PRIMER NOTE

Isolation and cross-species amplification of microsatellite loci in the freshwater minnow Zacco pachycephalus (Teleostei: Cyprinidae) for diversity and conservation genetic analysis

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Abstract

Asian minnows of the genus Zacco are dominant fish in various freshwater ecosystems. Two species, Zacco pachycephalus and Z. platypus, occur in Taiwan and are favourite targets for local sport anglers. The introduction of Zacco fish into nonindigenous habitats in Taiwan has become a conservation issue. We developed eight polymorphic microsatellites for Z. pachycephalus (average \( H_e = 0.779 \)) and these microsatellites were applicable to Z. platypus, which showed a comparable polymorphic level (average \( H_e = 0.784 \)). These loci can be used as genetic markers for identifying conservation units and studying population differentiation for both Zacco species.

Keywords: conservation unit, Cyprinidae, microsatellite, Zacco pachycephalus, Zacco platypus

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Asian minnows of the genus Zacco are widespread cyprinids inhabiting streams and rivers throughout Japan, Korea, China and Taiwan (see Ashiwa & Hosoya 1998 and references therein; Chen 1998). Although Zacco are small fish, they are the dominant fish species in some freshwater ecosystems and are targets for sport angling in some areas, such as Taiwan. Records indicate two species of Zacco occurring in Taiwan (Wang et al. 1997, 1999), Zacco pachycephalus and Z. platypus. The endemic Z. pachycephalus is widespread in the western part of Taiwan and did not occur in eastern Taiwan until about two decades ago (Chen & Fang 1999). This fish was introduced to rivers in eastern Taiwan by sport anglers to create extra fishing ranges. Furthermore, we noticed that Z. pachycephalus from southwestern Taiwan were morphologically distinct from those from elsewhere. In contrast, Z. platypus is restricted to two major rivers in northern Taiwan but is widespread throughout Japan, Korea and China. Recently, anglers’ legendary reports indicated that a ‘new’ morph of Z. platypus was common in some reaches of its native rivers. Two things are of importance in the conservation of the Zacco species in Taiwan. First, since the pressure caused by sport angling on the fish populations is high, conservation units (Crandall et al. 2000) must be unequivocally defined for both species. Second, the ‘new’ morph of Z. platypus could be an invasive entity from its distributional ranges outside Taiwan and this must be resolved before taking any conservation action. We developed microsatellite primers for Z. pachycephalus in an attempt to identify a valid conservation unit and to apply to its sympatric congers to resolve the issue of suspected introduction of nonindigenous Z. platypus.

Originally, we attempted an enrichment method (http://bioserver.georgetown.edu/faculty/hamilton) but were unable to obtain satisfactory results due to the problems reported by Koblizkova et al. (1998). We were also hampered by the frequent failure of polymerase chain reaction (PCR) amplifications with primers designed from flanking sequences of cloned loci. We ended up with one locus (Z128A) of workable primers. Therefore, we switched to constructing partial genomic libraries. Genomic DNA for constructing the partial libraries was prepared according to procedures in Sambrook et al. (1989). Genomic DNA was digested with Sau3AI and fractioned on a 1% agarose gel. DNA in the size range 300–700 bp was isolated, purified.
with the GFX™ Band Purification kit (Amersham) and ligated into plasmids PUC18/BamHI/BAP (Amersham) according to the manufacturer’s protocols. Ligated plasmids were transformed into the competent ECOS 101 cells (Yeastern Biotech). Recombinant clones containing inserts were transferred to Hybond-N+ nylon membranes (Amersham) which were hybridized to a set of oligonucleotide probes, including (AC)₁₅, (AT)₁₅, (AG)₁₅ and (AAT)₁₀. Probes were labelled with the digoxigenin (DIG) Oligonucleotide 3′-End Labeling Kit (Roche). Hybridization was performed at 55 °C for 16 h in a standard hybridization buffer, consisting of 5x SSC, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulphate (SDS) and 1% Blocking Reagent (Roche). The membranes were washed twice, each for 5 min at 45 °C, with a solution of 2x SSC and 0.1% SDS and then twice, each for 15 min at 65 °C, with a solution of 0.1x SSC and 0.1% SDS. Chemiluminescent detection was performed with the DIG Luminescent Detection Kit (Roche). A total of 75 positive clones was sequenced on an automated sequencer (MegaBACE 500; Molecular Dynamics) in which 38 contained dinucleotide, trinucleotide or other types of repeats. The on-line program PRIMER 3.0 (Rozen & Skalentsy 2000) was used to design primers from flanking regions of microsatellite DNA loci.

Individual genotypes were determined by PCR. Each PCR reaction totalled 10 µL, containing 100 ng template DNA, 0.5 U Taq DNA polymerase (Promega), 10 mM Mg²⁺, 1 mM dNTP, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 and 0.2 µM of each primer, with the forward primer being end-labelled with fluorescent dye (FAM, HEX or TAMRA). Amplification was carried out by the thermal profile: 95 °C for 5 min followed by 25 cycles of 95 °C for 30 s, optimal annealing temperature (Table 1) for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 10 min. The PCR products were run on linear polyacrylamide gels with a MegaBACE 500 automated sequencer. ET-400 Size Standard (Amersham) was used as the size marker to determine the allele sizes. Most of the allelic PCR products differed in multiples of their repeat motifs. The individuals with ambiguous genotypes were amplified and scored at least twice to determine the allele sizes.

