VENUS 68 (3–4): 151–163, 2010

Molecular Markers Reveal Genetic Contamination of Endangered Freshwater Pearl Mussels in Pearl Culture Farms in Japan

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Abstract: Phylogenetic analyses using mitochondrial DNA (Cox2-Cox1) and nuclear DNA (ITS1) were performed on a critically endangered freshwater pearl mussel, *Hyriopsis schlegeli*, endemic to Lake Biwa, Japan, and a closely related Chinese species *H. cumingii,* which was introduced to Japan approximately 30 years ago. Using samples of typical *H. schlegeli* and *H. cumingii*, clear genetic differences between the two species were recognized in both molecular markers. Surveys of the genetic compositions of freshwater pearl mussels showed evidences of hybridization between the two species in culture farms in Lakes Kasumigaura and Biwa. Given that pearl culture farms are not completely isolated from natural water systems in Lake Biwa, these findings suggest that native *H. schlegeli* might be threatened by hybridization with *H. cumingii.* The wild population in Lake Anenuma, Aomori Prefecture, Japan would be the only remaining 'pure' population of *H. schlegeli* free from hybridization and therefore be very important for conservation, although it originated from escaped cultured mussels.

Keywords: conservation genetics, *Hyriopsis*, endemic species, endangered species, Lake Biwa

Introduction

Exotic species have greatly impacted native ecosystems in a variety of ways. Ecological influences such as threats to native species from predation and occupation of native niches have been well documented (Mack *et al*., 2000; Mooney & Cleland, 2001). However, hybridizations between native and exotic species are not easily recognized (Strauss *et al*., 2006), and may result in genetic contamination of the native species. The impacts of such hybridization would be great especially when the native species involved are endangered. Thus, immediate actions are required to assess the current status of hybridization by determining the genetic composition of both native and exotic species.

The freshwater pearl mussel *Hyriopsis schlegeli* (Martens, 1861) (Bivalvia: Unionidae) is endemic to Lake Biwa, central Japan (Fig. 1), and is now threatened (Kondo, 1994; Nakanishi & Sekino, 1995). This species has very large shells, and is known to produce freshwater pearls. Following the development of commercial freshwater pearl culture in Lake Biwa, *H. schlegeli* was introduced to other freshwater systems in Japan. Propagation of the species, however, was not successful in most water systems, with the exception of Lake Kasumigaura in the Kanto district (Kihira *et al*., 2003). Since the 1930s, Lake Kasumigaura has been stocked several times with mussels from Lake Biwa, and a large wild population of *H. schlegeli* was established as early as 1963 (Ogiwara, 2002). Freshwater pearl culture farms are currently in operation only in Lakes Biwa and Kasumigaura (Figs. 1 and 2).

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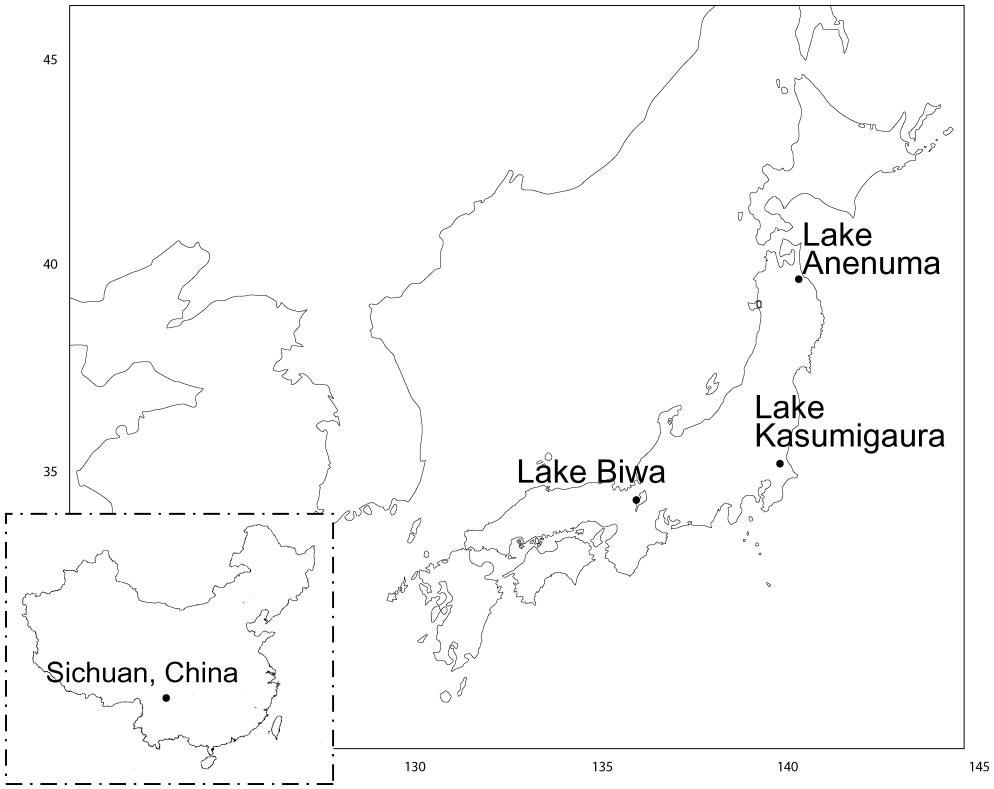

Fig. 1. Localities from which samples were obtained.

Although *H. schlegeli* was highly abundant in Lakes Biwa and Kasumigaura approximately five decades ago, a serious decline of wild mussels occurred in both lakes in the mid-1970s (Fig. 2). This might be the results of environmental changes and/or over-collection of seed mussels (Nakanishi & Sekino, 1995; Kondo, 2001; Ogiwara, 2002). Indeed, population crashes were so extensive that *H. schlegeli* is now counted as one of the critically endangered species under the category CR + EN in the Japanese Red Data Book (Ministry of the Environment, 2005).

