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PERMANENT GENETIC RESOURCES

Development of cytochrome *b* primers for mitotyping of barbels (*Barbus* spp.)

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Abstract

Primers were developed that allow for rapid, reliable and inexpensive screening of cytochrome *b* by analysis of single-stranded conformational polymorphisms. Twenty different haplotypes were identified from six species of Iberian *Barbus*. These primers proved useful for population and species level studies, and could also be valuable in population genetic and phylogenetic studies of other cyprinin fishes.

Keywords: Cyprininae, HBB674, LBB377, *Luciobarbus*, mitotype, SSCP

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Species of the genus *Barbus s.s.* are distributed throughout fresh waters of Eurasia and northern Africa (Howes 1987). Molecular markers have shed light on the taxonomy and evolution of this speciose genus, including recognition of cryptic species and frequent interspecific hybridization (e.g. Doadrio *et al*. 2002; Kotlík *et al*. 2002; Tsigenopoulos *et al*. 2002, 2003). In order to have diagnostic mitochondrial markers for studies of Iberian *Barbus*, we have developed primers for a species-specific region of the mitochondrial cytochrome *b* (cyt *b*). This region can be quickly and reliably screened for single-stranded conformational polymorphisms (SSCP). Primers were designed in regions of low variability using multiple sequence alignment of several species belonging to *Barbus* and *Luciobarbus* subgenera (Doadrio 1984) and placed to produce fragments shorter than 300–400 bp, maximizing efficiency in detection of variants (Sunnucks *et al*. 2000), while diagnosing the different species. The new primers (LBB377, 5′-CAGCCTTCGTYGGCTACGTACT-3′ and HBB674, 5′-AATCCRAGCAGGTCTTTGTAGG-3′) anneal between bases 377 and 695 of the cyt *b* gene, amplifying a 319-bp long fragment, and assaying 275 bp.

Initial polymerase chain reaction (PCR) amplifications were carried out in 25-μL reactions containing 1× PCR buffer, 0.5 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (Fermentas) and approximately 50 ng of template DNA in a Biometra Tgradient thermocycler. Cycling profile for PCR amplifications was 3 min at 94 °C (1 cycle), 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C (30 cycles) and 4 min at 72 °C (1 cycle). PCR products were visualized using 1.5% agarose gels (1× tris-borate-EDTA) stained with ethidium bromide. Fragment homology was confirmed by direct sequencing using the PCR primers on an ABI 3730 DNA Analyser.

Two sets of both primers were labelled on the 5′-end with TET and 6-FAM dyes for fluorescent SSCP analysis on an ABI PRISM 377 DNA sequencer. A fragment with a known sequence (to be used as internal standard) was amplified in 50-μL reactions containing 1× PCR buffer, 0.5 μM of each TET-labelled primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (Fermentas) with approximately 50 ng template DNA. Samples to be genotyped were amplified in 10-μL reactions containing 1× PCR buffer, 0.5 μM of each 6-FAM-labelled primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Fermentas) with about 20 ng of template DNA in an Eppendorf Mastercycler gradient machine. Cycling profile for both dye-labelled PCR amplifications was 3 min at 94 °C (1 cycle), 60 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C (30 cycles) and 4 min at 72 °C (1 cycle).

One microlitre of each 6-FAM-labelled sample was combined with 0.5 μL TET-labelled internal standard and 3.0 μL denaturing solution (100 mM NaOH, 50 mg/mL Blue Dextran and 50 mM EDTA, in deionized formamide), denatured at 94 °C for 3 min and kept on ice. One

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Table 1 Summary of cross-species screening of cyt *b* SSCPs using primers LBB377-HBB674. A total of 1381 specimens of six species of *Barbus* belonging to the two recognized subgenera were assayed. Subgenera: *Barbus**; *Luciobarbus*†. No. of differences and percentage of "*p*" distance reflect variation among all haplotypes at the intra- and interspecific levels. Haplotype codes, accession numbers and frequency distribution per species are also shown

