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Genetic Relationships and Origin of Two Geographic Groups of the Freshwater Threespine Stickleback, 'Hariyo'

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ABSTRACT—'Hariyo' comprise the southernmost freshwater populations of the threespine stickleback, *Gasterosteus aculeatus* species complex, distributed in central Honshu, Japan. Two geographic groups (Gifu and Shiga) of the 'hariyo' have been recognized as differing from each other in some morphological and ecological features. In order to elucidate the genetic characteristics and phylogenetic position of these populations, partial sequences of the mitochondrial cytochrome *b* gene were compared in 123 specimens from 10 'hariyo' populations, and eight other freshwater and anadromous populations of threespine stickleback in Japan. Phylogenetic analysis resolved 22 haplotypes in a single most-parsimonious tree. In contrast to previous allozyme analyses, the haplotype tree indicated that 'hariyo' populations are monophyletic. Each of two geographic groups of the 'hariyo' was also shown to be nearly monophyletic. The two geographic groups differed from each other in sequence by an average of 0.47% and from other populations by 0.70%. Based on a molecular clock, constructed from fossil records and mtDNA genetic distances of *Gasterosteus* and *Pungitius*, it was estimated that the two groups of 'hariyo' differentiated from the other threespine stickleback populations and also from each other in the middle Pleistocene. The results suggested that each of the two groups of 'hariyo' is a distinct evolutionarily significant unit with unique genetic features, as well as morphological and ecological characteristics.

Key words: *Gasterosteus aculeatus*, mitochondrial DNA (mtDNA), cytochrome *b* (cyt *b*), introgression, phylogeography

INTRODUCTION

The threespine stickleback, *Gasterosteus aculeatus*, is a widespread circumboreal, north-temperate species, principally having an anadromous life-style (Wootton, 1976). However, many freshwater (or landlocked) populations also occur, mainly in coastal regions, showing ecological and morphological traits distinguishing them from nearby anadromous populations. 'Hariyo' comprises a group of such freshwater populations, distributed in central Honshu, Japan, the southernmost part of the geographic range of the species in the western Pacific region (Ikeda, 1933; Mori, 1987a). 'Hariyo' has often been recognized as a distinct species or subspecies, because of its typical, freshwater-type morphological characteristics, i.e., small body size and degenerated lateral plates (*leiurus* type), and its distribution far from other anadromous and freshwater populations (Mori, 1987a). For instance, some authors have referred to 'hariyo' as *G. microcephalus* Girard, 1854 (e.g., Hosoya, 1993), although it is unlikely that the name is applicable to 'hariyo' in the context of phylogenetic systematics, owing to the type locality of this nominal species being in California, U. S. A.

The evolutionary origin of 'hariyo' is unclear. Some authors have suggested that the occurrence of threespine stickleback in central Honshu resulted from a southward invasion of ancestral populations during a glacial period, followed by the persistence of relict populations in low-temperature spring areas (Ikeda, 1933) . The origin of 'hariyo', however, may also be preglacial, since 'hariyo' are presently distributed on both the eastern (Nobi Plain, Gifu Prefecture) and western (eastern coast of Lake Biwa, Shiga Pref.) flanks of the Ibuki Mountains, which have comprised two different watersheds since the early Pleistocene (e.g., Kawabe, 1989). This geographic distribution pattern suggests that

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'hariyo' populations may have been subject to genetic divergence, and, in fact, ecological and morphological differences have been revealed between them; e.g., specimens from populations on Nobi Plain ('Gifu group') have a smaller body size, smaller number of lateral plates, smaller eggs and a larger clutch size than those on the eastern coast of Lake Biwa ('Shiga group') (Mori, 1987a, b). These differences imply significant genetic divergence between the two geographic 'hariyo' groups, and a long and unique evolutionary history for each. Considering that many freshwater *G. aculeatus* populations have been independently derived in various places (e.g., Hagen and McPhail, 1970), it is also possible that the two 'hariyo' groups do not share a common origin. Because 'hariyo' is now severely threatened owing to rapid destruction of their spring habitats through human activities (Mori, 1989), it is important to clarify evolutionarily significant units (ESUs; Moritz, 1994) for these populations, from the viewpoint of conservation.

Recently, several studies have been conducted on genetic population structures of threespine stickleback on both global and local scales, with the advance of allozyme electrophoresis and DNA sequencing techniques. According to the allozyme analyses of Haglund *et al*. (1992) and Higuchi and Goto (1996), *G. aculeatus* includes two highly divergent groups; one comprising populations around the Sea of Japan and the other along the western (Japan) and eastern Pacific and Atlantic coasts (North America and Europe). Comparing these studies, however, the estimated phylogenetic position of the two groups of 'hariyo' differed considerably, especially regarding the position of the Shiga population. On the other hand, population structures inferred from mitochondrial DNA (mtDNA) data presented a fundamental conflict with those inferred from allozyme data (Ortí *et al*., 1994; Yamada *et al*., 2001); i.e., the above-mentioned population structure was not evident from the mtDNA data, although relatively small sample sizes and insufficient data in the mtDNA studies failed to give a full picture of the Japanese populations. Yamada *et al*. (2001) also showed, in their mtDNA RFLP analysis, that the 'hariyo' populations had characteristic haplotypes, which implied the divergence of the 'hariyo' from other populations. However, because of the low resolution of the results and the limited number of