The decline in wild freshwater pearl mussels induced a shortage of seed mussels for commercial pearl culture. In Lake Kasumigaura, *Hyriopsis* mussels, referred to as an "improved variety" of *Hyriopsis* species, have been used for pearl culture since 1988. Although the origin of this improved variety is not clear, the mussel is more tolerant to water contamination than *H. schlegeli* (Ogiwara, 2002). The improved variety was transferred from Lake Kasumigaura to culture farms in Lake Biwa in 1992 and was used as seed mussels for pearl culture (Kondo, 2001) (Fig. 2). The present cultured mussels in Lake Biwa are likely dominated by the improved variety, however the taxonomic and genetic identity of this variety has not been clarified. Machii (1995) suggested that it might be a natural hybrid between *H. schlegeli* and the related Chinese species *H. cumingii* (Lea, 1852), whereas Kondo (2001) suggested that it could be *H. cumingii* itself based on morphological features.

Hyriopsis cumingii is endemic to China and is the most important mussel for freshwater pearl production there (Liu, 1979; Wang *et al*., 2008). This species was probably introduced to Lake Kasumigaura from China sometime between the mid-1970s and the mid-1980s, although the introduction was not clearly documented (Fig. 2). According to Dong *et al*. (2007), *H. schlegeli*

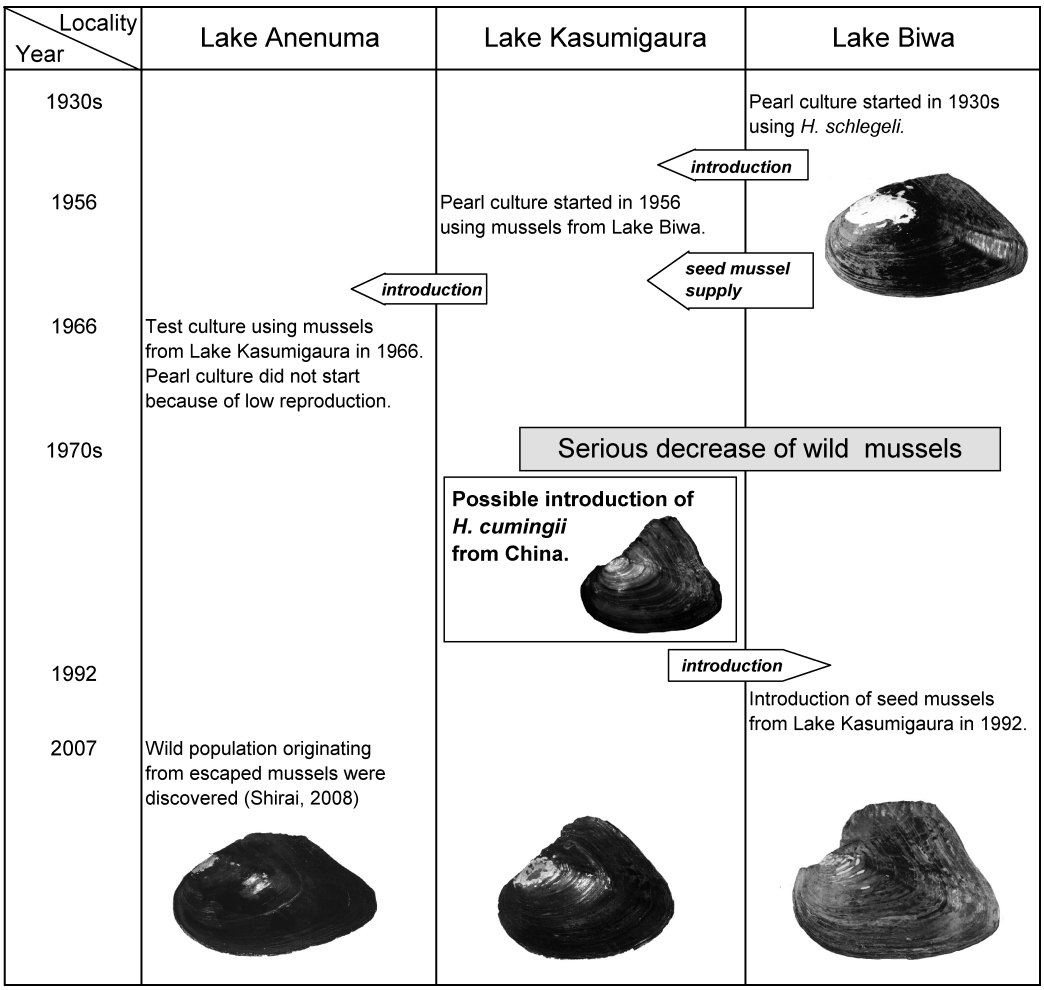

Fig. 2. A brief history of freshwater pearl aquaculture of *Hyriopsis* species in Japan.

and *H. cumingii* can be artificially crossed and produce hybrids. If the mussels transferred into Lake Biwa from Lake Kasumigaura in 1992 included *H. cumingii* or their hybrids, genetic contamination of *H. schlegeli* may have occurred at culture farms in Lake Biwa. This might have resulted in subsequent genetic contaminations of *H. schlegeli* in its natural habitat in Lake Biwa, given that most pearl culture farms are built adjacent to open water. It is necessary to clarify the genetic composition of native and cultured *Hyriopsis* mussels in Lakes Kasumigaura and Biwa. Previous studies using allozyme markers, however, did not provide sufficient evidence to distinguish the two species (Sakai *et al*., 1997; Kondo, 2001).

Shirai (2008) recently identified a wild population of *H. schlegeli* in Lake Anenuma, adjacent to Lake Ogawara, in Aomori Prefecture, northern Japan (Fig. 1), and suggested that the wild mussels originated from ones that escaped from a test aquaculture farm established there in 1966. Given that the test culture in Lake Anenuma used seed mussels from Lake Kasumigaura and took place before the introduction of *H. cumingii*, the large wild population of *H. schlegeli* in Lake Anenuma might not have been influenced by the introduction of *H. cumingii* (Shirai, 2008; Fig. 2).

