Universal PCR primers for S7 ribosomal protein gene introns in fish

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Lessa (1992) introduced intron-targeted PCR, in which a non-coding intron was amplified using primers designed from highly conserved exon sequences. Introns appear to harbour a much greater degree of genetic polymorphism within and between species than exons. On the other hand, length and nucleotide sequence of exons, and exon–intron arrangement can be highly conserved between considerably distant animal taxa. These characteristics may allow us to design sets of primers based on exon sequences to amplify flanking intron regions. Such sets of primers might function in very distant species. This study introduces two pairs of primer sets which were designed for amplifying the 1st and 2nd introns of the S7 ribosomal protein gene in fish. These primers were applied to distant fish species in order to determine their universality, and polymorphism in the amplified fragments was investigated.

The DNA sequence data of the S7 ribosomal protein gene of puffer fish (*Fugu rubripes*), frog (*Xenopus laevis*) and human were derived from Cecconi *et al.* (1996), Mariottini *et al.* (1993) and Annilo *et al*. (1995), respectively. Exons 1, 2 and 3 of these species were aligned to determine conserved sequence regions. Because exon 1 of humans showed very poor homology with exon 1 of other species, data from puffer fish and frog were used for aligning exon 1. By contrast, highly conserved regions among these distant species were observed in exons 2 and 3. Two sets of primers were designed from the conserved sequence regions. The primer sequences to amplify the 1st intron (RP1) were 5'-TGGCC-TCTTCCTTGGCCGTC-3' (S7RPEX1F) and 5'-AACTCGTCT-GGCTTTTCGCC-3' (S7RPEX2R), and those for the 2nd intron (RP2) were 5'-AGCGCCAAAATAGTGAAGCC-3' (S7RPEX2F) and 5'-GCCTTCAGGTCAGAGTTCAT-3' (S7RPEX3R). The PCR reaction mixture contained 0.2 U of *Taq* DNA polymerase (Perkin Elmer Cetus), 0.2 mM of each dNTP, 1 μL of the manufacturer's supplied 10× buffer, 2 mM $MgCl_2$, 10 pmol of each primer and 10–50 ng of template DNA, in a final volume of 10 μL. Amplification was carried out with an initial denaturation at 95 °C for 1 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min). PCR products and those digested by endonuclease were electrophoresed on a 2.5% agarose gel (Biogel) in TBE buffer (50 mM Tris, 1 mM EDTA, and 48.5 mM boric acid).

Using the standard phenol–chloroform method, crude DNA was extracted from frozen or ethanol-preserved muscles of chum salmon (*Onchorhyncus keta*), tuna (*Thunnus* spp.) and puffer fish (*Fugu rubripes*), each of which belonged to a different order. Results from PCR amplifications of RP1 and RP2 are shown in Fig. 1, where amplification of a single fragment was eminent in all species. Amplified fragments of salmon, tuna and puffer fish were all different in length with respect to each other, while no length difference was observed among eight tuna species (data not shown).

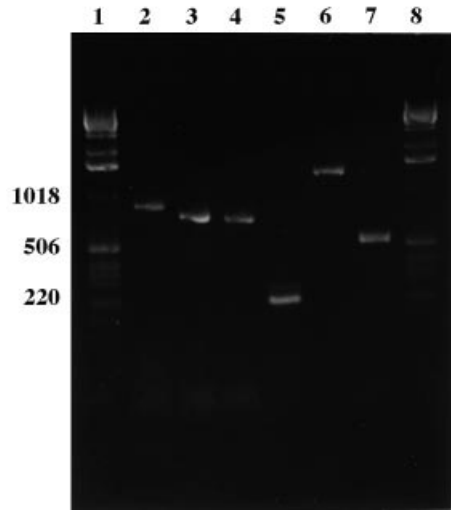

Fig. 1 Result of PCR amplification targeting the 1st (lanes 2–4) and 2nd introns (lanes 5–7) of the S7 ribosomal protein gene of three distant fish species. Lanes: 1 and 8, molecular weight marker (Gibco BRL 1 kb ladder); 2 and 5, chum salmon (*Onchorhyncus keta*); 3 and 6, yellowfin tuna (*Thunnus albacares*); 4 and 7, puffer fish (*Fugu rubripes*).

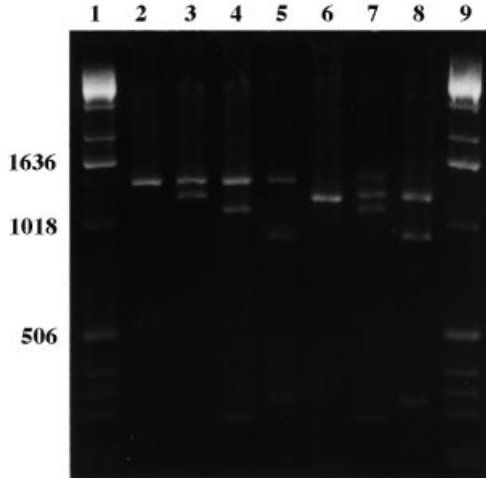

Fig. 2 *Hha*I restriction profiles observed in the 2nd intron of the S7 ribosomal protein gene of yellowfin tuna. Lanes 1 and 9, molecular weight marker (Gibco BRL 1 kb ladder). Deduced genotypes are: lane 2 AA; lane 3, AB; lane 4, AC; lane 5, AD; lane 6, BB; lane 7, BC; and lane 8, BD.