Table 1. Specimens of threespine stickleback used for mtDNA analyses

Sample code	Life-style[1)]	Group[2)]	Number of specimens	Locality
Gifu-hariyo				
G-1*	F	P	12	Yamayoke River, Ibi R. system; Nanno
G-2	F	P	6	Tsuya R., Ibi R. s.; Nanno
G-3**	F	P	6	Creek, Ibi R. s.; Ogaki
G-4	F	P	12	Naka R., Ibi R. s.; Ikeda
G-5	F	P	6	Spring pool, Nagara R. s.; Ijira (Shinsei)
Shiga-hariyo				
S-1	F	J/P	6	Spring pool, Lake Biwa; Moriyama
S-2	F	J/P	6	Spring pool, L. Biwa; Notogawa
S-3	F	J/P	6	Inukami R., L. Biwa; Hikone
S-4	F	J/P	5	Jizo R., L. Biwa; Samegai
S-5	F	J/P	12	Small stream, L. Biwa; Santo
Other populations				
I-1	A	J	6	L. Shinji-ko; Matsue, Shimane
I-2	F	P	6	Spring pool; Ono, Fukui
I-3***	F	P	2	Spring pool; Aizu, Fukushima
I-4	A	J	4	Koyoshi R.; Honjo, Akita
I-5	F	P	6	Spring pool; Otsuchi, Iwate
I-6	F	P	4	L. Towada; Akita
I-7	F	P	6	Nishikitappu R.; Tomakomai, Hokkaido
I-8	F	P	12	L. Harutori; Kushiro, Hokkaido
Outgroup				
Pungitius p. pungitius			1	Creek; Kushibiki, Yamagata

[1)] Inferred from localities and body size data in the breeding season; F=freshwater, A=anadromous.

[2)] Genetic group in Higuchi and Goto (1996), inferred from locality; J=Japan Sea group, P=Pacific-Atlantic group, J/P=indeterminable because of inconsistency between Higuchi and Goto (1996) and Haglund *et al*. (1992).

* Reared in an artificial pond from 1993 to1998; ** from 1983 to1987; *** from 1987 to 1999.

populations examined in the previous studies, further genetic data are needed for determining the relative positions of the 'hariyo' populations in the *G. aculeatus* species complex.

To elucidate the population structure and origin of 'hariyo', we examined genetic variation within 'hariyo', and the relationships between 'hariyo' and other populations of threespine stickleback, using specimens sampled from the species' entire geographic range in Japan. Because mtDNA analysis is often sensitive enough to detect intraspecific genetic variations (Avise *et al*., 1987; Avise, 2000), mtDNA sequence data were used in this study. The use of mtDNA sequence data also enabled the present results to be compared with those of a previous study on global population structures of the threespine stickleback using the same approach (Ortí *et al*., 1994).

MATERIALS AND METHODS

A total of 77 specimens from 10 'hariyo' populations (5 each from Gifu and Shiga) and 46 specimens from eight other threespine stickleback populations were used for the mtDNA analyses. One specimen of the ninespine stickleback, *Pungitius pungitius pungitius*, from Yamagata, Japan, was used as the outgroup taxon (Table 1, Fig. 1). Populations G-1 (from Nanno, Yamayoke River) and I-3 (Aizu) had been reared in small artificial ponds over five and twelve years, respectively, having been founded from initial population sizes of fewer than 100 individuals. Population G-3 (Ogaki) originated from a stock once reared in a small pond, and population G-5 (Ijira) had been transplanted from a natural population in Shinsei, Gifu (Nagara R. system). The samples of threespine stickleback included both anadromous (I-1 and I-4) and freshwater types (remainder). The genetic groups ('Japan Sea group' and 'Pacific–Atlantic group') proposed on the basis of allozyme data by Haglund *et al*. (1992) and Higuchi and Goto (1996) were inferred based on locality and assigned to each population (Table 1).

Total genomic DNA was isolated from a piece of muscle tissue stored in 99% ethanol. Tissue was digested by proteinase K at 37°C or 55°C, and DNA purified by standard phenol / chloroform extraction and ethanol precipitation, or using DNA extraction kits (GenElute Mammalian Genomic DNA Kit, Sigma). PCR amplification of a portion of the mitochondrial cytochrome *b* gene (cyt *b*) was conducted in a total volume of 25 μl containing 2.5 μl of 10 x PCR buffer II (Applied Biosystems; ABI), 0.2 mM each dNTP, 0.5 μM of each primer, 2 mM $MgCl_2$, 0.6 units of *Taq* DNA polymerase (ABI) and 1 μl template. The primers used were L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') (Meyer and Wilson, 1990) and H15341 (5'-TTTGATCCTGTTTCATGGAGRAA-3') (Aoyama *et al*., 2000) for the first (5'-) half of the cyt *b* region and L15285 (5'-CCCTAACCCGVTTCTTYGC-3') (Inoue *et al*., 2000) and H15915 (5'-ACCTCCGATCTYCGGATTACAAGAC-3') (Aoyama *et al*., 2000) for the 3'-half of the cyt *b* region.