In this study, we used mitochondrial and nuclear DNA markers, which are expected to have

higher resolutions than allozyme markers. The objectives of this study were to clarify: 1) the genetic differences between *H. schlegeli* and *H. cumingii*, and 2) the genetic composition of wild and cultured mussels in Japan for the conservation of *H. schlegeli*, which is an endangered endemic species in Japan.

Material and Methods

Samples

Samples were obtained from wild and cultured *Hyriopsis* mussels in three Japanese lakes (Table 1, Fig. 1). A total of nine wild samples were collected from Lake Anenuma in 2007 and seven cultured samples from Lake Kasumigaura were kindly provided by the Yanase Pearl Culture Company (Inashiki, Ibaraki Prefecture) in 2007. 40 cultured mussels were purchased from the Lake Biwa Freshwater Pearl Cooperative Association (Omihachiman, Shiga Prefecture) in 1994 and 1998 and preserved in a freezer in Baika High School (Toyonaka, Osaka Prefecture). Of the 40 mussels, 20 were born in 1990–1991 in the culture farm in Lake Biwa, before the introduction of mussels from Lake Kasumigaura, and were therefore not genetically influenced by *H. cumingii*. The remaining 20 mussels were born in 1996–1997 after the introduction, and thus might have been genetically influenced by *H. cumingii* or its hybrids with *H. schlegeli*. Finally, four samples of genuine *Hyriopsis cumingii* were obtained from Sichuan province, China.

For use as an outgroup in phylogenetic analyses, a sample of *Pronodularia japanensis* (Lea, 1834) was collected in Sakata City, Yamagata Prefecture in northern Honshu in 2007.

DNA extraction

The CTAB method by Winnepenninckx *et al*. (1993) was used to extract total DNA from an approximately 5-mm^3 piece of the foot muscle of each specimen. Fresh samples were used if possible, but in some cases, frozen or ethanol-fixed samples were used. Samples were boiled prior to DNA extraction according to Ueshima (2002).

Mitochondrial DNA (Cox2-Cox1) and nuclear ribosomal DNA (ITS1 region) sequencing

Sequences of the coding and non-coding regions of the mitochondrial DNA (mtDNA) Cox2-Cox1 genes and the internal transcribed spacer region 1 (ITS1) of nuclear ribosomal DNA (nrDNA) were determined using direct sequencing methods. It was expected that male mussels would have two highly diverged mitochondrial genomes because of doubly uniparental inheritance (DUI) that is known for some bivalves including Unionidae (Hoeh *et al*., 1996; Liu *et al*., 1996). In Unionidae, a unique protein-coding extension is known in the male mitotype of the Cox2 gene, which is approximately 600 bp longer than the female one (Curole & Kocher, 2002; Curole & Kocher, 2005). Based on the different length of amplified fragments, the PCR products of female mitochondrial Cox2-Cox1 were separated on agarose gels and used for nucleotide sequencing. The primer pair used to PCR was CoxII.2 (Curole & Kocher, 2002) and HCO2198 (Folmer *et al*., 1994). The ITS1 region of nrDNA was amplified using the universal primers ITS2 and ITS5 (White *et al*., 1990).

PCR reactions were performed in a mixture containing 18.4 μL of sterile distilled water, 0.125 μL of *ExTaq* DNA polymerase (5 U/μL; TaKaRa Bio, Shiga, Japan), 2.5 μL of 10 × ExTaq buffer, 2.0 μL of dNTP solution (2.5 mM each), 0.5 μL of each primer (5 mM), and 1.0 μL of genomic DNA (10–30 ng) in a final volume of 25 μL. Amplification was performed in a PCR Thermal Cycler Dice (TaKaRa Bio) under the following conditions: an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 45 or 55°C for 45 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The lower (45°C) annealing temperature was used for the mitochondrial genes and the higher (55°C) for the nrDNA.

Table 1. Wild and cultured samples of *Hyriopsis* used in this study and haplotypes identified by molecular markers. N designates numbers of samples. Haplotype designations of mtDNA and nrDNA are shown in Figs. 3 and 4. Biwa* indicates samples collected in Lake Biwa before the introduction from Lake Kasumigaura in 1992.

Locality	collection year	mtDNA haplotype	nrDNA haplotype	n
Anenuma [n = 9]: Wild mussels of Lake Anenuma	2007	X1	S1	5
		X1	S2	1
		X2	S1	1
		X7	S2	2
Biwa* [n = 20]: Cultured mussels of Lake Biwa, before the introduction of mussels from Lake Kasumigaura (collected samples were born in the culture farm before 1992)	1994	X1	S1	2
		X1	S2	5
		X1	S3	5
		X4	S3	2
		X5	S2	1
		X6	S1	1
		X7	S1	1
		X7	S2	1
		X7	S3	2
Biwa [n = 20]: Cultured mussels of Lake Biwa, after the introduction of mussels from Lake Kasumigaura	1998	X1	S1	8
		X5	S3	1
		X6	S3	1
		X7	S1	1
		X1	C1	3
		X1	C3	1
		X1	S1/C1	1
		X1	S2/C4	1
		X1	S3/C4	1
		X7	S1/C3	2
Kasumigaura [n = 7]: Cultured mussels of Lake Kasumigaura, after introduction of *H. cumingii* from China	2007	X1	C1	2
		X1	C3	1
		X3	C3	1
		Y1	S2	1
		Y1	C1	1
		Y1	C2	1
China [n = 4]: *Hyriopsis cumingii* collected in Sichuan, China	1999	Y2	C1/C3	1
		Y3	C1	1
		Y4	C1	1
		Y5	C1	1

