Lack of mitochondrial gene flow between populations of the endangered amphidromous fish *Plecoglossus altivelis ryukyuensis* inhabiting Amami-oshima Island

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**ABSTRACT:** The Ryukyu-ayu, *Plecoglossus altivelis ryukyuensis*, is an amphidromous fish that is endemic to Amami-oshima Island in southernmost Japan. Its abundance, however, has been appreciably reduced during the last two decades such that the subspecies is now considered to be endangered. The variation of the mitochondrial DNA control region was investigated among specimens of the extant populations in the eastern and western parts of Amami-oshima Island (Sumiyo Bay and Yakeuchi Bay areas, respectively), using restriction fragment length polymorphism analysis in order to estimate the extent of gene flow between the two areas. Of a total of 165 fish including temporally different samples, four haplotypes were detected and each area possessed the two haplotypes. However, the common haplotypes shared between these areas were not observed, which indicates that recent gene flow has not occurred between these populations. The nucleotide divergence between populations was much higher than the nucleotide diversity within each population, and the neighbor-joining phylogram among haplotypes showed that the haplotypes are associated with their geographic area. These results suggest that the two populations of Ryukyu-ayu on Amami-oshima Island have been historically formed.

**KEY WORDS:** amphidromous, conservation unit, endangered fish, gene flow, isolation, mitochondrial DNA, PCR-RFLP, Ryukyu-ayu.

**INTRODUCTION**

The ayu in the Ryukyu Archipelago, known as the Ryukyu-ayu, is described as a distinct subspecies, *Plecoglossus altivelis ryukyuensis*, based on the morphological and substantial genetic differentiation from the typical subspecies populations *P. a. altivelis* of the Japanese Archipelago and the Korean Peninsula.1-4 This endemic fish has an annual and semelparous life history, wherein an amphidromous migration between rivers and the sea occurs.5 Hatching occurs through late autumn to early winter in the lower reaches of streams, and larvae drift downstream to the sea where they feed on zooplankton until migrating upstream the next spring.5-8

Intensive surveys have failed to find this subspecies on Okinawa Island, leading to the conclusion that Okinawa Island populations have become extinct by the habitat destruction (e.g. river improvement or changing water conditions).9 Thus, only Amami-oshima is its extant habitat. Recent surveys of the distribution and abundance of this fish on the island have revealed that it is distributed within two areas: (i) the Sumiyo Bay area in the south-eastern part; and (ii) the Yakeuchi Bay area in the south-western part.3-11 Each year, the juveniles migrate upstream and spawning sites appear in the four rivers (Kawauchi, Sumiyo, Yakugachi, and Yanma) in the area of the Sumiyo Bay, while stable upstream migration and spawning sites in the Yakeuchi Bay area have been observed in only the Kawauchi River.3,12 Recently, these populations also have faced habitat loss caused by river improvement and typhoons. In view of their threatened condition, these populations are deemed to be endangered13 and to require further investigation to establish protective measures.

An effective strategy for the conservation of a particular species should, in part, be determined by information regarding its genetic structure, especially the spatial distribution of variability.14
One way in which evolutionary theory and genetics should complement ecologic approaches to conservation is the use of molecular approaches to augment studies of demography and metapopulation processes. Such integration will facilitate strategies that are consistent with ecologic concerns and the maintenance of evolutionary processes.

For the Ryukyu-ayu populations in Amami-oshima Island, relatively lower genetic variability than the other subspecies (P. a. altivelis), suggesting that founder or bottleneck effects have occurred and remarkable genetic differentiation between the populations of the Yakeuchi Bay and Sumiyo Bay have been revealed based on the data of mostly nuclear molecule markers (i.e. allozyme, minisatellite DNA fingerprinting, amplified fragment length polymorphism (AFLP) and microsatellite DNA). These results of genetic differentiation have been described as the frequencies of alleles shared between the two populations. The frequency difference of shared alleles suggests that the gene flow between the populations has been exactly more or less restricted, but a complete lack of present gene flow is not always supported without the detection of absence of shared alleles between populations. Therefore, it is unclear whether the present gene flow between the two extant populations of Ryukyu-ayu in Amami-oshima Island is low but is still present.