A battery of 4-bp cutter endonucleases was applied to PCR products of yellowfin tuna (*Thunnus albacares*) in order to investigate intraspecific restriction site polymorphism.

Restriction site polymorphisms were observed in both RP1 and RP2 fragments, where the total length of restriction fragments in all endonuclease digestions never exceeded twice the size of the uncut PCR product. Relatively simple restriction patterns obtained by *Hha*I digestion of the RP2 fragment are shown in Fig. 2, which allow us to interpret the presence of four alleles. Observed and expected heterozygosities were 0.213 and 0.239 for a Pacific Ocean sample (n = 38), 0.390 and 0.435 for an Indian Ocean sample (n = 39), and 0.434 and 0.416 for an Atlantic Ocean sample (n = 42), respectively. These genetic variations were comparable with the results of allozyme analysis obtained by Ward *et al*. (1994), and all populations analysed in this study were found to be in accordance with the Hardy–Weinberg equilibrium. These results support the use of polymorphic intron within the S7 ribosomal protein gene as Mendelian marker, at least in tunas.

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Genetic distinction of scorpionflies (*Panorpa vulgaris*) by microsatellites

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The scorpionfly *Panorpa vulgaris* represents an interesting model for studying sexual selection and life history. The scorpionflies mate promiscuously in both sexes (Sauer *et al*. 1998). There is evidence that the promiscuous mating behaviour of the females is forced selectively by their inability to determine the quality of their potential mates prior to mating (Sauer *et al*. 1998). Females appear to discriminate among males of varying phenotypic quality (nutrition status) only by mating longer with high-quality males. Similarly, lifetime mating duration is the decisive proximate determinant of male fitness. To achieve meaningful interpretations on sexual selection and lifetime history, genetic relationships must be determined unambiguously. Multilocus DNA fingerprinting provides high interindividual resolution power but is not suitable for population genetic studies in insects where limited amounts of DNA are obtained. Here we describe three highly informative microsatellites with individual exclusion probabilities of > 0.80 for *P. vulgaris*.

P. vulgaris specimens were collected in the field near Freiburg (Germany) or used after breeding. For DNA preparation, larvae or adult *P. vulgaris* were grinded with a pestle in 450 μL of 6% DTAB. A volume of 450 μL of DTAB and 20 μg of RNase A was added and incubated for 15 min at 68 °C. After chloroform extraction (900 μL) the supernatant (≈ 500 μL) was poured into a 2 mL Eppendorf tube containing 100 μL of 5% CTAB, 90 μL of H_2O. By inversing the tube a DNA/CTAB precipitate formed. After centrifugation (2 min, 10 000 ***g***) the pellet was dissolved in 300 μL of 1.2 M NaCl and the DNA precipitated with 750 μL of 99.5% ETOH. After washing (70% ETOH) the DNA was redissolved in 10 mM Tris/1 mM EDTA. All other procedures followed the methods outlined in Sambrook *et al*. (1989).

For library construction, restriction enzyme-digested DNA of the selected size was ligated into the vector pBluescript KS+^{TM} and *Escherichia coli* SURE cells were transformed. Individual bacterial colonies were collected and gridded automatically by a Biomek^{TM} workstation (Beckman). A total of 10 464 clones were generated, i.e. > 900 from unfractionated *Sau*3AI digests, > 6700 from 200 to 1000 bp long *Sau*3AI fragments and > 2800 from *Rsa*I fragments of 100–1000 bp. A total of 98% of clones were recombinant as judged from PCR amplifications of inserts. Nylon membranes were screened with 19 simple repetitive oligonucleotides. Thirty-two clones generating strong hybridization signals revealed simple repeats upon sequencing, and PCR primers were designed (Table 1).

The PCR reactions (10 μL), containing approximately 50 ng of template DNA, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 1–3 mM $MgCl_2$, 200 μM dNTP, 1 μM of each primer, and 0.5 U of *Taq* polymerase (Beckman), were performed in a Crocodile III^{TM} thermocycler (Appligene). The initial denaturation time was 5 min at 95 °C, thereafter 30 s. Annealing time was 60 s with the following temperatures: 2Pv at 60 °C, 5Pv at 54 °C, 7Pv at 56 °C. In the first two cycles the temperature was raised 6 °C and 3 °C above the final annealing temperature. Elongation lasted for 60 s at 72 °C and 5 min in the final step. Fluorochrome- (6-FAM, TET, HEX) labelled fragments were analysed automatically using an ABI 377 sequencer (GENESCAN program).

The highly polymorphic microsatellites 5Pv and 7Pv represented perfect $(AT)_n/(TA)_n$ blocks; 2Pv is a cryptic $(CT)_n$ repeat. As the original 2Pv 5′ primer (double underlined in sequence no. 2, Table 2) did not always amplify both parental alleles, a null allele was suspected. Therefore new primers (Table 1) were synthesized and alleles of different lengths were sequenced (Table 2). Obviously the amplification failure was due to a 1 bp insertion in sequence no. 1 at the 3′ end of the primer attachment site. In addition, an 8 bp (GCG-