Genetic Structure in Wild Populations of Ayu *Plecoglossus altivelis* in Korea and Japan

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Abstract

We investigated the genetic structure of Korean and Japanese ayu *Plecoglossus altivelis* populations by examining 669 individuals from 14 populations using three microsatellite loci. Genetic variation did not differ significantly among the populations examined in terms of allelic number and heterozygosity. Korean populations were genetically close to each other, implying that persistent gene flow has occurred in these populations. This suggests that eastern populations in Korea form a single large population and all of the Korean populations are distinct from the Japanese populations. Pairwise population F_{ST} estimates, principal component analyses, and a neighbor-joining tree showed that genetic separation between the southern and pooled eastern coast populations was probably influenced by restricted gene flow. Hierarchical analysis of molecular variance (AMOVA) revealed a weak but significant genetic structure among three ayu groups (eastern and southern coasts of Korea and the Japan coast), and no genetic variation within groups. The estimated genetic population structure and potential applications of microsatellite markers may aid in the proper management of ayu populations.

Key words: Ayu, Microsatellites, *Plecoglossus altivelis*, Population structure

Introduction

The ayu, *Plecoglossus altivelis*, is widely distributed in Korea and Japan and is an ecologically important inland fish (Han et al., 2003). Two different ecological forms of ayu, an amphidromous form that normally migrates between rivers and the sea and a landlocked form, have different life histories. Both forms exist in Japan, whereas only the amphidromous form is found in Korea (Iguchi et al., 1999; Ikeda and Taniguchi 2002). Recently, the number of ayu returning from the sea has declined, possibly because of environmental degradation in rivers caused by industrial and uncontrolled development. Therefore, knowledge of wild populations is essential for effective natural resource management and the conservation of native aquatic biodiversity (Ryman et al., 1995). Genetic

variation is important for the long-term survival of natural populations because it confers the ability to adapt to environmental changes, thereby increasing fitness (Frankel and Soulé, 1981). A lack of genetic variation caused by inbreeding can be detrimental to fitness. Estimates of genetic variation in wild populations, monitored using appropriate molecular markers, are important for preventing undesirable changes in production. Hence, the biological and genetic characteristics of ayu populations should be evaluated to maintain genetic variation.

To date, isozymes have been widely used as markers in studies of ayu population genetics. Taniguchi et al. (1983) studied genetic variability and differentiation among amphidromous, landlocked, and hatchery populations of ayu in Ja-

pan. Seki and Taniguchi (1985) and Nishida (1985) studied genetic divergence among amphidromous ayu populations in Japan. Although the variability of isozymes is beneficial for population genetic analyses of ayu, isozyme analysis requires careful collection and handling of tissues (Park et al., 1993). Furthermore, the resolution of isozymes is most effective at regional levels (Han et al., 2003). Han et al. (2003) found substantial gene flow that was sufficient to genetically homogenize 11 natural Korean ayu populations. In addition, a limited number of studies have applied genetic analyses to Korean amphidromous ayu populations. Seki et al. (1988) and Sawashi et al. (1998) showed genetic divergence between Korean and Japanese ayu populations, but they only studied four populations in Korea.

Microsatellites are highly polymorphic nuclear loci that have been used successfully in studies of population genetics, pedigree analysis, parentage assignment, and linkage mapping. Among the many types of DNA markers, microsatellites are particularly useful because they are evenly distributed in genomes, have a codominant Mendelian manner of inheritance, and are easily genotyped via PCR. Takagi et al. (1999) demonstrated the great potential of microsatellites as indicators of genetic variability and divergence among ayu populations, finding higher levels of polymorphism than were obtained during previously isozyme analyses.

The present study investigated genetic variation and population structure in natural populations of *P. altivelis* collected from Korea and Japan by analyzing microsatellite loci.

Materials and Methods

Fish samples

Samples of ayu were collected from 10 rivers located in eastern and southern Korea in 1998 (Table 1, Fig. 1). Ayu samples were also collected from the Namdae River and the Wangpi River in 1997. The Kochi River and the Biwa River populations in Japan were studied previously by Takagi et al. (1999) and were compared with the Korean populations. Wild fish were caught at a single location at each site within a few days using a pot, frozen with dry ice, and stored at -20°C until use.