			Intraspecific		Interspecific		Haplotypes	
Species	Sample size	No. of haplotypes	No. of differences	Percentage of '*p*' distance	No. of differences	Percentage of '*p*' distance	Letter code & accession no.	Frequency
B. bocagei†	287	3	1–3	0.364–1.091	4–28	1.455–10.182	*A*: AM748071	2.1%
							B: AM748072	97.6%
							Q: AM748073	0.3%
B. comizo†	441	4	1–2	0.364–0.727	4–30	1.455–10.909	*C*: AM748074	98.2%
							D: AM748075	1.1%
							G: AM748076	0.5%
							O: AM748077	0.2%
B. graellsii†	29	1	NA	NA	6–22	2.182–8.000	*X*: AM748089	100%
*B. haasi**	25	1	NA	NA	22–30	8.000–10.909	*Z*: AM748091	100%
B. microcephalus†	145	7	1–3	0.364–1.091	6–27	0.727–9.818	*I*: AM748082	0.7%
							J: AM748083	9.0%
							K: AM748084	2.8%
							L: AM748085	1.4%
							M: AM748086	83.4%
							N: AM748087	2.1%
							P: AM748088	0.7%
B. sclateri†	454	4	1–4	0.364–1.455	9–30	3.273–10.909	*E*: AM748078	1.8%
							F: AM748079	12.1%
							H: AM748080	85.9%
							R: AM748081	0.2%

microlitre of each sample was loaded onto a vertical acrylamide : bis-acrylamide gel (37.5:1, 8%). The gel was run at the settings for wattage-limiting module as GS Run 60 W C Chiller for 12 h using filter set C on an ABI PRISM 377 sequencer. Gel temperature was kept constant at 24 °C using a Betta Tech cooling system. Data were analysed using GENESCAN 3.1.2. Samples were aligned using the internal standard and scored by comparison with known haplotypes included on each gel. New SSCP haplotypes and some previously identified haplotypes were sequenced using primer LCB1 (Brito *et al*. 1997), allowing for characterization of the entire SSCP fragment and assessment of the protocol. This protocol was also found to work for other cyprinins, as indicated by amplification of *Carassius auratus* and *Cyprinus carpio*.

Overall, SSCP and sequence analysis of 1381 specimens of six Iberian *Barbus* species, belonging to the two subgenera, identified 20 haplotypes (Table 1). Pairwise '*p*' distances ranged from 0.364% to 10.909%. The list of 48 polymorphic sites can be found in Table 2. Scoring of haplotypes was straightforward and accurate, especially those belonging to different species. Only the most common (*M*) and a rare (*L*) haplotype of *Barbus microcephalus* exhibited very similar conformations.

These results demonstrate the utility of SSCP analysis with these primers for population and species level studies, as both very similar and divergent haplotypes were correctly identified. This methodology allows for rapid screening and reliable assessment of individual mitotypes, particularly in ongoing studies of population genetics and (female-mediated) introgressive hybridization in *Barbus*. Additionally, these primers may be useful in studies of other cyprinins, as suggested by the successful amplifications in *Carassius* and *Cyprinus*. The new primers could also prove useful as internal primers in phylogenetic and barcoding studies in combination with available primers (e.g. Sevilla *et al*. 2007).

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Table 2 List of the 48 polymorphic sites for each of 20 haplotypes found in the six species of *Barbus* screened. Dots represent matching identity to the first sequence. Accession numbers AM748071-AM748089 and AM748091