PCR conditions comprised 30–35 cycles of denaturation (94°C, 15 sec), annealing (48°C, 15 sec) and extension (72°C, 30 sec) on a GeneAmp 2400 or 9700 thermal cycler (ABI). Amplified double-stranded DNA was purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced directly using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (ABI) on an automated DNA sequencer

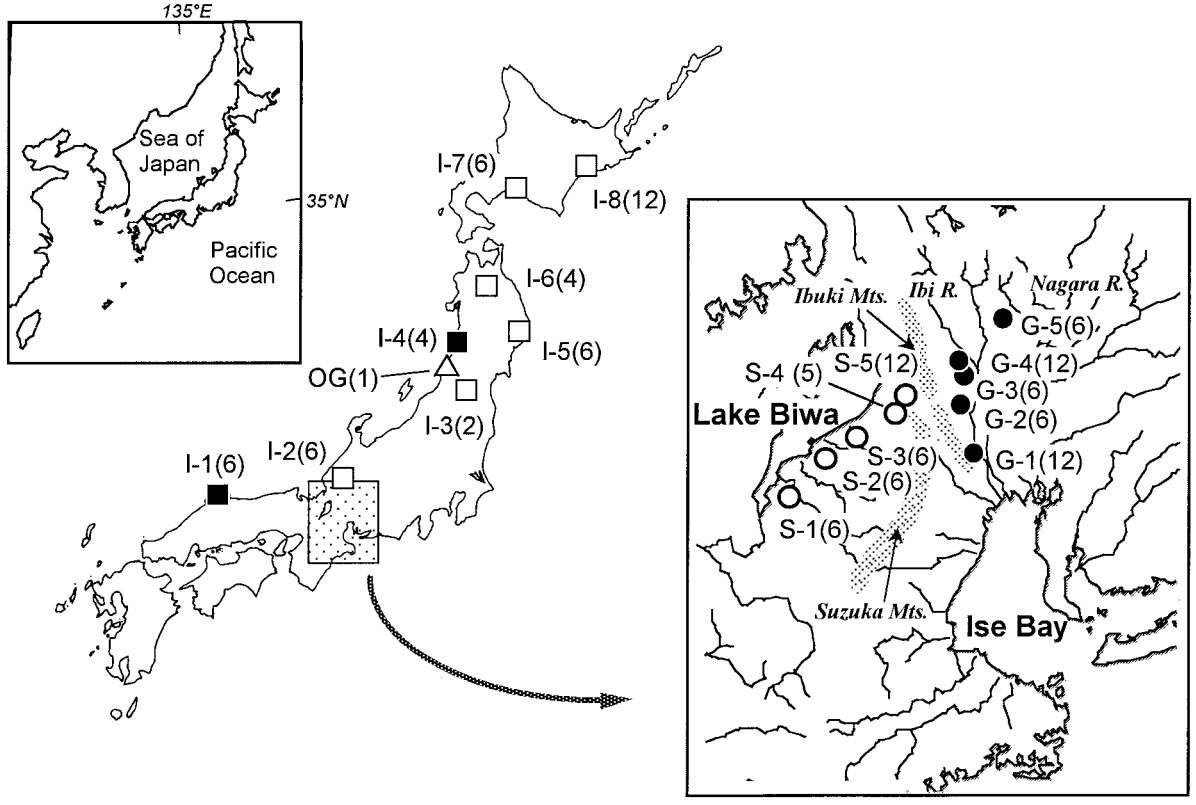

Fig. 1. Sampling localities and codes of 'hariyo' and other threespine stickleback populations. ●, Gifu group of 'hariyo'; ○ Shiga group of 'hariyo'; ■, anadromous populations; □, freshwater populations; △, outgroup (*Pungitius pungitius pungitius*). Number of specimens examined in parentheses.

(ABI 373S or GA310, ABI). Primers used for sequencing were the same as those for PCR.

Because our preliminary experiments had revealed greater variability in the 3'-half of the cyt *b* region, sequences in this region were determined for all specimens. Subsequently, for randomly selected one or two specimens with each haplotype (detected in the first experiment), the 5'-half sequences of the cyt *b* were also determined, then combined sequences defining haplotypes for phylogenetic analysis. Because of lower variability in the 5'-half of the cyt *b*, possible undetected haplotypes with substitutions in this part in the specimens which were not examined were ignored in the analysis. The 5'-half sequence data alone were compared with those from global populations in Ortí *et al*. (1994), which used data from the same sequence region. The nucleotide sequences of all haplotypes detected were deposited with DDBJ / EMBL / GENBANK (accession numbers AB094606–AB094628).

The DNA sequences obtained were edited with the multiple-sequence editor DNASIS (Hitachi). Haplotype diversity (h) and nucleotide diversity values (π, based on pairwise differences) for the 3'-half sequences were estimated to quantify genetic variation, and population structure examined by Φ-statistics and AMOVA (Excoffier *et al*., 1992), using ARLEQUIN ver 2.001 (Schneider *et al*., 2001). Corrected genetic distances between haplotypes were calculated based on the general-time-reversible (GTR) model with six substitution-type parameters (Lanave *et al*., 1984; Rodríguez *et al*., 1990), using PAUP*4.0b8 (Swofford, 2001). Phylogenetic relationships among haplotypes were inferred by the maximum-parsimony (MP) (non-weighted) and maximum-likelihood (ML) methods (F81 [Felsenstein, 1981] and HKY85 [Hasegawa *et al*., 1985] models), using PAUP*, and shown as an unrooted network (for Japanese haplotypes only) or a tree rooted by the outroup (for haplotypes from global populations). One thousand bootstrap replications were conducted so as to evaluate the robustness of the internal branches of the MP tree (Felsenstein, 1985).