PCR products were purified using the GENECLEAN III Kit (Qbiogene, Inc., Carlsbad, CA, USA). Cycle sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), using profiles of 28 cycles of 95°C for 10 s, 48°C for 8 s, and 60°C for 4 min for mtDNA, and 25 cycles of 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min for nuclear ITS. Both strands were sequenced using the same primers

used for the PCR as well as the following internal primers: forward UnioFCo1F (5'-GATTCGAGCAGAGTTGGGTC-3') and reverse UNIOFCOIR (Curole & Kocher, 2005) or HyFCo1R (5'-CCCCCAATTATCATAGGTATCAC-3') primers for the Cox2-Cox1 regions of mtDNA. Nucleotide sequences were determined using an ABI PRISM 377 automated sequencer (Applied Biosystems) with the standard settings. DNA sequences were edited and aligned using Sequencing Analysis ver. 3.4.1 (Applied Biosystems) and AutoAssembler ver.2.1.1 (Applied Biosystems). Multiple alignment was performed with Clustal X version 1.83 (Thompson *et al.* 1997) using default parameters, and the obtained alignment was manually corrected to maximize

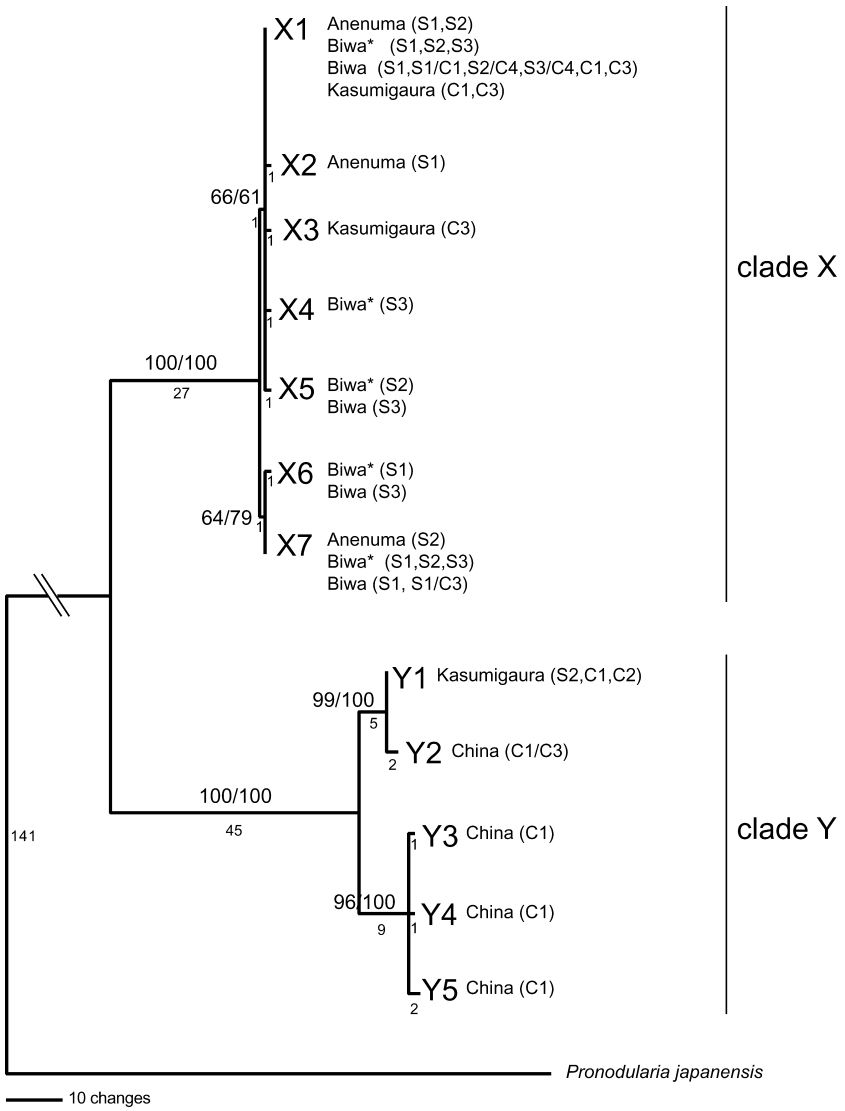

Fig. 3. The strict consensus tree of the six most parsimonious trees obtained from partial sequences of Cox2-Cox1 of mtDNA. The numbers above the branches are bootstrap supports obtained by MP and NJ analyses (MP/NJ). Numbers below branches represent branch length. NrDNA haplotypes relevant to the mtDNA haplotypes are shown after the haplotype names in parentheses. Biwa* indicates samples collected in Lake Biwa before the introduction from Lake Kasumigaura in 1992.

sequence similarity using Se-Al Sequence Alignment Editor version 2.0a11 (Rambaut, 2002).

Direct sequencing of nuclear ITS1 detected two different peaks overlapping within a single sequence profile in some samples. They were presumed to be caused by heterozygotes in ITS1 locus. In order to segregate alleles for these samples, we performed SSCP (single-stranded conformation polymorphism) analysis mostly following the method of Watano *et al.* (2004). PCR products were diluted 1:10 in denaturing formamide buffer, heated to 95°C for 3 min, and then immediately chilled on ice. They were loaded on nondenatured acrylamide gel containing 2% glycerol and run for 10 h at 300 V at 20°C in 1 × TBE. After electrophoresis, the DNA bands were detected by silver staining methods. Each band was cut out from the gel and the DNA of the band was purified by ethanol precipitation. Using the extracted single-stranded DNA as a template, PCR reactions were performed using the same PCR primers that were used in the initial PCR reaction. PCR products were then treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) to remove excess primers and dNTPs, and used as a sequencing template as described above.