The high mutation rates of mitochondrial DNA (mtDNA) can produce intraspecific polymorphism and deep interspecific divergence in relatively short evolutionary times. Maternal transmission and the absence of recombination can result in an effective population size at equilibrium that is reduced to 25% of that estimated by nuclear markers, and therefore has a greater sensitivity to divergence of distinct genetic populations due to random genetic drift. The control region, the unique long non-coding nucleotide sequence, is the most variable portion of mtDNA. Restriction fragment length polymorphisms (RFLPs) or single nucleotide polymorphisms (SNPs) of this region can provide useful information for the genetic structure and historical demography of the populations.

In our previous study we reported that the polymerase chain reaction (PCR)-RFLP haplotypes of the mtDNA control region were not shared between the two populations of the Ryukyu-ayu and suggested that there is little or no gene flow between the populations. However, this suggestion has not been necessarily conclusive because of the small sample sizes and limited sampling locations (21 and 8 samples from each river in the Yakeuchi Bay and Sumiyo Bay areas, respectively), as was the case for the other studies that reported the genetic differentiation between the two populations.

Here, we sampled the Ryukyu-ayu from all four rivers (Kawauchi, Sumiyo, Yakugachi, and Yanma) in the Sumiyo Bay and the one river (Kawauchi) in the Yakeuchi Bay on Amami-oshima Island again, and used PCR-RFLP of mtDNA to elucidate the lack of present gene flow between the two populations. Consequently, we show that recent mitochondrial gene flow between the two populations of the Ryukyu-ayu on Amami-oshima Island has not occurred, and we discuss the status of these populations for the conservation of this endangered amphidromous fish through the analysis of phylogenetic relationships among haplotypes detected.

**MATERIALS AND METHODS**

**Sampling**

Sampling locations are shown in Fig. 1. Specimens of the Ryukyu-ayu P. a. ryukyuensis were collected from four rivers of the Kawauchi (KAW; 33 specimens), the Sumiyo (SUM; 30), the Yankma (YAN; 30), and the Yakugachi (YAK; 33) running into Sumiyo Bay in the south-eastern part of Amami-oshima Island, and the Kawauchi River (WAU; 33) running into the Yakeuchi Bay in the south-western part of the island in June–July 2000. All specimens were in the adult or semi-adult stages swimming in the midstream of the rivers, and were caught by a casting net. This sampling was carried out under the
permission of the Uken and the Sumiyo Village Offices in Amami-oshima Island, and the Department of Fishery Management in the Kagoshima Prefectural Office. Specimens were transported in dry ice to Laboratory of Applied Population Genetics, Tohoku University and were kept at −50°C until genomic DNA extraction.

Total genomic DNA was extracted following a method slightly modified from that of Asahida et al. A portion of caudal fin was placed in 500 μL TNES-urea (10 mM Tris/HCl pH 7.5, 1.5 M NaCl, 10 mM EDTA, 0.5% SDS and 8 M urea) and 10 μL of proteinase K (50 μg/mL final concentration). After a gentle shake the mixture was incubated overnight at 37°C. The DNA was purified by a successive extraction with phenol: chloroform: isomylalcohol (25: 24:1) and chloroform:isomylalcohol (24:1), respectively. The DNA was precipitated with 3 M sodium acetate trihydrate and a double volume of 99% ethanol. The precipitate was decanted, washed with 70% and 99% ethanol, and air-dried. The DNA pellet was resuspended in 50 μL TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0) and preserved in 4°C until use.

