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Paralog-Specific Primers for the Amplification of Nuclear Loci in Tetraploid Barbels (*Barbus*: Cypriniformes)

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Thirty paralog-specific primers were developed, following an intron-primed exon-crossing strategy, for *S7* and *growth hormone* genes in *Barbus* (subgenera *Barbus* and *Luciobarbus*). We found that paralog-specific amplification requires the use of only one paralog-specific primer, allowing their simultaneous use with universal exon-primed intron-crossing primers of broad taxonomic applicability. This hybrid annealing strategy guarantees both specificity and generality of amplification reactions and represents a step forward in the amplification of duplicated nuclear loci in polyploid organisms and members of multigene families. Assays of several representative taxa identified high levels of segregating single nucleotide polymorphisms (SNPs) and nucleotide diversity within each of these subgenera. Additionally, several insertions–deletions (indels) that are diagnostic across species are found in intronic regions. Therefore, these primers provide a reliable source of valuable nuclear SNP and indel data for population and species level studies of barbels, such as applied conservation and basic evolutionary studies.

Key words: Barbus, *growth hormone*, Luciobarbus, *paralogous*, *SNP*, *S7*

Nuclear sequence data have been used with increasing frequency in population genetic, phylogenetic, and more recently in phylogenomic studies. Consequently, several PCR primers have become available, including several sets for cypriniform fishes (e.g., Li et al. 2007, 2010; Chen et al. 2008; Tao et al. 2010). Single nucleotide polymorphisms (SNPs) and insertions–deletions (indels) have become preferred genetic markers due to their availability across the genome, reliable detection, and information content (e.g., Ryynänen et al. 2007; Coates et al. 2009). However, most available "universal" nuclear primers have been developed for the amplification of single-copy genes, usually using an exon-primed intron-crossing strategy (EPIC; Lessa 1992; Slade et al. 1993; Palumbi and Baker 1994). EPIC primers are designed in conserved exonic regions to allow generality of use while crossing more variable intronic regions, hence their "universality." This primer design strategy explicitly focuses on single-copy genes due to complexities generated by the presence of paralogous copies. Such strategy results in the loss of information potentially provided by analysis of members of multigene families and different paralogs found in organisms with duplicated genomes. Members of multigene families and paralogs in polyploids arisen from recent genome duplication show high sequence similarity, especially protein-coding exons. Consequently, non-specific amplification is expectable, making universal EPIC primers unsuitable for their study. In such cases, using an intron-primed exon-crossing (IPEC) strategy similar to that used by Ryynänen and Primmer (2006) in salmonids is recommended. IPEC primers are designed in variable intronic regions that differ between paralogous loci, such as indels and SNPs, to allow paralog-specific amplification. As opposed to EPIC, the trade-off in IPEC primers lies between paralog-specific amplification and amplification that is only species specific and hence potentially of less general use.

As in many polyploid organisms, the risk of simultaneous amplification of paralogous loci is clear in tetraploid *Barbus s.s.*, which have undergone an additional round of genome duplication relative to other teleost fishes (e.g., Ráb and Collares-Pereira 1995). This genus is composed of dozens of species and is widespread across freshwaters of Europe, southwestern Asia, and northern Africa (Howes 1987), where it is an important component of local faunas. Furthermore, many species have very similar morphologies, a feature that has convoluted not only their taxonomy