RESULTS

Sequence variation

In the first experiment, DNA segments of 629 base pairs (bp) of the mitochondrial cyt *b* gene were amplified by PCR, 576 bp of them being successfully sequenced for all 124 specimens. A total of 25 nucleotide positions (4.3% in 576 bp) varied in the ingroup (*G. aculeatus* including 'hariyo'), these variations defining 22 haplotypes (a–v), which differed from each other by 1–9 (0.2–1.6%) positions.

For one or two specimens with each of the 22 haplotypes, the first half of the cyt *b* sequence was also deter-

Table 2. Variable nucleotide sites from 1093 bp sequences of the partial mitochondrial cytochrome *b* gene in the threespine stickleback

Haplotype	1 1 1 1 2 3 3 4 4 5 5 6 6 6 6 6 7 7 7 7 7 8 8 8 9 9 9 9 9 9 9 9 0 0 8 0 5 6 9 1 4 9 0 8 3 4 3 4 5 6 7 0 3 3 4 5 4 5 8 0 1 2 2 4 4 7 8 1 2 5 4 4 0 0 4 0 7 4 1 5 1 8 3 6 7 9 9 3 9 5 1 7 0 9 5 3 3 4 0 9 0 3 9 7	Samples[1] (N[2])
a	G G T A C A G T T C A T A T C T A G A G T A C C G A C G C T A C G C A	G-2(1), G-4(1), S-5(2)
b	. . . . . G	G-1(12), G-2(3), G-3(6), G-4(6), G-5(3)
c	. . . . . G . . . . . G . . T .	G-2(2), G-4(4)
d	. . . . . G . . . . . T .	G-4(1)
e	A . . . . . G . . . . . C . . . . . T .	G-5(3)
f	A . . . . . G . . . . . C . A . . **G** T . . . . . **A** T .	S-1(4), S-4(2)
g	A . . . . . G . . . . . C . A . . **G** T . . . . . T .	S-1(2), S-4(3), S-5(10)
h	A . . . . **G** . . . . . G . T . . . . C . . . . **G** T . . . . . T .	S-3(6)
i	A . . . . **G** . . . . . G . . . . . C . . . . **G** T . . . . . T .	S-2(6)
j	A **A** C G . . . C . . . . G . . . . A . A C G . . . . T . . . . . T .	I-2(6)
k	A . . . . . C C . . . G . . . . A . A C . . . . . T . . . . . T .	I-1(1)
l	A . . . . . C . . . . G . . . . . A C . . T . . T . . C . . . T .	I-6(4)
m	A . . . . . C . . . . G . . . . . A C . . T . . T . **T** . . . . T .	I-8(1)
n	A . . . . . C . . . . G . . . . . A C . . T A . T . . . . . T .	I-8(1)
o	A . . . . . C . T . . G . . . . . A C . . T . . T . . . . . T .	I-1(2), I-4(2), I-8(1)
p	A . . . . . C C . . . G . . . . . A C . . . . . T . . . . . T .	I-4(1), I-5(6), I-8(1)
q	A . . . . . C C . G . G . . . . . A C . . . . . T . . . . . T .	I-1(1)
r	A . . . T . . C . . . . G . . . . . A C . . . . . T **A** . . G T . T .	I-3(2)
s	A . . . . . A C . . . . G . . C . . . A C . . . . . T . . . . . T .	I-4(1), I-7(6), I-8(5)
t	A . . . . . A C . . . G G . . C . . . A C . . . . . T . . . . . T .	I-1(1), I-8(2)
u	A . . . . . A C . . . . G C . C . . G A C . . . . . T . . . . . T G	I-8(1)
v	A . . . . . A C . . . . G . . C G . . A C . . . . . T . . . . . T .	I-1(1)

Dots indicate same state as in first haplotype.

Numbers refer to position beginning from 5' end.

Underlined and bold letters indicate transversions and non-synonymous substitutions, respectively.

[1] Sample codes correspond to Fig. 1; [2] number of specimens with the same mtDNA haplotype.

mined, a total of 1093 bp being revealed (Table 2). The determined sequences corresponded to the sequence comprising the 75th and following nucleotides of the 747-bp sequence in Ortí *et al*. (1994: fig. 2), the first 674 bp of the former overlapping with the latter (32 out of 35 variable sites in Ortí *et al*. [1994] included). Base composition of the ingroup (A: 24.6%, C: 27.4%, G: 15.3%, T: 32.7% on average) was not remarkably biased and did not differ significantly from that of the outgroup (chi-square test, $P>0.1$). The uncorrected average sequence difference between the ingroup and outgroup was 16.2% (range, 15.9–16.6%), and the average GTR distance between them estimated as 0.189 (0.185–0.193).

A total of 35 nucleotide positions (3.2% in 1093 bp) varied in the ingroup (*G. aculeatus* including 'hariyo') (Table 2), 26 (74% in variable sites) of which were third codon positions, eight (23%) first positions and one (3%) second position; 29 (83%) nucleotide positions involved synonymous substitutions. The ratio of transitions to transversions estimated on the haplotype tree (see below) was 17.5 (35 / 2) for the ingroup.

Relationships between 'hariyo' and other populations

Only one MP tree was obtained for the 22 haplotypes of 1093 bp from the Japanese threespine stickleback with one outgroup haplotype (Fig. 2), being completely consistent with the ML trees based on both F81 and HKY85 models (not shown). The former was strongly supported by a high consistency index (CI=0.946), with only two homoplastic synonymous transitions, although the bootstrap values were not so high (59–81% for major clades) for a relatively small number of variable sites.

