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PRIMER NOTE

Isolation and characterization of microsatellite loci in the cichlid fish *Pseudotropheus zebra*

M.J.H. VAN OPPEN, C. RICO, J.C. DEUTSCH,* G.F. TURNER† and G.M. HEWITT
*Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK, *Imperial College, Silwood Park, Ascot SL5 7PY, UK, †Department of Biology, University of Southampton, Soton SO16 7PX, UK*

We have isolated and characterized a series of $(GT/CA)_n$ and $(CT/GA)_n$ microsatellite loci from the malawian cichlid fish *Pseudotropheus zebra* BB (Boulenger), with the aim of developing a PCR system to analyse individual loci on the A.L.F. sequencer (Pharmacia). A size-selected genomic library was constructed as described in Rico *et al*. (1994b) using a DNA mix from 20 unrelated individuals from Nkhata Bay, Lake Malawi. Amongst ≈ 4500 recombinants were 42 GT and 17 CT positives, suggesting that a GT repeat occurs roughly every 35 kb and a CT repeat roughly every 85 kb in the *P. zebra* genome. We have sequenced 45 of these clones as described in Rico *et al*. (1994a) and designed primers from both flanking regions for nine clones using the software program OLIGO™ Macintosh version 4.0 (National Biosciences). PCR conditions were optimized for seven of the nine loci (Table 1). PCR was carried out in 11-μL reactions consisting of 1 μL template DNA (≈ 20 ng), 1.1-μM of each primer (the forward primer was fluorescein-labelled), 200-μM of each dNTP, 1 or 3-mM $MgCl_2$ (Table 1), 0.2 μg BSA, 1 × NH_4 reaction buffer (Bioline, London, UK) and 0.25 units of BioTaq (Bioline). The mixture was overlaid with 10 μl mineral oil. The PCR profile used on an OmniGene Thermal Cycler (Hybaid) was as follows: an initial denaturing step of 3 min 94 °C was followed by 7 cycles of 30 s at 94 °C, 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C, followed by 23 cycles of 30 s at 89 °C, 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C. With this PCR profile genomic DNA and PCR products will be denatured and amplified during the first seven cycles, while mainly PCR products will be denatured and reamplified during the subsequent 23 cycles, resulting in fewer non-specific products. PCR products were diluted to the appropriate concentration (0–12× diluted) with dilution buffer (consisting of 600 μL Dextran-Blue loading dye [6 mg of Dextran-Blue/mL deionised formamide] and 900 μL of deionised formamide) and mixed with internal size markers prior to electrophoresis. Size markers were made as follows: 100 ng of M13mp18 + was used as DNA template using fluorescein-labelled universal primer and an unlabelled self-designed reverse primer (Table 2). PCR was carried out in 100 μL reactions, containing 0.5 μM of each primer, 200 μM of each dNTP, 1 × KCl reaction buffer containing $MgCl_2$ (Bioline) and 1.25 units of BioTaq. The reaction mixture was overlaid with a drop of mineral oil and amplified in a Perkin Elmer Cetus thermocycler using an initial denaturing step of 3 min at 94 °C followed by 28 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (Table 2) and 1 min at 72 °C. The last cycle had a 5 min instead of a 1 min extension time. PCR products were then run on 1% TBE-agarose gels stained with EtBr and the band of the correct size was excised from the gel. The piece of excised gel was placed in a punctured 0.5-μl microfuge tube filled with cotton wool. The tube was then placed in a 1.5 μL microfuge tube and spun in a microcentrifuge for 2–5 min. The DNA in TBE buffer was collected from the bottom of the 1.5-μL tube while the agarose remained in the cotton wool. One microlitre of this DNA was used for reamplification using the same PCR profile as before except that the steps were shortened to 30 s instead of 1 min. Between 0.5 and 1 μL of these PCR products was used to size the various alleles. PCR products (two loci were combined where possible) and size markers were run on short A.L.F. plates using Sequagel Extended (National Diagnostics) at 1000 V, 60 mA, 50 W, 48 °C and 1.25 s sampling time. A single gel was used for three consecutive runs. Running time varied between 30 and 55 min, depending on the size of the alleles. Alleles were sized using the software program Fragment Manager™ version 1.2 (Pharmacia).

Number of alleles and expected heterozygosities varied considerably among the different loci (Table 1). As expected, the longest repeats were most polymorphic. Repeats < 7 were monomorphic. Most of the polymorphic loci are useful in resolving population structure (M. J. H. van Oppen *et al*. unpubl. data).

Several additional cichlid microsatellite primers have recently been developed by other groups (Kellogg *et al*. 1995; Lee & Kocher 1996; Parker & Kornfield 1996; Zardoya *et al*. 1996).

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Correspondence: M. J. H. van Oppen. Tel: +(0)1603-592-947, Fax: +(0)1603-592-250. E-mail: m.oppen@uea.ac.uk

Table 1 *Pseudotropheus zebra* BB. Repeat sequence for the seven loci, optimal annealing temperature (T_A), optimal $MgCl_2$ concentration, primer sequences, size range of alleles, number of alleles (A), expected heterozygosity (H_E), sample size (n) and EMBL accession number

Locus	Repeat array of the cloned allele	T_A (°C)	$MgCl_2$ (mM)	Primer sequences (5′-3′) *F* = forward primer, R = reverse primer	Size range (bp)	A	H_E	n	Accession number
Pzeb1	$(GT)_{59}$	54	1	F: GCGAAAACAGGGCATGCATACAG R: AGCAGTGGATGAGTCATACTTAC	134–242	47	0.96	96	X99783
Pzeb2	$(GT)_{41}$	54	1	F: TTCGGTAGACTGATGCTTTCATA R: AAAGCCAAAGGGTGTGAACTGA	204–286	29	0.95	99	X99784
Pzeb3	$(GT)_{11}$	54	1	F: GAGCCTGCAAACCTTACTGTAAA R: AAGCTACACAAATTCCACTCATA	314–338	11	0.74	91	X99785
Pzeb4	$(GT)_4(TTGT)_2CTGCCT(GC)_{10}$ $GC(GT)_2CT(GT)_5GC(GT)_2$	56	1	F: GCTTGTTTTGGGTTGGTTTTGT R: ATGGACACGTGGACTCAAAGAC	123–145	10	0.77	98	X99786
Pzeb5	$(CT)_2TTT(CT)_8CA(CT)_2GTCT$	53	1	F: TGCAGCTGCTTTAAATTGCTTCA R: CTGGCAAGTTCTGGACTGACAC	121–137	6	0.34	98	X99787
Pzeb6	$(GT)_6(TG)_2(TC)_2$	46	1	F: TCAAATCAAAAGTACATTGT R: AACGCTATTCTGGACACGATTC	129	1	0	90	X99788
Pzeb7	$(GT)_6T(GT)_2$	53	3	F: GATCAATCAAAGTACATGGTTGT R: TTAGCCTCAAGTGACATAAATGA	156	1	0	20	X99789

Table 2 Primer sequences of reverse primers used in combination with universal primer to synthesise size markers and the annealing temperature (T_A) used for PCR.

Size marker (bp)	Primer sequence (5′-3′)	T_A (°C)
100	CTATGACCATGATTACGAATTC	50
154	GGCTCGTATGTTGTGTGGAATTGT	56
200	GTTAGCTCACTCATTAGGCACCC	53
259	CTGGCACGACAGGTTTCCCGACTG	59
312	CGCCCAATACGCAAACCGCCTCTC	60
353	GCTGTTGCCCGTCTCGCTGGTGAA	60

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