DNA extraction and microsatellite genotyping

For each ayu sample, DNA was extracted from a fin-clip following a slight modification of the methods described by Taggart et al. (1992). Fin tissue was placed in 700 μL TNES-Urea (10 mM Tris-HCl pH 7.5, 1.5 M NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 4 M Urea) and 5 μL of proteinase K (50 μg/μL final concentration). The mixture was then shaken gently and incubated overnight at 37°C. DNA was purified by successive extractions with phenol : chloroform

: isoamylalchol (25:24:1) and chloroform-:-isoamylachol (24:1), respectively. DNA was precipitated with 3 M sodium acetate trihydrate and a double volume of 99% cold ethanol. The precipitate was decanted, washed with 70% ethanol, and air-dried. The DNA pellet was resuspended in 100 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.2) and stored at 4°C prior to PCR analysis.
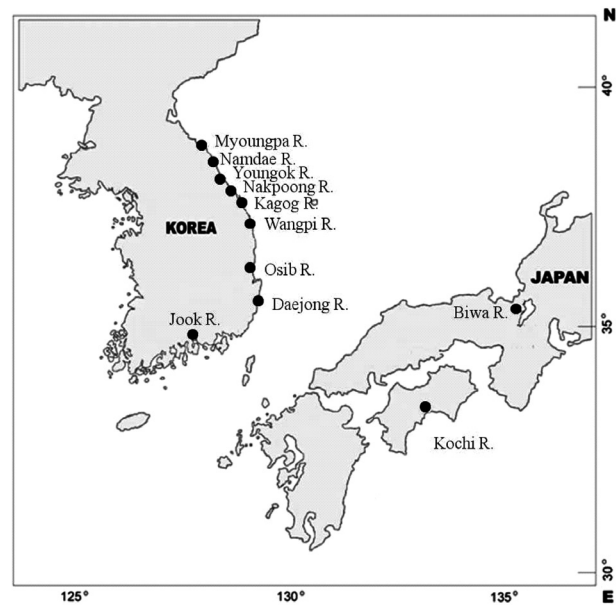

Fig. 1. Sampling locations of 14 ayu populations analyzed in this study (see Table 1 for site names).

Table 1. Sample sites, sample number and sampling date of ayu in the present study

Location	No. of type sample	Sampling date
Korea		
East Sea coast		
Myoungpa R.	Amphidromous: 61	Jun 26, 1998
Puk R.	Amphidromous: 50	Jun 28, 1998
Namdae R. (A)	Amphidromous: 40	Aug 18, 1997
Namdae R. (B)	Amphidromous: 51	Jul 22, 1998
Youngok R.	Amphidromous: 32	Aug 29, 1998
Nakpoong R.	Amphidromous: 22	Jun 17, 1998
Kagok R.	Amphidromous: 39	Jun 19, 1998
Wangpi R. (A)	Amphidromous: 37	Aug 14, 1997
Wangpi R. (B)	Amphidromous: 51	Jun 08, 1998
Osib R.	Amphidromous: 52	Aug 08, 1998
Daejong R.	Amphidromous: 37	Sep 11, 1998
South Sea coast		
Jook R.	Amphidromous: 37	Jul 08, 1998
Japan		
Biwa R.	Landlocked: 80	Apr 20, 1998
Kochi R.	Amphidromous: 80	Aug 28, 1998

For the microsatellite analysis, three primers, *Pal-1*, *Pal-2*, and *Pal-5* (Table 2), were screened using the annealing temperatures and PCR cycles described by Takagi et al. (1997). The forward primer from each primer set was 5-fluorescent labeled with one of three dyes: 6-FAM, HEX, or NED (PE Applied Biosystems, Foster City, CA, USA . PCR amplification of six microsatellite loci was conducted using an RTC 200 instrument (MJ Research, Wiltham, MA, USA in 10 mL of solution containing 10-50 ng DNA, 1× ExTaq buffer, 0.2 mM dNTPs, 10 pmol of each primer, and 0.25 U Taq DNA polymerase (Takara, Ohtsu, Japan . The amplification protocol included an initial denaturation for 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at the optimal annealing temperature (the annealing temperature for each locus is listed in Table 2), and 1 min at 72°C, with a final extension step of 5 min at 72°C. The sizes of fluorescence-labeled allele fragments were measured on an ABI PRISM 3130XL automated sequencer, followed by analysis with GeneMapper version 3.7 (Applied Biosystems).