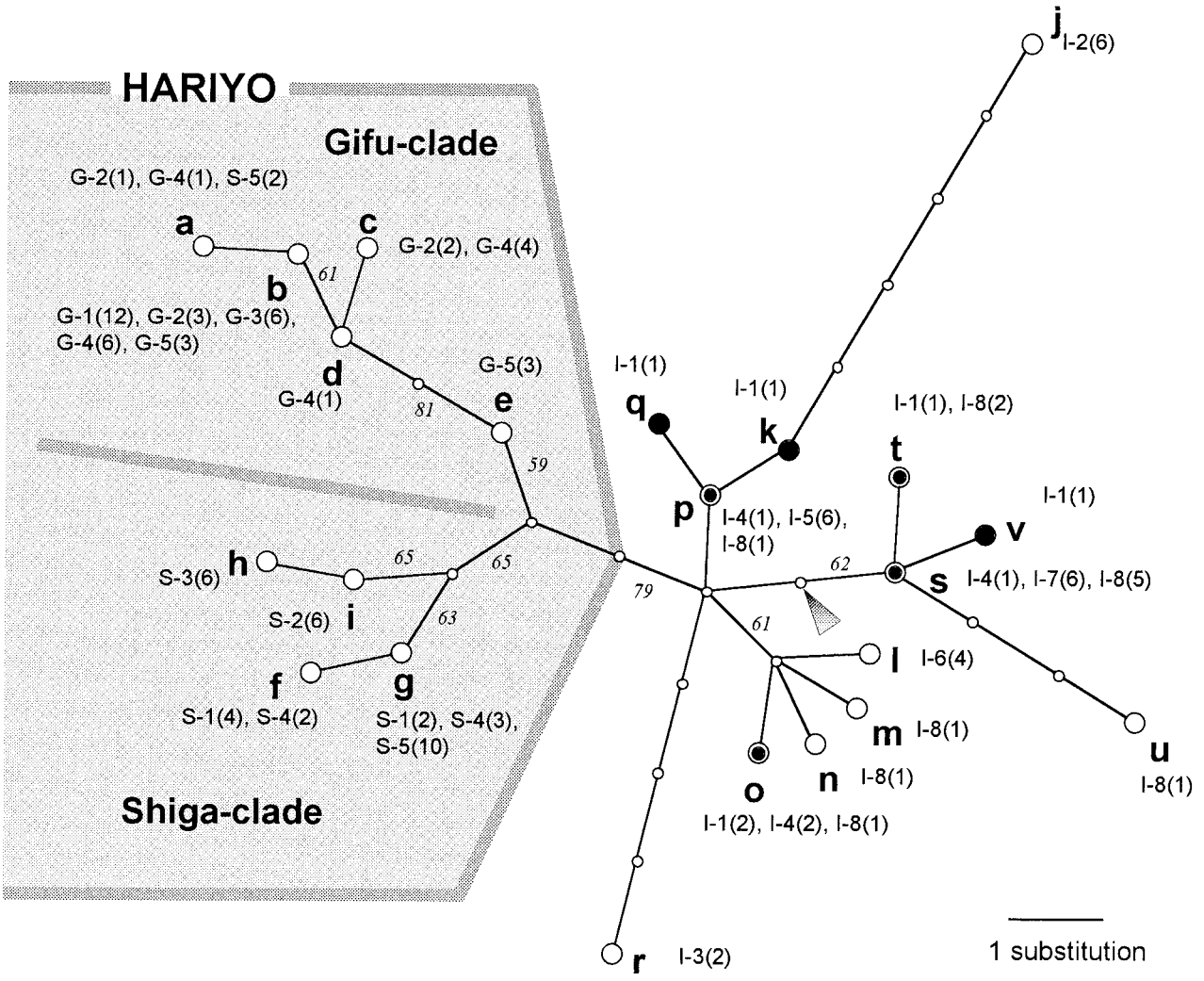

Fig. 2. Maximum-parsimony tree of haplotypes (a–v) detected from 'hariyo' and other threespine stickleback populations (tree length=37, CI=0.946). Sample codes correspond to those in Fig. 1 and Table 1. A 1093-bp sequence of each haplotype was determined for one or two specimens, and 576-bp for the others. The MP tree was calculated using the former sequences. Number of specimens with the same mtDNA haplotype given in parentheses. ○, from freshwater populations; ●, from anadromous populations; ◉, from both freshwater and anadromous populations. Triangle indicates root position estimated using a data set in Ortí *et al*. (1994). Bootstrap probabilities (>50%) of 1000 resamplings shown on internodes.

All 22 haplotypes from Japan detected in the present study were included in the 'Japanese-clade' (Ortí *et al.*, 1994) on the MP tree based on 672-bp sequence data common to the two data sets (Fig. 3). The root of the Japanese clade was located between haplotypes a–r and s–v.

Haplotypes from the Gifu and Shiga groups of 'hariyo' (a–h) formed a monophyletic group, differing from the haplotypes of the other populations (i–v) by 4–12 (0.366–1.10%, 0.697±0.147 SD % on weighted average) substitutions (0.00367–0.0111, 0.00703±0.00149 SD in GTR distance), assuming 1093-bp haplotypes for all specimens (Fig. 2). Pairwise Φ_{ST} was significantly larger than zero in all pairwise comparisons between 'hariyo' populations and all others (permutation tests, $P<0.05$).

