AFLP Analysis of Genetic Diversity in Three Populations of Ayu Plecoglossus altivelis

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The genetic diversity among three populations of ayu was analyzed by using fluorescence-based AFLP. Eight fish were collected from each of three locations representing native populations of the amphidromous form (AM), the landlocked form (LL), and the subspecific population from the Kawauchi River on Amami-oshima Island (RK). A total of 745 DNA fragments were scored using 19 arbitrarily chosen primer combinations, based on the restriction enzymes Msel and EcoRI with three and two base pair extensions, respectively. The proportions of polymorphic genes and the average heterozygosity were, respectively, 0.550 and 0.107 for AM, 0.521 and 0.095 for LL, and 0.135 and 0.022 for RK. The band-sharing coefficients were 0.839 for AM, 0.853 for LL, and 0.970 for RK. Genetic variation in each population varied directly with population size. The RK population, which is considered in danger of extinction, showed very low polymorphism. Nei's genetic distance was 0.017 between AM and LL, 0.410 between AM and RK, and 0.414 between LL and RK. These results suggest that AFLP markers provide useful data for the evaluation of genetic variability and divergence in fish.

Key words: ayu, Plecoglossus altivelis, Ryukyu ayu, population genetics, genetic diversity, AFLP markers, fluorescent marker, molecular technique

Genetic diversity and the spatial patterns of genetic variation in nature are topics of great interest to conservation biologists. The application of molecular techniques has become increasingly important in the study of conservation biology. Many molecular markers have been used for population genetics, and each has merits and demerits. Evaluation of several types of markers is needed to determine the best method to address a particular question.

The ayu is an important commercial freshwater fish in Japan. Native populations include the amphidromous and landlocked forms of Plecoglossus altivelis altivelis and one subspecific population P. a. ryukyuensis. This subspecies exists only on Amami-oshima Island and is considered to be in an endangered condition for maintaining population size. In order to determine the status of the amphidromous and landlocked forms, it is also important to evaluate the genetic diversity within and among these populations. Previous studies have reported genetic differentiation between these three groups on the basis of biochemical and molecular markers, including allozymes, microsatellite DNA, mitochondrial DNA (mtDNA), and amplified fragment length polymorphisms (AFLPs) with a radioisotope. In the previous AFLP paper, it was suggested that the AFLP technique was useful for the analysis of genetic divergence. However, it was not possible to get enough results for analyzing genetic diversities of ayu because only one primer set was used. Further, it was necessary to get the useful equipment of radioisotope for marking DNA fragments in that method. In the present study, we extend the genetic diversity analysis of ayu based on many primer-sets using a fluorescence-based AFLP technique.

Materials and Methods

Sample Collection

Fish of the amphidromous form (AM) were collected from Tosa bay; the landlocked form (LL) from Lake Biwa, and the ryukyuensis subspecies from the Kawauchi River on Amami-oshima Island. Eight whole fish were sacrificed from each location and taken to the laboratory for DNA analysis. DNA was extracted from fin tissue using a standard SDS method.

AFLP Procedure

The restriction endonucleases, EcoRI and Msel, were used to cut the nuclear DNA at specific recognition sequences. An adapter sequence (~18 bp) was made according to the report by Vos et al. An adapter sequence was ligated to the genomic DNA restriction fragments, and primers with a sequence complementary to the adapter plus two or three additional bases were used to amplify a subset of the fragments from the digested/ligated genomic DNA (Table 1). Two hundred and fifty ng of genomic DNA were digested in one hour at 37°C in a 16 μl reaction containing 5U EcoRI, 5U Msel, 1 x NEBuffer 2 (Bio-Rad Laboratories; 50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, 1 mM DTT, pH 7.9), and 4 μg/ml BSA (Bovine Se-
run Albumin Solution, 10 mM KPO₄, 0.1 mM NaCl, 0.25 mM EDTA, 1 mM 2-Mercaptoethanol, 50% Glycerol, pH 7.0. After a 4 μl reaction containing 10U T4 DNA ligase, 1 × NEBuffer 2, 1 mM ATP, 50pmol EcoRI and 5pmol MseI adapter was added, and incubated overnight at room temperature (~24°C). The digested/ligated DNA was diluted (1:10) with TE (10 mM Tris-HCl; 0.1 mM EDTA; pH 8.0) and stored at -20°C.