Data analyses

The genetic diversity of each location was estimated by the number of alleles per locus and observed (Ho) and expected (He) heterozygosities, which were calculated using FSTAT version 2.9.3 (Goudet, 2001) and GENEPOP version 1.2 (Raymond and Rousset, 1995). The inbreeding coefficient, F_{IS}, was calculated in an analysis of variance framework following Weir and Cockerham (1984) using GENEPOP. Departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were calculated using GENEPOP version 1.2 (Raymond and Rousset, 1995). Tests for the occurrence of null alleles were performed with MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004). Pairwise F_{ST} values were used to estimate genetic differentiation between population pairs according to Slatkin (1995) using FSTAT. An analysis of molecular variance (AMOVA) was employed to define the grouping of genetic variation in hierarchical arrangements using Arlequin version 3.11 (Excoffier et al., 2005). Genetic relationships among populations were assessed by principal component analysis (PCA) based on the covariance matrix of gene frequencies using PCA-GEN 1.2 (Goudet, 1999).

In addition, after constructing a genetic distance matrix based on a set of gene frequencies in different populations, which was estimated according to Nei (1972), a NJ tree was constructed for each replicated genetic distance matrix via bootstrapping of 1000 replications using NEIGHBOR in the PHYLIP version 3.5 software package (Felsenstein, 1993).

Results and Discussion

We examined 669 individuals from 14 ayu populations in Korea and Japan using three microsatellite DNA markers. Allele size in base pairs (S), the total number of alleles (A_T), and observed (H_O) and expected (H_E) heterozygosities for the three loci (*Pal-1*, *Pal-2*, and *Pal-5*) for each population are shown in Table 3. The number of alleles per locus for *Pal-1* and *Pal-2* revealed high levels of polymorphism, ranging from 10 to 19 and from 10 to 17, respectively. Average Ho and He values ranged from 0.579 in Nakpoong to 0.765 in Wangpi, respectively, and no linkage disequilibrium was found in the Korean and Japanese populations. These results suggest that all of the microsatellite loci were polymorphic, with differences being detected in the number of alleles and observed heterozygosity in the examined Korean ayu populations. Four of the 12 Korean populations and one Japanese population (landlocked) showed significant deviation from the observed allele frequencies for HWE, suggesting that null alleles were present at some loci, as determined by MICRO-CHECKER.

Pairwise F_{ST} estimates in ayu based on the microsatellites are given in Table 4. Korean populations had high genetic distances when compared with landlocked (Biwa) populations. In addition, high genetic distance was observed between landlocked (Biwa) and amphidromous (Kochi) populations in Japan. The observed results suggest there was low or restricted gene flow between the 13 amphidromous populations (12 Korean populations and one Japanese population) and the landlocked ayu population. This genetic differentiation between the two ecological forms of ayu in Japan has been detected in allozymes (Seki et al., 1985), mitochondrial DNA (Iguchi et al., 1999), and microsatellite markers (Takagi et al., 1999). Multi-locus pairwise estimates

Table 2. Nucleotide sequence of 3 microsatellite PCR primers repeat motif and amplification condition in Korean and Japanese populations

Locus	Repeat motif[*]	Primer sequence (5′-3′)	Annealing temp. (°C)[†]	Alleles number	Size (bp)[‡]
Pal-1	(GT)	F: TGTTTGGGAAGTGGGTGCGGG	52	22 (15)	102-152
		R: AGAAATCCACATCAACATCC			(104-132)
Pal-2	(GT)	F: TCACACTCCCTCACTGGGAC	52	20 (14)	146-190
		R: TTCAGCACACACATTATCTCAC			(158-188)
Pal-5	(CA)	F: TGGCTGTGCTTTATGTGGTC	52	4 (2)	207-213
		R: GGTGGTAGTATGTGGTGTTC			(207-213)

[*]Core repeat motif cloned *Plecoglossus altivelis*, [†]PCR annealing temperature were optimized for *Plecoglossus altivelis*, [‡]Estimated size of the PCR fragment when compared with M13 sequence fragment of known length.