Phylogenetic analyses

Phylogenetic relationships among the haplotypes of mtDNA and nrDNA sequences were inferred using maximum parsimony (MP) and neighbor-joining (NJ) methods using PAUP* 4.0 beta 10 (Swofford, 2002). For the MP analysis, all characters were unordered and equally weighted. The MP tree was constructed using the branch and bound search option with 100 random addition replicates, the tree bisection reconnection (TBR) branch-swapping algorithm, and the 'MulTrees' option. Branch support was assessed by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates using the branch and bound search option with the simple addition of sequence replicates. *Pronodularia japanensis* was used as an outgroup species for each analysis. Sequences of Cox2-Cox1 genes for *P. japanensis* were obtained from GenBank (accession no. AB055625, registered as *Inversidens japanensis*) and ITS region was sequenced in this study (accession no. AB507769). All sequences (haplotypes) of *Hyriopsis* samples were deposited in GenBank (accession nos. AB507749–AB507761 for mtDNA and AB507762–AB507768 for nrDNA).

Genetic diversity

DnaSP4.5 (Rozas *et al*., 2003) was used to calculate the mean number of nucleotide substitutions per site of mtDNA and nrDNA among groups (Dxy) and nucleotide diversity within groups (π).

Results

Mitochondrial DNA (Cox2-Cox1)

The 1027 bp nucleotide sequences of the female Cox2-Cox1 mtDNA region of *Hyriopsis*

Table 2. Haplotypes identified by nucleotide substitutions of ITS1. Substitution sites are denoted in the aligned sequences of 484 bp excluding indels.

	28	72	95	98	100	107	146	191	267
S1	G	C	G	G	G	A	C	G	C
S2	A	A	A	G	G	A	C	G	C
S3	G	A	G	G	G	A	C	G	C
C1	A	A	G	C	A	A	C	G	T
C2	A	A	G	C	A	T	G	G	T
C3	A	A	G	C	A	T	C	G	T
C4	G	A	G	C	A	T	C	A	T

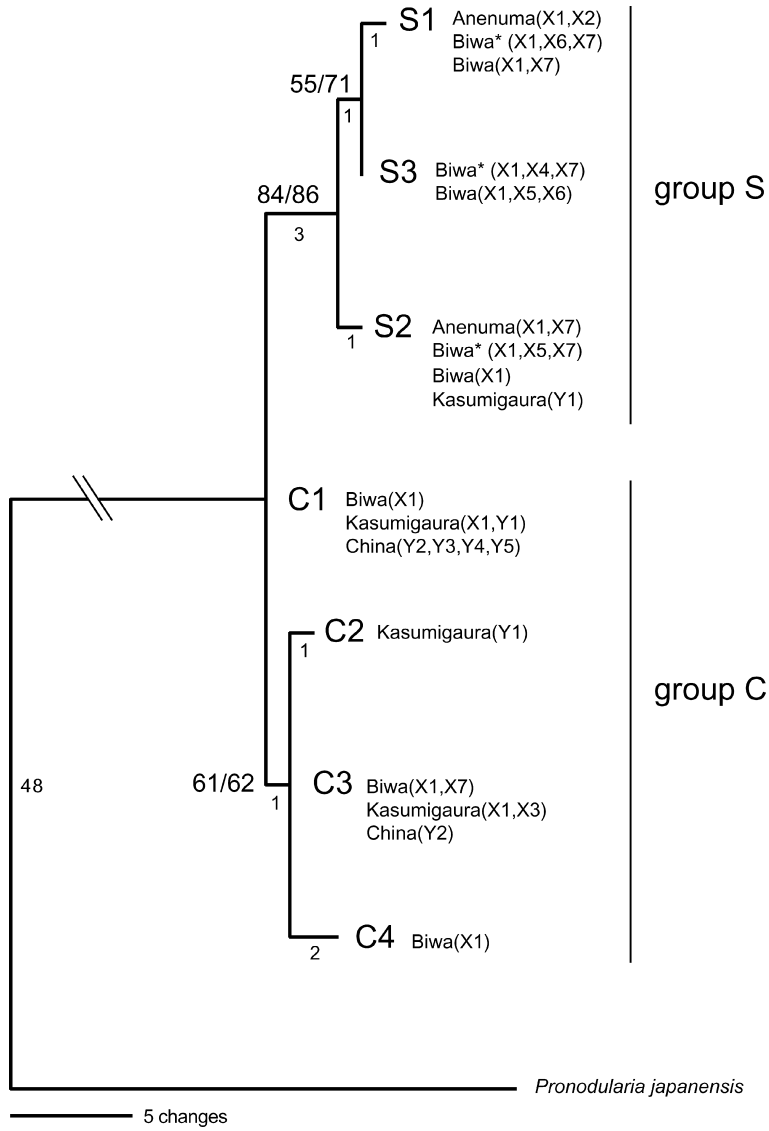

Fig. 4. The strict consensus tree of the two most parsimonious trees obtained from nucleotide sequences of the ITS1 region of nrDNA. The numbers above the branches are bootstrap supports obtained by MP and NJ analyses (MP/NJ). Numbers below branches represent branch length. MtDNA haplotypes relevant to the nrDNA haplotypes in each individual are shown after the haplotype names in parentheses. Biwa* indicates samples collected in Lake Biwa before the introduction from Lake Kasumigaura in 1992.

schlegeli and *H. cumingii* were determined in this study (269 bp for Cox2 region, 18 bp for non-coding region and 740 bp for Cox1 region). No indels (insertions and deletions) were found among sequences. In total, 12 haplotypes (X1–X7 and Y1–Y5) were recognized, containing 96 variable sites and 88 parsimony informative sites. MP and NJ analyses using the 12 haplotypes with an outgroup species produced trees with almost identical topologies (only the MP tree is shown; Fig. 3).