Five μl of diluted digested/ligated product were used as template for a 20 μl PCR reaction containing 1.5 mM MgCl₂, 200 μM of each dNTP, 30 ng of each primer (with a single base selective extension on the 3' end of each adapter), 1x PCR Buffer (Gibco BRL Inc.; 20 mM Tris-HCl pH 8.0, 50 mM KCl), and 0.4U Taq polymerase (Gibco BRL Inc.). DNA was amplified in a PTC-100 thermocycler (MJ Research, Inc.) using an initial 1.5 min denaturing step at 94°C followed by 23 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C. This (pre-amplification) product was diluted 1:10 with TE (10 mM Tris-HCl; 0.1 mM EDTA; pH 8.0) and stored at ~20°C.

Five μl of diluted digested/ligated product were used as template for a 20 μl PCR reaction containing 1.5 mM MgCl₂, 200 μM of each dNTP, 5 ng of EcoRI primer and 30 ng of MseI primer (with one or two additional base selective extensions of each pre-amplified primer), 1x PCR Buffer (Gibco BRL Inc.), and 0.4U Taq polymerase (Gibco BRL Inc.). DNA was amplified in a PTC-100 thermocycler (MJ Research, Inc.) using an initial 1.5 min denaturing step at 94°C followed by 23 cycles of 30 s at 94°C, 30 s at 65°C (0.7°C lower each cycle), and 1 min at 72°C. The final 24 cycles were: 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. Only the EcoRI primer was labeled with fluorochrome.

Twenty μl of loading buffer (98% formamide, 10 mM EDTA, 0.1% each of xylene cyanol and bromophenol blue) were added to the PCR product, which was then denatured for 4 min at 95°C, and immediately cooled on ice. Five μl of sample were loaded on a 3.5% polyacrylamide 7.5 M urea gel and run at 35 W until the xylene cyanol band was at the middle of the gel. Gels were scanned on a Fluorimager 595 (Molecular Dynamics Inc.), and visualized with MD’s Fragment NT analysis software.

### Data Analysis

AFLP gels were scored for the presence or absence of particular bands. Each band was assumed to be a discrete locus and names assigned using the nomenclature "eGA/mCAG-xxx"*, where e and m stand for the EcoRI and MseI restriction enzymes, respectively, GA and CAG represent the base extensions, and xxx the approximate size of the fragment in bp. Only the fragments in the size range of 100 and 600 bp were scored. Individuals with the band were scored as homozygous "11" and individuals without the band as homozygous "22". If there was any doubt, the individuals were scored at that locus. Presumably some individuals with a band and scored as 11 were heterozygous "12".

Genetic variation was assessed within each population on the basis of average heterozygosity and estimated proportion of polymorphic loci. The validity of this approach was based on the following assumptions: (i) Mendelian segregation of polymorphic fragments; (ii) allelic identity of same-size fragments appearing in the genetic profiles of multiple individuals; (iii) single-base substitutions as the mutational events leading to a lack of cutting and/or amplification at selected sites; (iv) allelic proportions adhering to Hardy-Weinberg expectations, and; (v) the existence of a single dominant (amplified) and recessive (null) allele at each locus. Average heterozygosity (H) was estimated using the Hardy-Weinberg equation, using the square root of the frequency of recessive homozygotes (individuals lacking a fragment) to estimate the frequency of the "2" allele. The proportion of polymorphic loci (P) was estimated as the number of loci at which the common allele had a frequency of less than 0.95 divided by the total number of individuals in the analysis. Genetic diversity among populations was estimated using Nei’s genetic distance for a small number of individuals and the band-sharing coefficients within each population.

### Results

**AFLP Patterns**

Scanned patterns depicting the AFLPs generated from a single primer combination are shown in Figs. 1 to 3. A total of 745 variable DNA fragments were scored using the 19 arbitrarily chosen primer combinations, with the number of discernible fragments per primer combination ranging from 6 to 59 (mean ± SE = 39.21 ± 12.47). High polymorphism was observed at some loci. For instance, the combination eGC/mCAT was highly polymorphic for fragments between 337 bp and 390 bp in the AM and the LL samples (Fig. 1), while the eGA/mCTT combination was highly polymorphic for fragments between 450 bp and 516 bp in all three populations (Fig. 2). The AM sample had different allele frequencies that showed strong differentiation from LL at two loci: eGA/mCCT-338* (frequencies of 0.064 for AM and 0.646 for LL) and eGG/mCAT-338* (frequencies of 0.065 for AM and 0.646 for LL).
Fig. 1. Representative AFLP from three populations of ayu using a single PCR-primer combination (EcoR1-GC and Msel-CAG). Fragment sizes were determined on the basis of a BIORAD Fluorescein Ladder (Size marker, M).

No. 1-8, Amphidromous forms; No. 9-16, Landlocked forms; No. 17-24, Ryukyu subspecies; M, size marker.