Table 3. Genetic variabilities at 3 loci of microsatellite DNA in Korean and Japanese ayu

Population		Locus			
		Pal-1	*Pal-2*	*Pal-5*	ALL
Korea					
Myoungpa	S	100-138	160-190	207-213	
	A_T	19	16	3	38
	H_O	0.830	0.939	0.370	0.705
	H_E	0.895	0.918	0.335	0.708
	P	NS	NS	NS	NS
	F_{IS}	0.072	-0.022	-0.107	0.004
Puk	S	100-150	156-182	207-213	
	A_T	18	13	3	34
	H_O	0.723	0.864	0.383	0.652
	H_E	0.889	0.861	0.376	0.705
	P	***	NS	NS	***
	F_{IS}	0.186	-0.003	-0.019	0.075
Namdae (A)	S	102-138	160-188	207-213	
	A_T	16	12	3	31
	H_O	0.816	0.923	0.225	0.650
	H_E	0.906	0.898	0.346	0.712
	P	*	NS	*	**
	F_{IS}	0.099	-0.028	0.349	0.087
Namdae (B)	S	104-138	160-190	207-213	
	A_T	18	13	3	34
	H_O	0.723	0.864	0.383	0.652
	H_E	0.889	0.861	0.376	0.705
	P	***	NS	NS	***
	F_{IS}	0.186	-0.003	-0.019	0.075
Youngok	S	104-136	160-186	207-213	
	A_T	14	11	3	28
	H_O	0.690	0.871	0.419	0.659
	H_E	0.873	0.836	0.402	0.700
	P	*	NS	NS	NS
	F_{IS}	0.210	-0.043	-0.042	0.057
Nakpoong	S	102-150	160-186	207-213	
	A_T	10	10	3	23
	H_O	0.824	0.722	0.273	0.579
	H_E	0.855	0.848	0.384	0.671
	P	NS	NS	NS	NS
	F_{IS}	0.037	0.184	0.290	0.137
Kagok	S	104-150	160-184	207-213	
	A_T	17	12	3	32
	H_O	0.750	0.852	0.308	0.642
	H_E	0.903	0.855	0.384	0.721
	P	**	NS	NS	**
	F_{IS}	0.170	0.004	0.198	0.109
Wangpi (A)	S	104-136	156-182	207-213	
	A_T	13	14	3	30
	H_O	0.882	0.931	0.242	0.677
	H_E	0.889	0.889	0.249	0.669
	P	NS	NS	NS	NS
	F_{IS}	0.008	-0.048	0.025	-0.013
Wangpi (B)	S	104-152	160-190	207-213	
	A_T	13	12	3	28
	H_O	0.868	0.951	0.444	0.765
	H_E	0.857	0.877	0.429	0.730
	P	*	NS	NS	NS
	F_{IS}	-0.014	-0.085	-0.036	-0.048
Osib	S	104-136	156-190	207-213	
	A_T	18	13	3	34
	H_O	0.723	0.864	0.383	0.652
	H_E	0.889	0.861	0.376	0.705
	P	*	NS	NS	NS
	F_{IS}	0.186	-0.003	-0.019	0.075

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Table 3. Continued

Population			Locus			
			Pal-1	*Pal-2*	*Pal-5*	Mean
	Daejong	S	104-132	160-188	207-213	
		A_T	12	12	3	27
		H_O	0.750	0.857	0.314	0.642
		H_E	0.858	0.728	0.345	0.679
		P	NS	NS	NS	NS
		F_{IS}	0.126	-0.036	0.090	0.055
	Jook	S	104-138	146-188	207-213	
		A_T	16	15	3	34
		H_O	0.765	0.806	0.324	0.626
		H_E	0.849	0.880	0.309	0.673
		P	NS	NS	NS	NS
		F_{IS}	0.100	0.085	-0.050	0.069
Japan						
	Biwa	S	96-140	160-204	207-219	
		A_T	18	17	3	38
		H_O	0.814	0.797	0.431	0.682
		H_E	0.898	0.876	0.504	0.761
		P	NS	NS	*	*
		F_{IS}	0.094	0.090	0.145	0.104
		S	104-140	160-194	207-213	
		A_T	16	17	2	35
	Kochi	H_O	0.814	0.848	0.317	0.687
		H_E	0.912	0.898	0.370	0.726
		P	NS	NS	NS	NS
		F_{IS}	0.014	0.056	0.144	0.053