The PCR-RFLP of mtDNA

The amplification for the mtDNA control region and detection of the RFLP were examined following the protocol described by Ikeda and Taniguchi. The fragment of approximately 1.5 kbp containing the control region was amplified. Primer sequences are as follows: L15923 (5′-TTAAAGCATCGGTCTTGTAA-3′) and H1067 (5′-TATAGTGGGTATCTAATCCCAGTT-3′). These primers bind to a part of the tRNA Th and 12SrRNA gene regions, respectively. For amplification, the following reagents were added to each microtube: 1.5 μL of template DNA, 5 μL of 10 × buffer (100 mM Tris/HCl pH 8.3, 30 mM MgCl₂, 500 mM KCl), 1 μL of each primer (100 pmol), 5 μL of 2 mM of each dNTP and 0.5 U of rTaq DNA polymerase (Takara, Otsu, Japan). Each sample was brought up to 50 μL with sterile de-ionized H₂O. Amplifications were performed using a Thermal Cycler 480 (Takara, Otsu, Japan) with the following protocol: 94°C for 1 min; 94°C for 1 min, 48°C for 1 min, 72°C for 1 min (30 cycles); 72°C for 5 min.

The amplified samples were subjected to endonuclease digestion using the four-base recognition enzymes Alu I, Hae III, Hha I,Msp I, Rsa I, Taq I, and the five-base recognition enzyme Hin f I (Nippon Gene, Toyama, Japan). Digestions were performed directly in the PCR buffer at 37°C (65°C for Taq I) for at least 5 h. The DNA fragments were separated in a 3.0% (W/V) NuSieve 3:1 agarose gel (BMA, Rockland, ME, USA), stained with ethidium bromide, and photographed. A composite mtDNA haplotype, consisting of seven letters that represent the fragment pattern generated by each of the restriction endonucleases, was compiled for each specimen.

Data analysis

Heterogeneity for haplotype distributions among sample locations was analyzed using the GENEPOP 3.1 package employing the Markov chain method to obtain unbiased estimates of log-likelihood (G)-based exact tests through 1000 iterations. A presence–absence matrix of fragments for each composite haplotype was constructed using the REAP 4.0 software package. This matrix was then used for a number of analyses in the REAP program: the number of base substitutions per nucleotide site (d) among composite haplotypes, haplotype diversity (h) for each sample location, and nucleotide diversity within each sample location (π) and divergence between the locations (dsg). An unrooted neighbor-joining phylogram of the haplotypes based on the d value matrix was constructed using the NEIGHBOR and DRAWTREE programs in the PHYLIP 3.5 package.

RESULTS

Representative restriction morphs and the fragment sizes in each endonuclease are shown in Table 1. Of the 165 Ryukyu-ayu specimens, four mtDNA haplotypes were resolved by the RFLPs for the Hin f I, Rsa I, and Taq I enzymes. The haplotype frequencies in each sample location are shown in Table 2 and Fig. 2. The four rivers in the Sumiyo Bay area (KAW, SUM, YAK, and YAN) possessed two common haplotypes (II and III). The two different generation samples collected from the Kawauchi River (WAU and WAU-93) in the Yakeuchi Bay area also had two haplotypes (I and IV), but a common haplotype between the two areas was not observed. The respective haplotypes of I, III, IV represent the haplotypes of 11, 14, 16, which we reported in the previous study. The haplotype II in the Sumiyo Bay area was newly detected in the present study. Log-likelihood (G)-based exact tests for heterogeneity of haplotype distributions pro-
duced highly significant differences overall among sample locations \((P = 0.000)\). However, the all pairs of samples within the each area, that is, each combination of the KAW, SUM, YAK, and YAN in the Sumiyo Bay area, and the WAU and WAU-93 in the Yakeuchi Bay area had no significant differences, with \(P\) values ranging from 0.053 to 1.000.