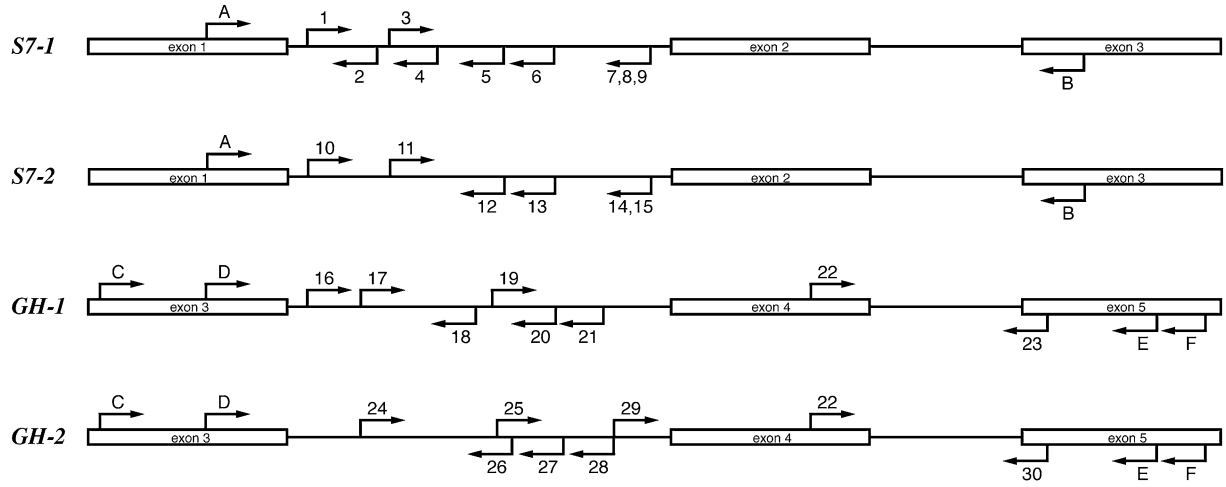

Figure 1. Schematic representation of the 4 loci and annealing sites of primers used for amplification and sequencing. Numbers refer to Table 1.

(Kottelat and Freyhof 2007) but also ecology and evolution studies. Due to the complete genome duplication of *Barbus*, most molecular studies on barbels have relied on mitochondrial DNA sequence data, allozyme electrophoresis, and more rarely on microsatellites (e.g., Machordom et al. 1995; Chenuil et al. 1997; Zardoya and Doadrio 1999; Callejas and Ochando 2000; Kotlík and Berrebi 2001, 2002; Doadrio et al. 2002; Tsigenopoulos et al. 2002, 2003; Kotlík et al. 2004; Gante et al. 2009; Lajbner et al. 2009; Schreiber 2009). Indeed, studies that include *Barbus* and employ nuclear sequence data use at best one of the paralogous loci (Mayden et al. 2009; Marková et al. 2010). Different authors studying polyploid taxa have often resorted to cloning individual amplicons to identify paralogs (e.g., Bart et al. 2010; Saitoh et al. 2010), but this is impractical and expensive for large-scale studies. Therefore, there is the need for primers that consistently and reliably amplify each of the paralogs individually in tetraploid *Barbus*.

Among available nuclear loci, *S7* ribosomal protein and *growth hormone* (*GH*) genes have proven useful in phylogenetic studies of cypriniform fishes (e.g., Johnson et al. 2004; Schönhuth et al. 2008; Mayden et al. 2009; Moyer et al. 2009). Here, we develop 30 paralog-specific primers for *Barbus S7* and *GH* genes for population and species level studies, mostly using an IPEC strategy to avoid simultaneous amplification of paralogs. During amplification, we adopted a hybrid annealing strategy, successfully combining universal EPIC and paralog-specific IPEC primers for targeted paralog amplification.

Materials and Methods

We made use of available EPIC primers to generate sequences of the duplicated loci in representatives of *Barbus* and *Luciobarbus* subgenera, after which we designed several paralog-specific primers. Initially, paralogous copies of *S7* spanning exons 1–3 were simultaneously amplified using EPIC primers S7RPEX1F and S7RPEX3R (Chow and Hazama 1998) in 25 μl reactions containing 1 × PCR buffer, 0.5 μM of each primer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1 U Taq polymerase, and approximately 50 ng of template DNA. Cycling profile for PCR amplifications was 3 min at 94 °C (1 cycle); 30 s at 94 °C, 45 s at 55 °C, and 90 s at 72 °C (25 cycles); and 10 min at 72 °C (1 cycle). This PCR product was diluted with water (1:99) and 0.5 μl used as template in a nested PCR as above with EPIC primers S7RPEX1F and S7RPEX2R (Chow and Hazama 1998). Cycling profile was 3 min at 94 °C (1 cycle); 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C (20 cycles); and 30 min at 72 °C (1 cycle). PCR products were then cloned with TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions. Paralogous *S7* copies were individually amplified and sequenced from single colonies using M13 forward and reverse primers.