Size in base pair of alleles (S), total number of alleles (A_T), Observed (H_O) and expected
(HE) heterozygosities, probability value estimates regarding deviation from Hardy-Weinberg equilibrium (P) and inbreeding coefficient (F_{IS}).
Departure from Hardy-Weinberg equilibrium: NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. F_{ST} values between samples (below diagonal) and probability of differentiation with *P* value in F_{ST} estimate (above diagonal)

	Myoungpa	Puk	Namdae (A)	Namdae (B)	Youngok	Nakpoong	Kagok	Wangpi (A)	Wangpi (B)	Osib	Daejong	Jook	Biwa	Kochi
Korea														
Myounpa		-	-	-	-	-	-	-	-	-	-	+	+	+
Puk	0.006		-	-	-	-	-	-	-	-	-	+	+	+
Namdae (A)	0.005	0.001		-	-	-	-	-	-	-	-	-	+	+
Namdae (B)	0.005	-0.010	0.001		-	-	-	-	-	-	-	+	+	+
Youngok	0.016	-0.001	0.003	-0.001		-	-	-	-	-	-	+	+	+
Nakpoong	0.011	0.007	0.013	0.007	0.007		-	-	+	-	-	+	+	+
Kagok	0.008	0.002	0.011	0.002	0.010	0.016		-	-	-	-	+	+	+
Wangpi (A)	0.002	-0.002	0.007	-0.002	0.007	0.001	0.011		-	-	-	+	+	+
Wangpi (B)	0.020	0.003	0.012	0.003	0.006	0.018	0.010	0.011		-	-	+	+	+
Osib	0.006	-0.010	0.001	-0.010	-0.001	0.010	0.002	-0.002	0.003		-	+	+	+
Daejong	0.008	-0.001	0.004	-0.001	-0.003	0.010	0.013	0.002	0.008	-0.001		+	+	+
Jook	0.011	0.012	0.006	0.012	0.014	0.026	0.020	0.019	0.022	0.012	0.016		+	+
Japan														
Biwa	0.109	0.107	0.114	0.107	0.122	0.131	0.086	0.128	0.092	0.107	0.129	0.128		+
Kochi	0.020	0.029	0.022	0.030	0.039	0.048	0.019	0.040	0.040	0.029	0.043	0.029	0.071	

+, significant; −, not significant in F_{ST}. Significance was tested at the 5%.

of F_{ST} showed that differences between Korean and Japanese amphidromous populations were significant, although they had low F_{ST} values (ranging from 0.019 to 0.048). These findings were also evident in the PCA scatter plots (Fig. 2). The first two axes together explained 78% of the total genetic variation. The first (PC1) and second (PC2) axes explained 66% ($P = 0.05$) and 12% ($P < 0.005$) of the variance among the populations, respectively, and Japanese and Korean populations were separated. This implies a distinct geographical difference between Korea and Japan. Genetic differentiation between Korean and Japanese ayu populations was confirmed by Seki et al. (1988). Iguchi et al. (1999) found that although a phylogenetic tree with one Korean and six Japanese ayu populations showed few associations with geographic locations, there was a larger extent of net nucleotide substitutions between Korean and Japanese samples.

The lack of differentiation (Table 4) and the PCA indicated (Fig. 2) that there were no geographical trends among the populations on the East Sea coast of Korea. Indeed, matrices of linearized genetic distance (F_{ST}) and geographical distance (G) in kilometers between samples within the eastern Korean populations were compared statistically to assess the effect of isolation by distance. The extent of isolation by distance could not be inferred from scatter plots of F_{ST} on G. This analysis found no significant correlations with distance for populations in eastern Korea (data not shown), suggesting there was a single large population. This is thought to represent the influence of a certain
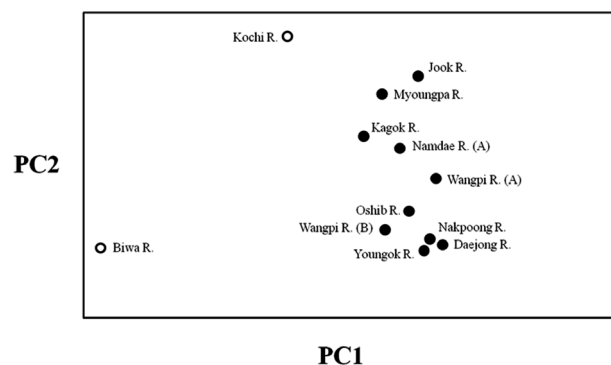
level of gene flow through migration. The lack of regional equilibrium with isolation by distance within the Korean populations suggests that they may still remain in an unstable condition because the equilibrium pattern with isolation by distance should require a sufficiently long period of time to achieve a stable condition (Hutchison and Templeton, 1999). However, pairwise population F_{ST} estimates between the Jook population (South Sea coast) and all other populations were weak (F_{ST} values for all pairs were lower than 0.05), but there was substantial differentiation in microsatellite variation with significant P values (< 0.05) compared to values for all other population pairs (Table 4), suggesting that significant population subdivisions existed at small spatial scales.

