



Development of codominant markers for identifying species hybrids *

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Abstract

Herein we describe a simple method for developing species-diagnostic markers that would permit the rapid identification of hybrid individuals. Our method relies on amplified length polymorphism (AFLP) and single strand conformation polymorphism (SSCP) technologies, both of which can be performed in any molecular biology facility using standard laboratory equipment. We demonstrate the utility of the AFLP-SSCP method by developing three taxon-specific markers that will be suitable for monitoring introgression in endangered Klamath basin suckers.

Introduction

Species hybridization has become a matter of increasing concern for conservation biologists. In addition to the technical complexities involved in the study of hybridization, this subject has added political implications as seen with the stalled approval of policy regarding species “intercrosses” under the Endangered Species Act (Allendorf et al. 2001; Federal Register 1996, 61: 4710–4713). In addition to concerns about unnatural hybridization due to habitat alterations and species translocations (Allendorf et al. 2001), the role natural hybridization plays in the evolution of new taxa is increasingly becoming recognized (Dowling and Secor 1997). In the past, hybrid identification was based largely on morphologic characters, which can be biased or inaccurate measures of hybrid status. Recent molecular advances have improved our ability to identify hybrid individuals and could greatly enhance our ability to manage susceptible populations. In particular, the availability of species-diagnostic, single-locus codominant markers would permit the

rapid identification of hybrid individuals and assist with long-term monitoring of introgression.

For phylogenetically similar hybridizing taxa, the identification and development of diagnostic molecular markers can be a labor-intensive process that usually requires a high level of technical expertise. The goal of this paper is to demonstrate a simple strategy for generating codominant markers that are useful as management tools for identifying hybrid individuals and can easily be applied in any molecular biology laboratory. We used the Amplified Fragment Length Polymorphism (AFLP) approach (Vos et al. 1995) as a starting point for developing species-diagnostic markers. For a review of the AFLP procedure and its applications see Mueller and Wolfenbarger (1999). The AFLP method is an effective technique for screening large portions of the genome for sequence polymorphism that requires several intricate steps. By sequencing individual AFLP bands that are diagnostic for each species though, a number of easily screened, PCR-based single-locus markers can be generated. Steps involved in AFLP include enzyme restriction of whole genomic DNA into small fragments, ligation of oligonucleotide adapters to the restriction ends of DNA fragments,

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selective PCR amplification of DNA fragments and electrophoresis. The presence of a diagnostic AFLP band indicates an intact restriction site at the first step of the AFLP procedure. Targeting polymorphisms at the restriction sites requires inverse PCR techniques (Triglia 2000) to identify flanking sequences suitable for development of new PCR priming regions. This can be technically challenging and markers developed from these sequences are not guaranteed to be codominant since factors other than restriction polymorphisms can cause AFLP polymorphisms. However, a follow up of the initial AFLP screen with single strand conformation polymorphism (SSCP) analysis (Orita et al. 1989) would allow for a more direct assessment of sequence polymorphisms. Fragments that are monomorphic in a standard AFLP analysis but species-diagnostic in AFLP-SSCP analysis can only arise due to nucleotide substitution differences within the AFLP priming region and therefore must represent codominant markers. By developing PCR primers specific to the codominant locus, an analysis of many individuals could rapidly be completed by SSCP, allele-specific PCR or, if the nucleotide substitution falls at a restriction site, by restriction fragment length polymorphism (RFLP) analysis.

The AFLP-SSCP marker development procedure was evaluated with suckers (Catostomidae) from the Klamath Basin, Oregon. Four sucker species are native to the Klamath River Basin: shortnose sucker (*Chasmistes brevirostris*); Lost River sucker (*Deltistes luxatus*); Klamath largescale sucker (*Catostomus snyderi*); Klamath smallscale sucker (*Catostomus rimiculus*). Morphologic and genetic evidence suggests that federally endangered shortnose and Lost River suckers (U.S. Department of the Interior 1988) have undergone extensive introgression and may be hybridizing with non-listed largescale and smallscale suckers (Miller and Smith 1981; Harris 1991; Harris and Markle 1993). A suite of single-locus codominant markers will aid in the identification of hybrid samples, enable a program for monitoring of introgression in these endangered species, and possibly aid in identifying taxa operating as 'conduits of gene exchange' (Dowling and Secor 1997).