Polymorphic position	381	384	396	405	425	429	432	441	444	450	456	459	465	468	469	474	477	480	489	492	494	501	504	507	516	522	531	537	543	547	549	552	561	564	567	570	573	588	591	594	597	606	609	612	618	636	639	651
Hap. A	A	A	C	T	A	A	C	A	C	A	G	A	A	G	A	A	T	A	A	T	A	A	A	A	T	C	A	A	T	G	A	T	C	A	C	C	A	C	A	G	C	C	G	G	A	T	C	C
Hap. B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap. C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Hap. D	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Hap. E	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	G	.	.	.	.	G	.	.	.	G	.	.	.	.	C	.	.	T	T	.	.	.	.	.	.	.	.	G	.	.	T
Hap. F	.	G	.	.	.	.	.	G	T	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	T	T	.	.	.	.	.	.	.	.	G	.	.	T
Hap. G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Hap. H	.	G	.	.	.	.	.	G	T	.	.	G	.	.	.	.	.	G	.	.	.	G	.	.	.	.	G	.	.	.	.	C	.	.	T	T	.	.	.	.	.	.	.	.	G	.	.	T
Hap. I	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	G	.	.	.	.	C	C	.	.	.	.	A	.	C	.	.	T	.	.	.	G	A	.	.	A	.	.	.	.	T
Hap. J	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	.	.	.	.	.	C	C	.	.	.	C	A	.	C	.	.	T	.	.	.	G	A	.	.	A	.	.	.	.	T
Hap. K	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	.	.	.	.	.	C	C	.	.	.	.	A	.	C	.	G	T	.	.	.	G	A	.	.	A	.	.	.	.	T
Hap. L	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	.	.	.	.	.	C	C	.	.	.	.	A	.	C	.	.	T	.	.	.	G	A	A	.	A	.	.	.	.	T
Hap. M	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	.	.	.	.	.	C	C	.	.	.	.	A	.	C	.	.	T	.	.	.	G	A	.	.	A	.	.	.	.	T
Hap. N	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	G	.	.	.	.	.	C	C	.	.	.	C	A	.	C	.	.	T	.	.	.	G	A	.	.	A	.	.	.	T	T
Hap. O	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Hap. P	G	.	.	.	.	.	.	.	T	G	.	C	.	.	.	.	G	.	.	.	.	.	.	C	C	.	.	.	.	A	.	C	.	.	T	.	.	.	G	A	.	.	A	.	.	.	.	T
Hap. Q	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
Hap. R	.	G	.	.	.	.	.	G	T	.	.	G	.	.	G	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	T	T	.	.	.	.	.	.	.	.	G	.	.	T
Hap. X	G	.	.	.	.	.	.	G	T	.	.	C	.	A	.	.	G	G	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	T	.	.	.	G	A	.	.	.	.	.	.	.	T
Hap. Z	.	G	T	C	.	C	.	G	T	.	A	.	G	A	.	.	C	G	.	C	.	.	.	.	C	T	.	G	.	.	T	C	T	.	.	.	G	A	G	A	.	T	A	A	.	C	T	T

References

Brito RM, Briolay J, Galtier N, Bouvet Y, Coelho MM (1997) Phylogenetic relationships within genus *Leuciscus* (Pisces, Cyprinidae) in Portuguese fresh waters, based on mitochondrial cytochrome *b* sequences. *Molecular Phylogenetics and Evolution*, **8**, 435–442.

Doadrio I (1984) *Relaciones filogenéticas y biogeográficas de los barbos (Barbus, Cyprinidae) de la Península Ibérica y aportes corológicos y biogeográficos a su ictiofauna continental*. PhD Thesis, University Complutense de Madrid, Madrid, Spain.

Doadrio I, Carmona JA, Machordom A (2002) Haplotype diversity and phylogenetic relationships among the Iberian barbels (*Barbus*, Cyprinidae) reveal two evolutionary lineages. *Journal of Heredity*, **93**, 140–147.

Howes GJ (1987) The phylogenetic position of the Yugoslavian cyprinid fish genus *Aulopyge* Heckel, 1841, with an appraisal of the genus *Barbus* Cuvier & Cloquet, 1816 and the subfamily Cyprininae. *Bulletin of the British Museum of Natural History (Zoology)*, **52**, 165–196.

Kotlík P, Tsigenopoulos CS, Ráb P, Berrebi P (2002) Two new *Barbus* species from the Danube River basin, with redescription of *B. petenyi* (Teleostei: Cyprinidae). *Folia Zoologica*, **51**, 227–240.

Sevilla RG, Diez A, Norén M *et al*. (2007) Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome *b* and nuclear rhodopsin genes. *Molecular Ecology Notes*, **7**, 730–734.

Sunnucks P, Wilson ACC, Beheregaray LB, Zenger K, French J, Taylor AC (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Molecular Ecology*, **9**, 1699–1710.

Tsigenopoulos CS, Kotlík P, Berrebi P (2002) Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian Peninsula and neighbouring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. *Biological Journal of the Linnean Society*, **75**, 83–99.

Tsigenopoulos CS, Durand JD, Ünlü E, Berrebi P (2003) Rapid radiation of the Mediterranean *Luciobarbus* species (Cyprinidae) after the Messinian salinity crisis of the Mediterranean Sea, inferred from mitochondrial phylogenetic analysis. *Biological Journal of the Linnean Society*, **80**, 207–222.