The haplotype \((h)\) and nucleotide diversity \((\pi)\) in each sample location are shown in Table 3. Haplotype diversity ranged from 0.186 (SUM) to 0.460 (YAK) with an average of 0.356 in the Sumiyo Bay area, and 0.304 and 0.257 with an average of 0.281 in the Yakeuchi Bay area. Significant differences were not observed among all pairs of the \(h\) values in each sample location tested using Student’s \(t\)-test with the Bonferroni correction for multiple comparisons (initial \(\alpha = 0.05\); 15 tests = 0.0033). However, the average value of nucleotide diversity

### Table 1  Fragment patterns produced by digestion of PCR products with seven restriction endonucleases

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>Restriction morph and fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu I</td>
<td>a: 560 + 260 + 170 + 120 + 100 + 90</td>
</tr>
<tr>
<td>Hae III</td>
<td>a: 770 + 510 + 110 + 80 + 60</td>
</tr>
<tr>
<td>Hha I</td>
<td>a: 830 + 670</td>
</tr>
<tr>
<td>Hinf I</td>
<td>a: 930 + 260 + 160 + 70 + 60</td>
</tr>
<tr>
<td></td>
<td>b: 930 + 260 + 200 + 70</td>
</tr>
<tr>
<td>Msp I</td>
<td>a: 440 + 210 + 150 + 130 + 110</td>
</tr>
<tr>
<td></td>
<td>b: 1120 + 260</td>
</tr>
<tr>
<td>Rsa I</td>
<td>a: 1380</td>
</tr>
<tr>
<td></td>
<td>b: 1120 + 260</td>
</tr>
<tr>
<td>Taq I</td>
<td>a: 1230 + 110 + 90</td>
</tr>
<tr>
<td></td>
<td>b: 1340 + 90</td>
</tr>
<tr>
<td></td>
<td>c: 1230 + 180 + 90</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction.

### Table 2  Distribution of mtDNA haplotype of the Ryukyu-ayu at each sample location on Amami-oshima Island

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Sumiyo Bay</th>
<th>Yakeuchi Bay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KAW  n=30</td>
<td>SUM  n=30</td>
</tr>
<tr>
<td>I (aaaaaac)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II (aaaaaba)</td>
<td>0.233</td>
<td>0.100</td>
</tr>
<tr>
<td>III (aaaaabc)</td>
<td>0.767</td>
<td>0.900</td>
</tr>
<tr>
<td>IV (aaaaab)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

KAW, Kawauchi River; SUM, Sumiyo River; YAK, Yakugachi River; YAN, Yanma River; WAU, Kawauchi River.

Composite designations are as follows: \(Alu I\), \(Hae III\), \(Hha I\), \(Hinf I\), \(Msp I\), \(Rsa I\), and \(Taq I\).

mtDNA, mitochondrial DNA.

### Table 3  mtDNA haplotype diversity \((h)\) and nucleotide diversity \((\pi)\) within sample location

<table>
<thead>
<tr>
<th>Area</th>
<th>Sample location</th>
<th>(h \pm SE)</th>
<th>(\pi \times 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sumiyo Bay</td>
<td>KAW</td>
<td>0.370 ± 0.084</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>SUM</td>
<td>0.186 ± 0.089</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>YAK</td>
<td>0.460 ± 0.061</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>YAN</td>
<td>0.409 ± 0.083</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Average ± SD</td>
<td>0.356 ± 0.119</td>
<td>0.049 ± 0.017</td>
</tr>
<tr>
<td>Yakeuchi Bay</td>
<td>WAU</td>
<td>0.304 ± 0.094</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>WAU-93</td>
<td>0.257 ± 0.110</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>0.281</td>
<td>0.106</td>
</tr>
</tbody>
</table>

mtDNA, mitochondrial DNA.
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The nucleotide divergence among sample locations \( \left( d_A \times 100 \right) \) in the Sumiyo Bay area ranged from 0.042 to 0.058 and averaged 0.052, and was 0.110 between temporally different samples of the Kawauchi River (WAU and WAU-93). These values were almost equal to the nucleotide diversity within each sample location. In contrast, the values of the pairs of sample locations belonging to the different areas ranged from 0.860 (YAN and WAU) to 0.875 (YAK and WAU-93) and averaged 0.867. These values were much higher than those within each area. The unrooted neighbor-joining phylogram among the four haplotypes showed association with each geographic area (Fig. 3).