The genetic structure of ayu populations in Korea and Japan was estimated by AMOVA (Table 5). The variation within three groups (East Sea coast, South Sea coast, and Japan coast) was 2.10% ($F_{CT} = 0.021$, $P < 0.05$), suggesting the possibility of substructure among the populations. Analysis of variation within the three groups found no genetic variation within the groups (0.40%; $F_{SC} = 0.004$, $P = 0.111$); 97.5% of the total variation was due to differences within populations ($F_{ST} = 0.025$, $P < 0.001$). The hierarchical pattern of genetic differentiation among groups of ayu on the Korean and Japanese coasts indicates there were weak but historical patterns of isolation and restriction on gene flow. Pairwise population F_{ST} estimates and AMOVA results were consistent with previous results based on isozyme data (Han et al., 2003), although few associations with geographic locations in amphidromous ayu populations were found in the present study. These results suggest that microsatellite loci can provide a powerful method for revealing genetic variation, with increased accuracy and resolution compared with isozyme markers.

The populations examined in this study were clustered using the neighbor-joining (NJ) method (Fig. 3). All of the Korean populations were separated from the two Japanese populations. The Jook River population was also separated from the cluster of the other Korean populations. Our findings support the pattern of genetic structure in wild ayu populations in Korea and Japan that has been revealed by genetic analyses (pairwise population F_{ST} estimates and AMOVA).

In conclusion, our results suggest that the ayu populations on the East Sea coast of Korea form a single population, and all of

Fig. 2. Principal component analysis plotting the relationships between Korean (●) and Japanese (○) ayu populations.

Table 5. Analysis of molecular variance (AMOVA) based on microsatellite DNA variation in amphidromous ayu

Source of variation	%	Φ	P-value
Among three regional groups of Amphidromous ayu	2.10	0.021	< 0.05
(East Sea [11], South Sea [1] coasts of Korea and Japanes population [1]			
Among populations within group	0.40	0.004	0.111
Within population	97.50	0.025	< 0.001

The percentage of variance (%), probability estimated from permutation (P), and the F-statistics (Φ) are given at each hierarchical level. Numbers in parenthesis correspond to populations sampled in Fig. 1.
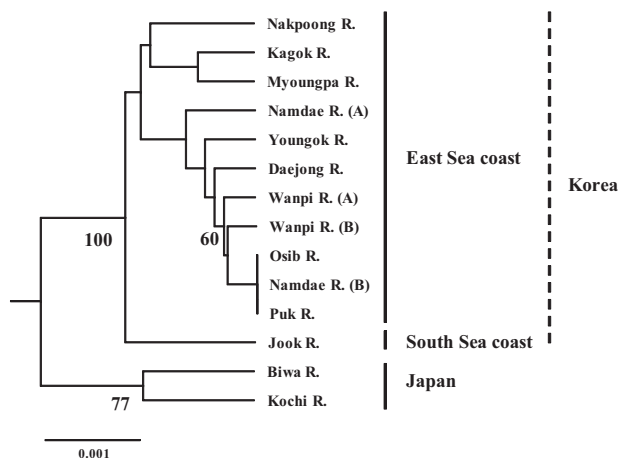
Fig. 3. Neighbor-joining tree of ayu populations. Nodal values for bootstrap support over 50% of the 1,000 replicated trees.

the Korean populations are distinct from the Japanese populations. The observed importance of genetic variation and genetic structure will provide a means for defining evolutionary and conservation units for the management and sustainable use of ayu resources. Further analyses of other genetic markers, such as maternally inherited mitochondrial DNA, and studies that include more populations from other areas of Japan would help identify gene flow patterns in ayu.

Acknowledgments

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