The MP analysis produced six most parsimonious trees with a tree length of 367, a consistency index of 0.924, and a retention index of 0.935. Two major clades were recognized within the strict consensus tree of the six trees, both of which were supported with very high bootstrap values. The first clade (hereafter clade X) consisted of haplotypes X1–X7, which were obtained from *Hyriopsis* samples from Lakes Biwa (both before and after introduction from Lake Kasumigaura), Anenuma, and Kasumigaura. The second clade (hereafter clade Y) consisted of haplotypes Y1–Y5, which were obtained from *H. cumingii* collected in China and *Hyriopsis* samples collected in Lake Kasumigaura. The nucleotide diversity (π) determined by Jukes and Cantor (Jukes and Cantor, 1969) was 0.00232 (+ –0.00039) for clade X and 0.01062 (+ –0.00255) for clade Y. The average number of nucleotide differences between the two clades was 81.543, and the mean number of nucleotide substitutions per site (Dxy) with Jukes and Cantor between clades was 0.08393 (+ –0.01830).

Nuclear ribosomal DNA (ITS1 region)

The 484–492 bp nucleotide sequences from the ITS1 region of *Hyriopsis* samples were determined in this study. The aligned length was 494 bp including 10 bp indels. The direct sequencing of nuclear ITS1 region exhibited two different peaks overlapping within a single sequence profile for six samples. This was likely caused by the presence of two allelic sequences in an individual, and each different allele was separated by PCR-SSCP methods. In total seven haplotypes were recognized by nine diagnostic sites (Table 2).

The MP analyses for the seven haplotypes identified the two most parsimonious trees with a tree length of 58, a consistency index of 0.983 and a retention index of 0.900 (Fig. 4). The strict consensus tree derived from the two trees revealed one clade (hereafter group S) comprising haplotypes S1–S3 supported with a bootstrap value of 84 and by three synapomorphic characters (substitution site 98, 100 and 267; see Table 2). The remaining haplotypes, C1–C4, did not form a clade in the MP analyses. Since they did comprise a clade in the NJ analyses with bootstrap support of 54, we designated the latter as group C.

Group S consisted of haplotypes obtained from *Hyriopsis* samples collected in Lakes Biwa (both before and after the introduction from Lake Kasumigaura), Kasumigaura, and Anenuma. Group C consisted of haplotypes obtained from *H. cumingii* collected in China, as well as from *Hyriopsis* samples collected in Lake Biwa (after the introduction from Lake Kasumigaura) and Lake Kasumigaura. The value of π determined using Jukes and Cantor was 0.00415 (+ –0.00146) for group S and 0.00415 (+ –0.00112) for group C. The average number of nucleotide differences between the two groups was 5.50, and the Dxy with Jukes and Cantor was 0.0115 (+ –0.00411). The haplotype compositions of all individuals are shown for each locality (Table 1).

Discussion

Genetic differences between* H. schlegeli *and* H. cumingii *based on mtDNA and nrDNA sequences

The phylogenetic analyses of partial nucleotide sequences of Cox2-Cox1 of female mtDNA and the ITS1 region of nrDNA revealed the presence of two distinct groups (clades X and Y in the mtDNA tree; groups C and S in the nrDNA tree). In both phylogenetic trees, *H. schlegeli* born in Lake Biwa before 1992 (i.e., before the introduction of mussels from Lake Kasumigaura) and *H. cumingii* collected in China were assigned to different groups (Figs. 3 and 4). This suggests that the two species can be clearly distinguished by species-specific markers of mtDNA and nrDNA. The differences between the two species (Dxy) were 8.4% in the Cox genes and 1.2% in the ITS region, which were slightly higher than the values (5.6% in Cox and 0.9% in ITS) reported between closely related freshwater mussels in North America (Campbell *et al*., 2008).

Our study provides new molecular markers that can be used to assess the genetic composition of the two species.

Molecular evidence of hybridization between H. schlegeli *and* H. cumingii *in pearl culture farms in Japan*

Our results suggest that hybridizations between *H. schlegeli* and *H. cumingii* have occurred in Japanese culture farms. Four samples from Lake Kasumigaura and four samples from Lake Biwa exhibited the mtDNA haplotype of *H. schlegeli* (X1 or X3) and nrDNA haplotypes of *H. cumingii* (C1 or C3). On the other hand, one sample from Lake Kasumigaura exhibited the mtDNA haplotype of *H. cumingii* (Y1) and nrDNA haplotype of *H. schlegeli* (S2) (Table 1). These results are likely caused by hybridization between the two species. Given that only female lineage of mtDNA were surveyed in this study, our results suggest that crossings between the two species have occurred in both directions in the cultured mussels of Lake Kasumigaura, which is consistent with the recent report of artificial reciprocal crosses between the two species (Dong *et al*., 2007). Five samples from Lake Biwa exhibited the mtDNA haplotypes of *H. schlegeli* and both nrDNA haplotypes of *H. schlegeli* and *H. cumingii* (S1/C1 in Table 1, for example). These results would represent heterozygotes produced by crossings between *H. schlegeli* and *H. cumingii* or their hybrids.

If we assessed the extent of genetic influence *of H. cumingii* using the proportion of its haplotypes in *Hyriopsis* mussels sampled in Japan, 43% of mtDNA and 86% of nrDNA were of *H. cumingii* origin in Lake Kasumigaura, on the other hand, 0% of mtDNA and 45% of nrDNA were of *H. cumingii* origin in Lake Biwa (after the introduction from Lake Kasumigaura). Haplotypes of *H. cumingii* origin were found more in Lake Kasumigaura than in Lake Biwa. This result might reflect the history of the introductions: *H. cumingii* was introduced to Lake Kasumigaura from China sometime between the mid-1970s and the mid-1980, and then *Hyriopsis* mussels were transferred to Lake Biwa from Lake Kasumigaura in 1992 (Fig. 2). Although some samples from Lakes Biwa and Kasumigaura may represent pure *H. schlegeli* and *H. cumingii* based on the two molecular markers (X1, 5, 6, or 7 with S 1 or 3 for *H. schlegeli*, and Y1 with C1 or C2 for *H. cumingii* in Table 1), these might have been caused by backcross involving their hybrids. Future studies with a larger number of molecular markers with higher resolution will be necessary to clarify their identity.