The haplotypes i–v from non-'hariyo' populations did not tend to be clustered with haplotypes from the same or geographically close populations, some in fact being shared by geographically distant populations (e.g., haplotype t shared by most distant populations I-1 and I-8). Genetic subdivision between populations corresponding to Japan Sea and Pacific–Atlantic groups (excluding 'hariyo') previously proposed by allozyme studies was not supported by the present mtDNA data (AMOVA: Φ_{CT}=–0.248, $P>0.9$).

Relationships between two 'hariyo' groups

A total of five haplotypes were detected in each of the Gifu (haplotypes a–e) and Shiga groups (a, f–i) of 'hariyo', respectively (Table 2). The haplotypes from each group were completely monophyletic (Gifu-clade and Shiga-clade; Fig. 2), with the exception of two specimens possessing haplotype a, which was included in the Gifu-clade, from population S-2 (Santo). The divergence of the two groups was significant, corresponding to 71% of total variance (AMOVA: Φ_{CT}=0.710, P=0.011). Excluding haplotype a of S-2, the average pairwise sequence difference and GTR distance between individuals of the two groups were 0.468±0.123 SD

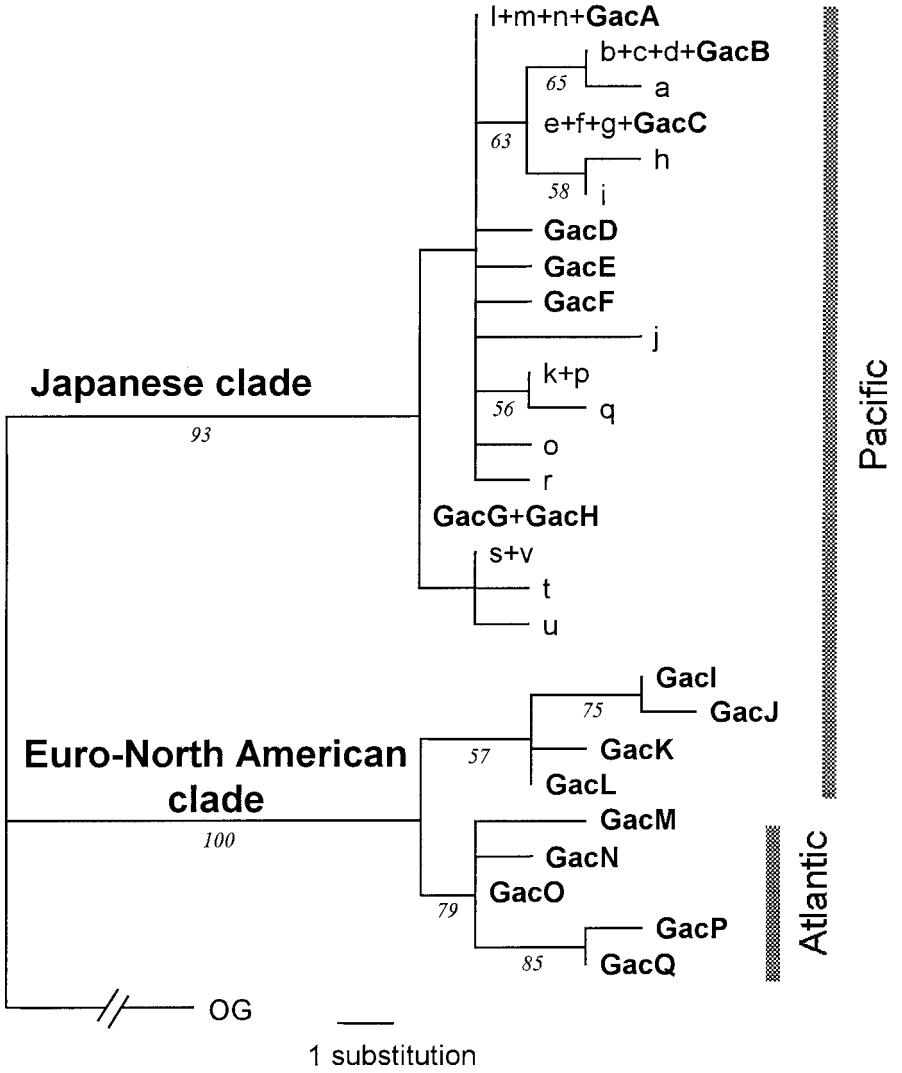

Fig. 3. Maximum-parsimony tree of haplotypes of 672-bp detected in the present study (a–v) and those from Ortí *et al.* (1994) (GacA–GacQ). The root position was estimated by the calculation including an outgroup. Bootstrap probabilities (>50%) of 1000 resamplings shown on internodes.

Table 3. Genetic diversity within populations of the threespine stickleback

Samples[1] (Type[2])	N[3]	No. of haplotypes	Haplotype diversity h±SD	Nucleotide diversity π±SD
G-1* (F)	12	1	0.000±0.000	0.00000±0.00000
G-2 (F)	6	3	0.733±0.155	0.00243±0.00199
G-3* (F)	6	1	0.000±0.000	0.00000±0.00000
G-4 (F)	12	4	0.682±0.102	0.00205±0.00159
G-5 (F)	6	2	0.600±0.129	0.00208±0.00177
S-1 (F)	6	2	0.533±0.172	0.00093±0.00102
S-2 (F)	6	1	0.000±0.000	0.00000±0.00000
S-3 (F)	6	1	0.000±0.000	0.00000±0.00000
S-4 (F)	5	2	0.600±0.175	0.00104±0.00114
S-5 (F)	12	2	0.303±0.148	0.00316±0.00220
I-1 (A)	6	5	0.933±0.122	0.00417±0.00303
I-2 (F)	6	1	0.000±0.000	0.00000±0.00000
I-3* (F)	2	1	0.000±0.000	0.00000±0.00000
I-4 (A)	4	3	0.833±0.222	0.00203±0.00192
I-5 (F)	6	1	0.000±0.000	0.00000±0.00000
I-6 (F)	4	1	0.000±0.000	0.00000±0.00000
I-7 (F)	6	1	0.000±0.000	0.00000±0.00000
I-8 (F)	12	7	0.833±0.100	0.00353±0.00240

[1] Sample codes correspond to Fig.1; [2] A = anadromous type, F = freshwater type;
[3] number of specimens.
* Populations which had undergone a period of artificial maintenance.