**DISCUSSION**

For the amphidromous Ryukyu-ayu populations on Amami-oshima Island, remarkable genetic differentiation between the Sumiyo Bay and Yakeuchi Bay areas has been reported based on the data using mainly nuclear markers.\(^3\,18\,20\) However, because these analyses were based on small sample sizes or comparison with limited localities, the extent of gene flow between the areas has not yet been examined. In the previous study we suggested that the PCR-RFLP haplotypes of mtDNA control region were not shared and there is little or no gene flow between the two populations of the Ryukyu-ayu although the sample sizes and number of locations were small.\(^24\) According to the examination of larger samples, the present study clearly shows that there is no mtDNA haplotype shared between the two areas and that gene flow has not occurred between the two populations.

This result indicates that the populations of the Sumiyo Bay and Yakeuchi Bay areas (in the following, we call these two populations eastern and western populations, respectively, based on Sawashi and Nishida\(^11\)) are completely isolated.

The populations of amphidromous ayu *P. a. altivelis* in the Japanese Archipelago, distributed in a continuous geographic condition and having a higher abundance to allow the juveniles to migrate to nearby rivers, did not show remarkable genetic differentiation, as opposed to that of the Ryukyu-ayu.\(^2\,4\,38\,40\) If we assume that there is no difference in the dispersal ability of the larvae in the ocean between the two subspecies, the factor affecting the isolation between the eastern and western populations of the Ryukyu-ayu may be a lack of rivers along the Oshima Channel large enough to allow the Ryukyu-ayu to settle and/or create spawning sites. Indeed, some surveys for the distribution of the Ryukyu-ayu on Amami-oshima Island did not observe juveniles or adults in those rivers (Sawashi and Nishida;\(^11\) Ikeda M *et al.* unpubl. data, 2000). The recent reduction of the population size, which leads to reduced chances for the dispersal of free-swimming larvae, might also be another factor affecting isolation.\(^11\)

The samples from four rivers in the Sumiyo Bay area did not have significant differences in haplotype distributions and diversity \( (h) \). As well, the nucleotide divergence \( (d_A) \) among samples was almost equal to the nucleotide diversity \( (\pi) \) within each sample. These results are not surprising because all of these rivers empty directly into Sumiyo Bay, and the Ryukyu-ayu may not home to a particular river. Consequently, there is probably a great deal of mixing between fish from different rivers within the eastern population.

The abundance of the western population of Ryukyu-ayu is smaller than that of the eastern population,\(^11,41\) thus we can consider that the recent population size of the western population is relatively small. From the data showing no heterogeneity for haplotype frequencies between the different generation samples (WAU and WAU-93), the population size might not have been changed at least since 1993. However, it seems that the value of nucleotide diversity \( (\pi) \) in the western population does not always support stable population size in the long term. The value was much higher than that within the eastern population, which was caused by the distant relatedness between the two haplotypes (I and IV) in the western population (Table 3; Fig. 3). This result suggests that the western population might have experienced demographic growth (generation of the new haplotypes) and subsequent drastic bottlenecks (loss of the haplotypes). In contrast, low nucleotide...
diversity between haplotypes in the eastern population with a large abundance might be indicative of the small founder and/or genetic bottleneck events followed by recent population expansion.

Molecular markers are also an important tool for identifying population units that merit separate management and have a high priority for conservation. Moritz has distinguished two types of conservation units, namely management units (MU), representing populations that are currently demographically independent, and evolutionary significant units (ESU), which represent historically isolated sets of populations that are on independent evolutionary trajectories.\(^\text{15,16}\) The ESU are recognized by reciprocal monophyly for alleles (or haplotypes), whereas MU are recognized by significant divergence in allele frequencies. In the case of the two isolated populations of the Ryukyu-ayu on Amami-oshima Island, the higher nucleotide divergence and haplotype phylogram associated with each geographic area suggests that the eastern and western populations are each distinct ESU. These two isolated sets of populations may possess adaptations specific to local conditions, and conservation efforts might be directed towards preserving the genetic integrity of each population because failure to preserve distinctive stocks may reduce the evolutionary potential of this fish.

**ACKNOWLEDGMENTS**

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