Methods

Genomic DNA was extracted from fin and muscle tissue using the TNES-urea procedure (Belfiore and

May 2000). Each of the four study species (shortnose sucker, Lost River sucker, Klamath largescale sucker, and Klamath smallscale sucker) was sampled for 10 individuals and for each species the samples came from two populations (5 samples per population). Each sample was screened for variation with 64 AFLP primer combinations (Vos et al. 1995) as modified by Agresti et al. (2000). Amplified products were run on a 5% denaturing acrylamide gel and visualized fluorescently through single-primer labeling with fluorescein and detection with a Fluorimager 595 (Molecular Dynamics). Of the 64 AFLP combinations, sixteen were selected for further screening with SSCP based on the large number of monomorphic bands displayed on the AFLP denaturing gel (Figure 1a). These combinations were run on 0.5X MDE - SSCP gels and fluorescently detected using the agarose and Vistra Green™ overlay procedure of Rodzen et al. (1998) (Figure 1b). Variant alleles fixed for a particular species were excised from the gel matrix with a scalpel and eluted in 500 μ l of TLE (10 mM Tris; 0.1 mM EDTA) overnight at room temperature. Five μ l of the elution was used as PCR template for re-amplification using AFLP pre-amplification primers and conditions to prepare product for sequencing (Figure 1c). PCR primers were designed from this sequence using *PrimerSelect* software (Lasergene 5.1, DNASTAR Inc.) with an expected temperature of 60 °C and used to amplify the target locus in all individuals. The PCR product was run on an SSCP gel (Figure 1c) and the alternate allele was sequenced in the remaining species and aligned with the original sequence to verify sequence homology (suggesting that they represent allelic forms at the same locus). All sequences were analyzed for the presence of restriction sites at the polymorphism and, if present, RFLP analysis was carried out on all individuals (Figure 1d). If no polymorphic restriction sites were identified or if numerous polymorphic sites were present, all individuals were analyzed with denaturing acrylamide or SSCP gels.

To demonstrate the codominant nature of the markers, we calculated observed and expected heterozygosities (H_O and H_E) and deviations from Hardy-Weinberg (H-W) expectations ($\alpha = 0.05$) for species that were polymorphic at a specific locus (not fixed for either allele). All analyses were carried out with GENEPOP 3.0 (Raymond and Rousset 1995).