Additionally, the eGT/mCAA-226* band was found only in AM (Fig. 3).

Genetic Variation within Populations
The genetic variation observed for each population is shown at Table 2. For the AM sample, 298 of the 542 fragments scored were polymorphic based on the 0.95 criterion, yielding an estimate of polymorphism of $P=0.550$. The average heterozygosity for these fragments was relatively high ($H=0.107$). For the LL sample, 276 of the 530 fragments scored were polymorphic ($P=0.521$), and with an average heterozygosity of 0.095. Finally, the RK sample had the lowest level of diversity, with 47 of the 348 fragments scored being polymorphic ($P=0.135$) with an average heterozygosity near zero (0.022).

Population Structure
Assuming single base substitutions for each band difference and picking up only monomorphic loci (fixed bands) in each population, genetic divergence on RK was estimated as the number of allelic substitutions between RK and the other two populations divided by the total number of monomorphic loci in RK and the other two populations. The results were 183/334 (54.8%) for AM and 193/350 (55.1%) for LL. However, there was only one allelic substitution between AM and LL (0.4%); the eGT/mCAA-226* band was found only in amphidromous in-

Table 2. Genetic diversity within populations as reflected by proportions of polymorphic loci ($P$) and average heterozygosity ($H$)

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of variable bands</th>
<th>No. of fixed bands</th>
<th>$P$</th>
<th>$H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphidromous</td>
<td>298</td>
<td>244</td>
<td>0.550</td>
<td>0.107</td>
</tr>
<tr>
<td>Landlocked</td>
<td>276</td>
<td>254</td>
<td>0.521</td>
<td>0.095</td>
</tr>
<tr>
<td>Amami</td>
<td>47</td>
<td>301</td>
<td>0.135</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Table 3. Genetic distance between populations from AFLP data (above the diagonal), band-sharing coefficients within population (on the diagonal), and substitution-bands/fixed bands between populations (below the diagonal)

<table>
<thead>
<tr>
<th></th>
<th>Amphidromous</th>
<th>Landlocked</th>
<th>Amami</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphidromous</td>
<td>(0.839)</td>
<td>0.017</td>
<td>0.410</td>
</tr>
<tr>
<td>Landlocked</td>
<td>1/234</td>
<td>(0.853)</td>
<td>0.414</td>
</tr>
<tr>
<td>Amami</td>
<td>183/334</td>
<td>193/350</td>
<td>(0.970)</td>
</tr>
</tbody>
</table>

Nei's genetic distance estimates are shown in Table 3. The distance was only 0.017 between the AM and the LL while the distance estimates for the RK and the AM or the LL were 0.410 or 0.414, respectively. The average band-sharing coefficients were 0.839 within the AM, 0.853...
within the LL, and 0.970 within the RK.

Discussion

Using AFLP markers, we have determined that the three populations show a similar level of genetic differentiation expected from previous studies. The subspecific population from Kawauchi R. showed the greatest differentiation from the other two populations. There was only one fixed difference, eGT/mCAA-226*, between the AM and LL populations, indicating that this locus may be an excellent marker for identifying amphidromous individuals in a mixed population of amphidromous and landlocked individuals. More individuals need to be analyzed to confirm this result.

The results of this study are similar to those from previous studies using other genetic markers, such as allozymes, microsatellite DNAs, mtDNAs, and AFLPs. Particularly regarding the relationship between variability and population size, the Amami-oshima Kawauchi River population showed very low genetic variability and its limited population size has resulted in the landlocked population being considered in danger of extinction. Takagi et al. suggested by using one primer-set at an AFLP marker that the population in the Kawauchi River, located at the west coast of Amami-oshima Island, is genetically different from the population in the Yanma River, located on the east coast of the island. Using many primer-set of AFLP may prove useful in examining not only genetic diversity between two populations, but also genetic variability in each population.

The amphidromous population showed greater genetic variability than the landlocked population. These results were different from the previous paper using one primer-set of AFLP markers. It was suggested that many primer-sets of AFLP must be used for estimation of genetic variability in each population. Even though several authors have reported similar results from the analysis of allozymes and microsatellite DNAs, the advantage of using AFLP markers is that loci are easy to detect and many loci can be scored on a single gel. Thus the techniques should have merit in the study of genetic diversity which should be based on the analysis of many loci. However they have the demerit that a scanning system for reading fluorescent markers is needed.

It is also suggested that AFLP markers provide a valuable tool for studies to understand genetic relationships at both the intraspecific and the interspecific level.

Acknowledgments

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