Conservation of H. schlegeli

Hyriopsis schlegeli is one of the representative relict endemic species in Lake Biwa (Takahashi, 1989), and its fossils are found in the Pleistocene Kobiwako (Palaeo-Lake Biwa) Group (Matsuoka, 1987; Nishino and Watanabe, 2000). On the other hand, the Chinese species *H. cumingii* has a wide distribution range throughout mainland China (Liu, 1979). The two species are considered closely related, given that they are morphologically similar and able to produce artificial hybrids (Dong *et al*., 2007). Although previous studies did not provide molecular markers to distinguish them (Sakai *et al*., 1997; Kondo, 2001; Wu *et al*., 2008), our molecular data, in contrast, have shown clear differences between the two species in both mtDNA and nrDNA markers (Figs. 3 and 4). *H. schlegeli* has accumulated original genetic differences from its sister species since isolation of the population to Lake Biwa.

Hyriopsis schlegeli is now considered a critically endangered species, and conservation of the species is very important. Our findings of crossings between *H. schlegeli* and *H. cumingii* in culture farms in Lake Biwa, however, raise an alarm for the conservation of *H. schlegeli*. Given that culture farms are built adjacent to natural water systems, mussels with non-native genes that escape from the farms may cause genetic contamination of wild *H. schlegeli* in Lake Biwa. Indeed, *H. cumingii*-like individuals have been collected outside farms in Lake Biwa, which

suggests that the wild *H. schlegeli* may be threatened by *H. cumingii* or their hybrids. Further genetic assessments of wild population of *H. schlegeli* in Lake Biwa are necessary, although collecting wild *H. schlegeli* samples is extremely difficult.

Our results also revealed that mussels of Lake Anenuma found by Shirai (2008) exhibit only native haplotypes of *H. schlegeli* in both mtDNA and nrDNA markers. The composition of haplotypes was similar to that of the Lake Biwa mussels born before 1992 (Table 1). These results suggest that the mussels from Lake Anenuma are free from the genetic influence by *H. cumingii*. Although it originated with escaped animals from the test culture farm in the 1960s, the wild population in Lake Anenuma would be the only remaining 'pure' and abundant population of *H. schlegeli* free from hybridization, and would therefore be very important for conservation.

Acknowledgments

We are grateful to Drs. Y. Watano, T. Asakawa, K. Takayama, N. Wakita, and other members of our laboratory at Chiba University for their valuable advice, and Mr. M. Matsuda and Dr. K. Nakai of Lake Biwa Museum for their useful information. Many thanks are also given to those who provided samples, especially Dr. S. Fukuhara of Baika High School, Osaka, Mr. K. Yanase of the Yanase Company, Ibaraki, Japan and Dr. A. Shi of Sichuan University, Chengdu, Sichuan, China. We also thank anonymous reviewers for their useful comments. This study represents a portion of the doctoral dissertation of A. Shirai prepared under the supervision of T. Kajita, which was partly supported by Research Assistant Programs of the Graduate School of Science, Chiba University.

References

Campbell, D., Johnson, P., Williams, J. D., Rindsberg, A. K., Serb, J. M., Small, K. K. & Lydeard, C. 2008. Identification of 'extinct' freshwater mussel species using DNA barcoding. *Molecular Ecology Resources* 8: 711–724.

Curole, J. P. & Kocher, T. D. 2002. Ancient sex-specific extension of the cytochrome c oxidase II gene in bivalves and the fidelity of doubly-uniparental inheritance. *Molecular Biology and Evolution* 19: 1323–1328.

Curole, J. P. & Kocher, T. D. 2005. Evolution of a unique mitotype-specific protein-coding extension of the cytochrome c oxidase II gene in freshwater mussels (Bivalvia: Unionoida). *Journal of Molecular Evolution* 61: 381–389.

Dong, Z.-G., Li, X.-Y. & Li, J.-L. 2007. Tolerance of alien species *Hyriopsis schlegeli*, native species *Hyriopsis cumingii*, and their hybrids to three ecological factors. *Chinese Journal of Ecology* 26: 1080–1084. (in Chinese with English summary)

Ministry of the Environment (ed.) 2005. *Threatened Wildlife of Japan, Red Data Book 2nd ed. Volume 6, Land and Freshwater Mollusks*. 402 pp., 12 pls. Japan Wildlife Research Center, Tokyo. (in Japanese with English summary)

Felsenstein, J. 1985. Confidence limits on phylogenies – an approach using the bootstrap. *Evolution* 39: 783–791.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299.

Hoeh, W. R., Stewart, D. T., Sutherland, B. W. & Zouros, E. 1996. Multiple origins of gender-associated mitochondrial DNA lineages in bivalves (Mollusca: Bivalvia). *Evolution* 50: 2276–2286.

Jukes, T. H. & Cantor, C. R. 1969. Evolution of protein molecules. *In*: Munro, H. N. (ed.), *Mammalian Protein Metabolism III*, pp. 21–132. Academic Press, New York.