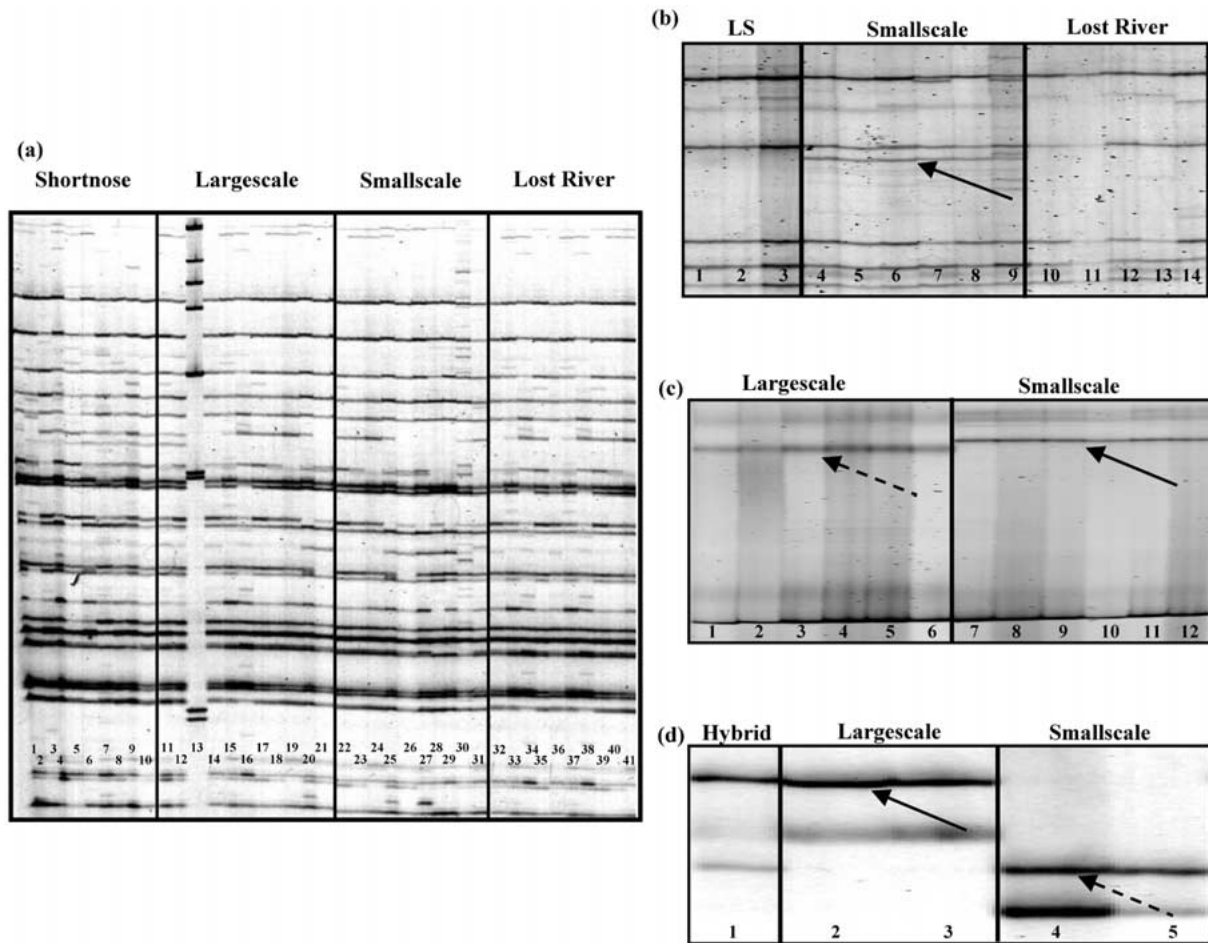


Figure 1. Development of codominant *Catostomus rimiculus* marker, Cri1 (named after the species for which it is diagnostic). (a) AFLP reactions for 10 representatives of each of the four Klamath basin sucker species are visualized on a denaturing acrylamide gel: shortnose (lanes 1–10), largescale (lanes 11, 12, 14–21), smallscale (lanes 22–31), and Lost River (lanes 32–41) suckers, size ladder (lane 13). This gel demonstrates a large number of AFLP bands shared by the four species. Illustrations b–d are enlarged to permit easy visualization of each banding pattern and do not show all samples that were analyzed. (b) AFLP reactions from figure (a) are secondarily screened with an SSCP gel to detect species-specific sequence polymorphisms in all samples. This gel shows amplicons for largescale (LS) (lanes 1–3), smallscale (lanes 4–9), and Lost River (lanes 10–14) suckers. For largescale, shortnose and Lost River suckers not shown, the banding pattern is identical to the pattern displayed by largescale and Lost River suckers shown in this figure. For smallscale suckers not shown, the banding pattern is identical to the pattern displayed by smallscale suckers. An arrow indicates a band unique to Klamath smallscale suckers. The band, which was not visible on the denaturing AFLP gel, was excised from the SSCP gel and sequenced. (c) PCR primers were designed and used to amplify this locus in largescale (lanes 1–6) and smallscale (lanes 7–12) suckers as visualized on an SSCP gel. For largescale, shortnose and Lost River suckers not shown, the banding pattern was identical to the pattern displayed by largescale suckers. For smallscale suckers not shown, the banding pattern is identical to the pattern displayed by the smallscale suckers shown. The alternate allele indicated with the dashed arrow was excised, sequenced and aligned with the original Klamath smallscale allele indicated with the solid arrow. The sequences were examined for nucleotide polymorphisms and the presence of restriction sites. (d) RFLP of the amplicon is demonstrated for largescale (lanes 2–3) and smallscale (lanes 4–5) suckers. The solid arrow indicates the unrestricted band present in largescale suckers and the dashed arrow indicates the restricted band present in smallscale suckers. For largescale, shortnose and Lost River suckers not shown, the RFLP banding pattern is identical to the pattern displayed by largescale suckers. For smallscale suckers not shown, the RFLP banding pattern is identical to the pattern displayed by the smallscale suckers shown. Lane 1 shows a shortnose \times Lost River sucker morphologic hybrid possessing both species-specific alleles.