Eight microsatellites were found to be polymorphic among Z. pachycephalus and all loci were applicable to Z. platypus (Table 1). The sample sizes and polymorphic levels are given in Table 2. The number of alleles per locus ranged from 11 to 30 and from six to 45 for Z. pachycephalus and Z. platypus, respectively. For Z. pachycephalus, the observed and expected heterozygosity ranged from 0.272 to 0.650 and from 0.620 to 0.962, respectively. The level of polymorphism in Z. platypus was comparable to Z. pachycephalus: the observed and expected heterozygosity ranged from 0.272 to 0.650 and from 0.620 to 0.962, respectively. The observed genotype frequencies were tested against Hardy–Weinberg expectation for both species. The individuals collected from one river were treated as a population totalling, therefore, 112 locus-population cases for Z. pachycephalus and 48 cases for Z. platypus. For Z. pachycephalus 85% of the cases and for Z. platypus 81% of the cases were in agreement with the expectation. For those 16 cases which deviated from Hardy–Weinberg expectation in Z.

### Table 1: Repeat motif, primer sequences and annealing temperature (Tₐ) for eight microsatellite loci of Zacco pachycephalus

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Repeat motif</th>
<th>Primer sequences</th>
<th>Tₐ (°C)</th>
<th>Z. pachycephalus</th>
<th>Z. platypus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z128A</td>
<td>(GT)₁₄</td>
<td>L: 5'-TGCGCTGATGACTGACGCTGGTTT</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCTGACACTCCTCGG</td>
<td></td>
<td>Z. pachycephalus</td>
<td>Z. platypus</td>
</tr>
<tr>
<td>ZD181</td>
<td>(GA)₁₈</td>
<td>L: 5'-CTGACGACAGGCTCAGCATG</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CTGTTTCTAGCTCTGGTCC</td>
<td></td>
<td>Z. pachycephalus</td>
<td>Z. platypus</td>
</tr>
<tr>
<td>ZD366</td>
<td>(GT)₁₂AT(GT)₆</td>
<td>L: 5'-GTGACAGATGCTGATATTTTGCG</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GGTTCCATATTTCCTACACCA</td>
<td></td>
<td>Z. pachycephalus</td>
<td>Z. platypus</td>
</tr>
<tr>
<td>ZD992</td>
<td>(CT)₁₂</td>
<td>L: 5'-GGTTCCATATTTCCTACACCA</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>ZD1021</td>
<td>(GT)₁₅</td>
<td>L: 5'-GGTTCCATATTTCCTACACCA</td>
<td>60</td>
<td>60</td>
<td></td>
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<tr>
<td>ZD582</td>
<td>(GT)₁₃</td>
<td>L: 5'-GGTTCCATATTTCCTACACCA</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>ZD331</td>
<td>(GT)₁₂(CT)₆</td>
<td>L: 5'-GGTTCCATATTTCCTACACCA</td>
<td>60</td>
<td>60</td>
<td></td>
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<tr>
<td>ZD657</td>
<td>(CA)₁₄(TC)₁₈</td>
<td>L: 5'-GGTTCCATATTTCCTACACCA</td>
<td>58</td>
<td>Z. pachycephalus</td>
<td>Z. platypus</td>
</tr>
</tbody>
</table>

*GenBank Accession nos were in the following order: AY332512, AY332513, AY332517, AY332518, AY332514, AY332515, AY332516 and AY332519. Cross-species amplification was tested on Z. platypus.
**Table 2** Characteristics of eight microsatellite loci in two *Zacco* species

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sample size</th>
<th>Allele range (bp)</th>
<th>No. of alleles</th>
<th>$H_O$</th>
<th>$H_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Z. pachycephalus</em></td>
<td><em>Z. platypus</em></td>
<td><em>Z. pachycephalus</em></td>
<td><em>Z. platypus</em></td>
<td><em>Z. pachycephalus</em></td>
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<tr>
<td>Z128A</td>
<td>302</td>
<td>103</td>
<td>106–136</td>
<td>106–119</td>
<td>14</td>
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<tr>
<td>ZD181</td>
<td>302</td>
<td>103</td>
<td>207–271</td>
<td>203–238</td>
<td>30</td>
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<tr>
<td>ZD366</td>
<td>302</td>
<td>103</td>
<td>223–359</td>
<td>229–252</td>
<td>23</td>
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<tr>
<td>ZD992</td>
<td>302</td>
<td>103</td>
<td>185–246</td>
<td>183–222</td>
<td>22</td>
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<tr>
<td>ZD1021</td>
<td>302</td>
<td>103</td>
<td>179–213</td>
<td>189–249</td>
<td>11</td>
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<tr>
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<td>302</td>
<td>103</td>
<td>189–242</td>
<td>199–215</td>
<td>16</td>
</tr>
<tr>
<td>ZD331</td>
<td>302</td>
<td>103</td>
<td>271–310</td>
<td>267–304</td>
<td>17</td>
</tr>
<tr>
<td>ZD657</td>
<td>302</td>
<td>103</td>
<td>246–331</td>
<td>246–357</td>
<td>30</td>
</tr>
</tbody>
</table>

Sample size, size range of polymerase chain reaction products, number of alleles, observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$).

*pachycephalus*, 10 involved populations from eastern Taiwan (all $H_O$ less than $H_E$), probably reflecting a founder effect during the introduction. The other cases were not concentrated in any population or locus and could result from null alleles. For the eight deviated cases in *Z. platypus*, no obvious pattern was observed and could include segregating null alleles.

We applied nonmetric multidimensional scaling analysis (Kruska & Wish 1978) with Sorenson distances (Krebs 1989) among individual genotypes. The results showed that the ‘new’ morph of *Z. platypus* is indeed a distinct genetic entity, probably an accidental human introduction. In addition, no conspicuous genetic differentiation was observed for the morphological differences in southwestern *Z. pachycephalus*. A full account of the population genetics for both species will be published elsewhere.

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**References**