Kihira H., Matsuda, M. & Uchiyama, R. 2003. *Freshwater Mollusks of Japan 1. Freshwater Mollusks in Lake Biwa and the Yodo River*. 160 pp. Pisces, Tokyo. (in Japanese)

Kondo, T. 1994. *Hyriopsis schlegeli*. *In*: *Basic Record on Rare Aquatic Wild Organisms in Japan. Vol. I, Part I Mollusca*, pp. 25–32. Fishery Agency, Tokyo. (in Japanese)

Kondo, T. 2001. *Hyriopsis schlegeli* (Martens, 1861). *In*: *Report on Projects to Promote Conservation Acts of Rare Aquatic Organisms*, pp. 19–26. Japan Fisheries Resource Conservation Association, Tokyo. (in Japanese)

Liu, Y. Y. 1979. *Freshwater Mollusk Economic Fauna of China*. 134 pp. Science Press, Beijing. (in Chinese)
Liu, H. P., Mitton, J. B. & Wu, S. K. 1996. Paternal mitochondrial DNA differentiation far exceeds maternal mitochondrial DNA and allozyme differentiation in the freshwater mussel, *Anodonta grandis grandis. Evolution* 50: 952–957.
Machii, A. 1995. *Shinju-monogatari* [*Pearl Tales* (trans. auct)]. 191pp. Shokabo, Tokyo. (in Japanese)
Mack, R. N., Simberloff D. & Londsdale, W. M. 2000. Biotic invasions: causes, epidemiology, global consequences, and control. *Ecological Applications* 10: 689–710.
Martens, E. von. 1861. Die Japanischen Binnenschnecken im Leidener Museum. *Malakozoologische Blätter* 7: 32–61.
Matsuoka, K. 1987. Malacofaunal succession in Pliocene to Pleistocene non-marine sediments, Central Japan. *The Journal of Earth Sciences, Nagoya University* 35: 23–115.
Mooney, H. A. & Cleland, E. E. 2001. The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences, USA* 98: 5446–5451.
Nakanishi, M. & Sekino, T. 1995. Recent drastic changes in Lake Biwa bio-communities, with special attention to exploitation of the littoral zone. *Geo Journal* 40: 63–67.
Nishino, M. & Watanabe, N. C. 2000. Evolution and endemism in Lake Biwa, with special reference to its Gastropod mollusk fauna. *In*: Rossiter, A. & Kawanabe, H. (eds.), *Ancient Lakes*: *Biodiversity, Ecology and Evolution. Advances in Ecological Research* 31: 151–180.
Ogiwara, T. 2002. "The present condition of aquaculture of freshwater pearl mussel in Lake Kasumigaura". *Proceedings of Shijimi Symposium*: 13–17. (in Japanese)
Rambaut, A. 2002. *SE-AL Sequence Alignment Editor, version 2.0a11*. University of Oxford, Oxford.
Rozas, J., Sanchez del Barrio, J. C., Messeguer, X. & Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19: 2496–2497.
Sakai, H., Ujiie, M., Mizutani, E. & Ikeda, I. 1997. Allozyme comparison between Japanese and Chinese Limnetic Pearl Mussels. *Journal of National Fisheries University* 46: 101–104.
Shirai, A. 2008. *Hyriopsis schlegeli* (Martens, 1861) (Bivalvia: Unionidae) from Anenuma, Aomori Prefecture: new locality for a species previously thought endemic to Lake Biwa. *Chiribotan* (*Newsletter of the Malacological Society of Japan*) 39: 25–29. (in Japanese)
Strauss, S. Y., Lau, J. A. & Carroll, S. P. 2006. Evolutionary responses of natives to introduced species: what do introductions tell us about natural communities? *Ecology Letters* 9: 357–374.
Swofford, D. L. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0.* Sinauer Associates, Sunderland, Massachusetts.
Takahashi, S. 1989. A review of the origins of endemic species in Lake Biwa with special reference to the goby fish, *Chaenogobius isaza*. *Journal of Paleolimnology* 1: 279–292.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin F. & Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882.
Ueshima, R. 2002. Simple methods for DNA Preservation in Molluscan specimens. *Venus* 61: 91–94. (in Japanese)
Wang, J.-L., Li, J.-L., Wang, G.-L. & Bai, Z.-Y. 2008. Sequence variation of Ribosomal DNA Internal Transcribed Spacer 1 of *Hyriopsis cumingii* from the Five Freshwater Lakes of China. *Journal of Lake Science* 20: 208–214. (in Chinese with English summary)
Watano, Y., Kanai, A. & Tani, N. 2004. Genetic structure of hybrid zones between Pinus pumila and P. parviflora var. pentaphylla (Pinaceae) revealed by molecular hybrid index analysis. *American Journal of Botany* 91: 65–72.
White, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. *In*: Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (eds.), *PCR Protocols*, pp. 315–322. Academic Press, San Diego.
Winnepenninckx, B., Backeljau, T. & Dewachter, R. 1993. Extraction of high-molecular-weight DNA from mollusks. *Trends in genetics* 9: 407.
Wu, H.-S., Ouyang S., Wu, X.-P., Zhap, D.-X., & Ruan, L.-Z. 2008. The taxonomical status of *Hyriopsis cumingii* and *Hyriopsis schlegerii* inferred from ITS-1 Sequences. *Journal of Nanchang University (Engineering & Technology)* 30: 234–237. (in Chinese with English summary)

(Received June 9, 2009 / Accepted February 8, 2010)

分子データから示された琵琶湖固有の絶滅危惧種イケチョウガイの移入種ヒレイケチョウガイによる遺伝的撹乱

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要　約

琵琶湖固有の絶滅危惧種イケチョウガイと中国からの移入種ヒレイケチョウガイの交雑の実態を探るため，分子マーカーを用いて解析を行った。ミトコンドリアの Cox 遺伝子と核 rRNA 遺伝子の ITS1 領域の塩基配列を用いた系統解析の結果，両種はいずれの分子マーカーでも区別されることが分かった。遺伝的組成を調べたところ，琵琶湖と霞ヶ浦の淡水真珠養殖場では両種の交雑に由来すると考えられる個体がみつかった。琵琶湖の養殖場は自然環境とは完全には隔離されていないため，逸出した養殖個体とイケチョウガイの野生個体との交雑が懸念される。姉沼の野生集団は移植された養殖個体の逸出に由来するものの，唯一交雑の影響を受けていない純粋なイケチョウガイの集団であると考えられるため，保全上非常に重要である。