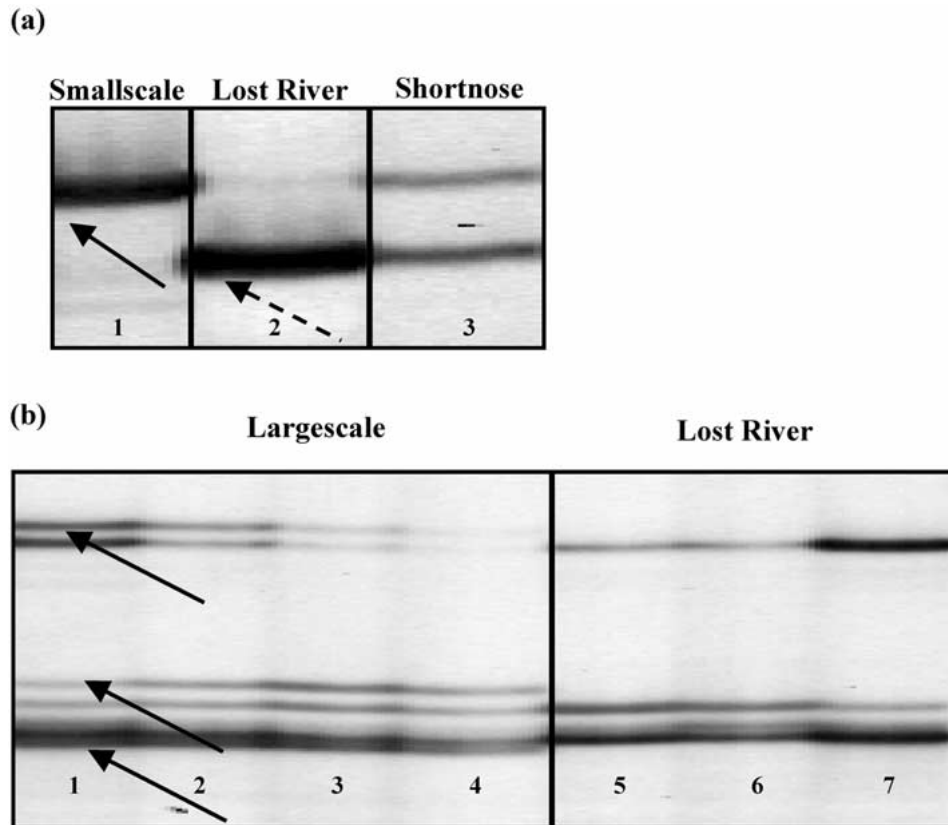


Figure 2. Demonstration of co-dominant *Catostomus rimiculus* (Cri2) and dominant *C. snyderi* (Csn1) markers. (a) A sequence deletion in Klamath smallscale suckers produces two alleles in Cri2, which are visualized on a polyacrylamide gel. The larger allele indicated by the solid arrow in lane 1 is fixed in smallscale suckers and the smaller allele indicated with the dashed arrow in lane 2 is fixed in Lost River suckers. Both alleles are present in the shortnose sucker in lane 3. For smallscale suckers not shown, the banding pattern is identical to the pattern displayed by the single smallscale sucker sample shown here. For Lost River suckers not shown, the banding pattern is identical to the pattern displayed by the single Lost River sucker shown here. The shortnose and largescale suckers not shown are polymorphic for these two alleles. (b) Both Csn1 alleles are resolved with an SSCP gel. One allele consists of three bands specific to largescale suckers as indicated by solid arrows (lanes 1–4), which are not present in the second allele found in Lost River suckers (lanes 5–7). For largescale suckers not shown, the banding pattern is identical to the pattern displayed by the largescale suckers. For Lost River, shortnose, and smallscale suckers not shown, the banding pattern is identical to the pattern displayed by the Lost River suckers shown.

Results and discussion

Three taxon-specific markers were developed using the AFLP-SSCP method. Markers Cri1 (Figure 1) and Cri2 (Figure 2a) are diagnostic for Klamath smallscale suckers and Csn1 is diagnostic for Klamath largescale suckers. For Cri1, smallscale suckers possess a single-nucleotide polymorphism (SNP) that is not present in shortnose, largescale, and Lost River suckers (GenBank AF335378 and AF335379). A *Tsp* 451 restriction site is present at the smallscale sucker SNP, producing a species diagnostic RFLP marker. We used this marker to confirm the hybrid origin of a shortnose \times Lost River sucker identified morphologically

by gill raker and vertebral counts (Figure 1). Locus Cri2 (Figure 2a) has a sequence deletion in Klamath smallscale suckers that produces a size polymorphism in this two-allele system (GenBank AF335380 and AF335381). Lost River suckers and Klamath smallscale suckers are fixed for alternate alleles while shortnose and largescale suckers are polymorphic for both alleles. Neither shortnose ($H_O = 0.28$, $H_E = 0.31$, $P = 0.48$) nor largescale ($H_O = 0.25$, $H_E = 0.28$, $P = 0.38$) sucker genotype frequencies differed significantly from H-W expectations.

Locus Csn1 (Figure 2b) is diagnostic for Klamath largescale suckers and possesses a single nucleotide polymorphism (SNP) that is not present in the

three remaining species (GenBank AF335382 and AF335383). No polymorphic restriction sites were identified for this SNP in either sequence, so the locus was scored from the different SSCP allelic patterns. Lost River, shortnose and smallscale suckers were fixed for one allele while both alleles are present in Klamath largescale suckers. Genotype frequencies did not significantly differ from H-W equilibrium ($H_0 = 0.19$, $H_E = 0.19$, $P = 0.98$).

The AFLP-SSCP method described herein was used to develop three taxon-specific markers that will be exceptionally useful for monitoring introgression in endangered Klamath basin suckers. We have demonstrated that these markers can be used to test the hybrid origin of morphologically ambiguous individuals and that this method can be performed in any molecular biology facility using standard laboratory equipment. We recommend AFLP-SSCP as an efficient method of rapidly screening the genome for taxon-specific polymorphisms that can be developed into diagnostic markers with relative ease.

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