% (range, 0.274–0.732%) and 0.00471±0.00124 SD (0.00275–0.00738), respectively, assuming 1093-bp haplotypes for all specimens.

Genetic diversity within populations

Genetic diversity within populations of 'hariyo' was relatively high compared with other freshwater *G. aculeatus* populations (Table 3). Although populations G-1 and G-3, which experienced a period of artificial maintenance, were monomorphic, the other Gifu populations had an average h value of 0.672 (0.600–0.733) and π value of 0.219% (0.205–0.243%). Diversity indices of the Shiga populations were 0.287 (0.000–0.600) and 0.102% (0.00–0.316%), respectively. On the other hand, all freshwater non-'hariyo' populations were monomorphic (h=0, π=0), except for I-8 from Harutori (h=0.833, π=0.353%), which occurs sympatrically with an anadromous population (Mori, 1990). Anadromous populations I-1 and I-4 exhibited high variability (I-1: h=0.933, ≤ π=0.417%; I-4: h=0.833, π=0.203%).

DISCUSSION

Conflict between allozyme and mtDNA data

In order to clarify the global pattern of population structures of threespine stickleback, Ortí *et al*. (1994) used partial sequences of the mitochondrial cytochrome *b* gene, which overlapped in most part with the sequences examined in the present study. They determined that the mtDNA lineage ('Japanese clade'), consisting of all populations on the western Pacific coast and a few freshwater populations on the eastern Pacific coast, was highly divergent from other North American and European populations ('Euro-North American clade'), although the relationships within each clade were unclear. Our data are consistent with their result, all haplotypes in the present study being included in the 'Japanese clade'.

Previous allozyme studies, however, have shown a fundamentally different population structure, i.e., the existence of a 'Japan Sea group', which is highly derived from other Pacific (including Japan) and Atlantic populations ('Pacific–Atlantic group') (Haglund *et al*., 1992; Higuchi and Goto, 1996). Such a divergence between these two groups was detected in neither the present mtDNA data nor the analyses by Ortí *et al*. (1994). Higuchi and Goto (1996) showed that morphological differences supported the two groups revealed by the allozyme analysis and provided genetic evidence of hybridization between the two genetic groups. Based on these data, as claimed by Higuchi and Goto (1996) and Yamada *et al*. (2001), which recently supported the results of Ortí *et al*. (1994) by mtDNA RFLP analysis for western Pacific populations, the allozyme data are considered to be more representative of the organismal phylogeny, the mtDNA results having been caused by introgressive hybridization.

Yamada *et al*. (2001) also supposed that the direction of mtDNA introgression was from the Pacific–Atlantic group to the Japan Sea group, mainly based on the likely smaller population size of the latter, and that the original mtDNA of the latter has been completely displaced by that of the former. Our present result confirmed the mtDNA displacement, by direct comparison with the global data of Ortí *et al*. (1994), but did not provide any more evidence for the direction of the introgression.

Origin of 'hariyo'

Results of the allozyme analysis by Haglund *et al*. (1992) indicated that the Gifu and Shiga groups of 'hariyo' were polyphyletic, the former having been derived from the Pacific–Atlantic group and the latter from the Japan Sea group. In contrast, the allozyme analysis by Higuchi and Goto (1996) showed that both groups of 'hariyo' were included in the Pacific (–Atlantic) group, although the former were not monophyletic. The inconsistency between these studies may have resulted from differences in the kinds of loci analyzed, those in allele frequencies at the same loci and/or other artificial factors. These two allozyme studies, however, both concluded that the two groups of 'hariyo' were not monophyletic.

Our results, however, indicated monophyletic relationships between mtDNA haplotypes from the two groups of 'hariyo', as did also the limited data of Yamada *et al*. (2001). Although this implies a monophyletic origin of 'hariyo', such relationships might have also resulted from introgressive hybridization and displacement of either mtDNA by the other following secondary contact between the Gifu and Shiga groups, which had originated separately. Without further evidence from nuclear genes, it is impossible to determine the ancestral group(s) of the two groups of 'hariyo' from mtDNA data alone.

However, whether or not introgression has occurred, the present mtDNA data indicate that the two groups of 'hariyo' exchanged genes at least at the mtDNA level after diverging from the other threespine stickleback populations, subsequently separating and being isolated long enough for the fixing of diagnostic haplotypes. The divergence in nuclear genes must have occurred over a similar or longer time. Accordingly, we attempted to estimate the divergence time between the two groups of 'hariyo', and between them and other threespine stickleback populations, on the basis of fossil records (Bell, 1994) and genetic distances of mtDNA.

