficulties obstruct ecological studies of larval stages. Moreover, relationships (e.g., intraspecific competitions for space and food) between adults and younger individuals may affect the stability of populations (Seed, 1969; Kautsky, 1982; Seed and Suchanek, 1992). These interesting problems can not be examined without testing whether larger 'adults' and smaller 'young' individuals are members of the same species or not. Matsumasa and Nishihira (1994) reported a morphological difference between larger (shell length≥40 mm) and smaller (shell length<40 mm) shells of mussels collected from a rope substrate at Asamushi, and suggested that the growth pattern of the mussel shell changes with size in this gregarious animal. In this study, all Asamushi mussels ranging from 12.80 to 62.30 mm in shell length were identified as *M. galloprovincialis* by PCR product analysis. Therefore, the morphological difference in shell shape between two size groups would appear to be due to the change in growth pattern with size in a single species population, rather than a mixture of different species. Secondarily, no hybrids were detected for mussels of Asamushi by PCR product analysis in this study. This finding should be examined in more detail using other methods such as morphological, electrophoretic, and mitochondrial DNA analyses. An interesting future problem may be to determine whether the difference in shell morphology between Asamushi and Maruishi sites is due to genetic variation, environmental variation, or both.

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