Two specimens from Santo (S-2, Shiga group) shared haplotype a, included in the Gifu-clade, with specimens from the Gifu group. Although this might imply relatively recent, but restricted contact between the two groups, it may also have resulted from artificial transplantation (see Mori and Watanabe, 1990). Excluding the exceptional specimens from Santo, 0.47% sequence differences (GTR distance = 0.0047) existed, on average, between the Gifu and Shiga groups, and 0.70% sequence differences (GTR distance = 0.0070) between these groups and other populations. The divergence between *Gasterosteus* and *Pungitius* has been estimated at around 10 million years ago (Ma) (see Bell, 1994 and Ortí *et al*., 1994). Using this value with the genetic distances between the two genera (see Results), the divergence indices of the partial cytochrome *b* gene used in the present study are calibrated as 1.6% and 0.019 per million years in uncorrected sequence difference and GTR distance, respectively. The divergence times estimated using the two data are very close; 0.25–0.29 Ma between the two groups of 'hariyo' and 0.37–0.43 Ma between 'hariyo' and other populations. This suggests that both divergences occurred in the middle Pleistocene.

Although of uncertain precision, owing to the incompleteness of the fossil record and the rough calibration scale (1 substitution = ca. 0.06 Ma), the estimated divergence time of the two groups of 'hariyo' from each other and together from the other populations both appear to predate the most recent glaciation (Wülm = Wisconsin glaciation; 0.07–0.01 Ma). The ancient Lake Biwa system is considered to have been connected directly with an easterly-located freshwater system (Paleo-Lake Tokai-ko), including the present Nobi Plain (Gifu), until the early Pleistocene, after which the two areas were separated by the Ibuki and Suzuka Mountains (see Kawabe, 1989). This timing of watershed separation, isolating the Gifu and Shiga groups of 'hariyo', is roughly supported by the divergence time estimated by the mtDNA molecular clock.

Evolutionary status of 'hariyo'

The long period of isolation between the two groups of 'hariyo' suggests that each group is a distinctive evolutionary entity. This situation differs from those for most other threespine stickleback populations, which share haplotypes and probably interchange genes even at the present time, although some unique populations of the latter are known. For example, the Aizu population (I-3), which is distributed in an isolated area some 500 m above sea level, is also distinct (possibly at a level equivalent to 'hariyo') from the other populations in having a haplotype (r) differing by five substitutions from the closest haplotype among the remainder. The same situation applies to the Ono population (I-2), which is a 'Pacific' population distributed on the Sea of Japan side (Higuchi and Goto, 1996), characterized by a haplotype (j) differing by five substitutions from the next closest.

As well as the genetic divergence in mtDNA, many morphological and ecological differences between the two groups of 'hariyo', such as development of lateral plates, body size, egg size, fecundity and reproductive ecology, have been reported (Mori, 1987a, b, 1991). However, because these groups have usually been treated as a single phenotypic group or subspecies of *G. aculeatus*, artificial transplantation without consideration for localities has been carried out (Mori, 1989; Mori and Watanabe, 1990). In addition, it is known that fish from populations protected by law

have been illegally traded commercially with intentionally incorrect locality data. Although the taxonomic treatment of the two groups remains undetermined here, for the purposes of conservation, both the Gifu and Shiga groups of 'hariyo', which are both severely threatened by human activities, must be treated as evolutionarily significant units (ESU; Moritz, 1994) distinct from each other and from other *Gasterosteus* populations.

Genetic variation within populations

The comparisons of intra-populational genetic diversity revealed that freshwater populations of the threespine stickleback generally showed less diversity than anadromous populations, five of the six freshwater populations, excluding 'hariyo', being monomorphic in mtDNA sequences (see also table 1 in Taylor and McPhail, 1999), although the sample sizes were limited (N=2–12). This may have resulted from bottleneck effects, because many freshwater populations are, at least in recent years, restricted to small spring ponds or streams. A freshwater population in Harutori Lake has retained high genetic diversity, but this appears to have been maintained by gene flow from a cohabiting anadromous population (Mori, 1990). This explanation is consistent with the fact that allelic frequencies in allozyme data do not differ between the two populations (Higuchi *et al*., 1996).

In contrast with other freshwater populations, the 'hariyo' populations exhibited relatively high genetic diversity in mtDNA, six of the eight natural populations being polymorphic (h=0.303–0.733, π=0.09–0.35%). While the haplotype diversity values of these populations were smaller than those of two anadromous populations (h=0.833–0.933), the nucleotide diversity values of the former were as high as those of the latter (π=0.20–0.42%). This suggests that the number of haplotypes in the 'hariyo' populations has decreased due to some bottleneck effects, although some of the major haplotype lineages have been retained. Furthermore, their genetic diversity implies that the 'hariyo' populations have never been subjected to extreme bottleneck conditions and that the cool spring environments (lower than 20°C in summer) required by them have been maintained for a long time, at least since the middle Pleistocene.

On the other hand, two populations of 'hariyo', which had been kept in small artificial ponds (G-1, G-3), had apparently lost some genetic (mtDNA) diversity, although their original genetic diversity is unknown. In some cultured fishes, such as salmonids (Ferguson, 1995) and ayu (Iguchi *et al*., 1999), remarkable decreases in genetic diversity have been observed over successive hatchery generations, such observations suggesting that genetic diversity is easily lost by rearing in a small pond, even with several dozen founder individuals. This draws attention to the need for the preservation of genetic diversity, in so far as conservation activities for 'hariyo' and other threespine stickleback populations are concerned.

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