Likewise, paralogous copies of *GH* spanning exons 3–5 were simultaneously amplified using Multiplex PCR kit (Qiagen) in 10 μl reactions containing 0.5 μM of each primer and approximately 30 ng of template DNA using EPIC primers GHe3.min.3F, GHe3.min.63F, GHe5.173R, and GHe5.183R (Moyer et al. 2009). Cycling profile was 15 min at 95 °C (1 cycle); 60 s at 94 °C, 90 s at 56 °C, and 90 s at 72 °C (30 cycles); and 10 min at 72 °C (1 cycle). Products were visualized using 2% agarose gels stained with ethidium bromide. Because different paralogs show large size differences, we excised individual bands from the gel. DNA was eluted from individual gel slices by centrifugation for 40 s at 5000 revolutions per minute through QIAquick PCR purification kit columns (Qiagen) and different *GH* paralogs reamplified. Based on multiple sequence alignments, we designed 30 paralog-specific primers in regions that differ between loci, mostly in intronic regions following an IPEC strategy (Figure 1; Table 1).

We tested these newly designed primers in 186 specimens of 6 species of the subgenus *Luciobarbus* (*B. bocagei*, *B. comizo*, *B. graellsii*, *B. guiraonis*, *B. microcephalus*, and *B. sclateri*) and in

Table 1 Primers used for amplification of nuclear loci and their performance in subgenera *Barbus* and *Luciobarbus*

Primer name	No.	Sequence (5′-3′)	*Barbus*	*Luciobarbus*	Source
S7RPEX1F	A	TGGCCTCTTCCTTGGCCGTC	+	+	Chow and Hazama (1998)
S7RPEX3R	B	GCCTTCAGGTCAGAGTTCAT	+	+	Chow and Hazama (1998)
Bs7-1.i1.19F	1	CCCAGCTAAAGAGTTTTCAAATG	+	+	This study
Bs7-1.i1.202R	2	GCACATGGGGCCCAGTAAT	+	+	This study
Bs7-1.i1.281F	3	GATGGCCCATATTTGCGATT	+	+	This study
Bs7-1.i1.349R	4	CTCCAAGCATGTCGTTAGCAC	+	+	This study
Bs7-1.i1.548R	5	TGACATACAACCTAACCTACTG	−	+	This study
Bs7-1.i1.628R	6	GTCTGCAGAAATAAGTCACTGAA	+	+	This study
Bs7-1.i1.802Ra	7	TGTCTRATTTACTGACGCCCATG	−	+	This study
Bs7-1.i1.802Rb	8	TATCTAATTTATTAACGCCAATG	+	−	This study
Bs7-1.i1.802Rc	9	TRTCTMATTTATTAACGCCAATG	+	−	This study
Bs7-2.i1.19F	10	CCCAGCTAAAGAGTTATCAAGTT	+	+	This study
Bs7-2.i1.269F	11	GGTGGCCCATATTTGCGGTA	−	+	This study
Bs7-2.i1.552R	12	GATGACATACAACCTACTAACG	−	+	This study
Bs7-2.i1.628R	13	CTGCAGAAGCAAGCCATTAAG	+	+	This study
Bs7-2.i1.785Ra	14	TGTCTGATTTATTAACTCCCAAA	+	+/−	This study
Bs7-2.i1.785Rb	15	GAAACTGATTTATTAACTCCCAAA	−	+/−	This study
GHe3.min.3F	C	GACAACCTGTTGCCTGAGGAACGC	+	+	Moyer et al. (2009)
GHe3.min.63F	D	GCAACTCTGACTCCATTGAGGC	+	+	Moyer et al. (2009)
GHe5.173R	E	CAGTTGGAATCCAGGGATCTC	+	+	Moyer et al. (2009)
GHe5.183R	F	CTACAGGGTGCAGTTGGAATC	+	+	Moyer et al. (2009)
Bgh-1.i3.79F	16	GGGGTCTGTGGAAAAGTTTGG	+	+	This study
Bgh-1.i3.179F	17	ACCAATAGAATAAAGTAATGAAA	+/−	+	This study
Bgh-1.i3.432R	18	CCAACAGGAGTTTTCGATCTT	−	+	This study
Bgh-1.i3.479F	19	CAACTAACAAATTGTATCAATC	+/−	+	This study
Bgh-1.i3.536R	20	GTCATCCACTTGTGTGAAAGC	+/−	+	This study
Bgh-1.i3.661R	21	GTCAATCCAAGTACTGTGT	+	+	This study
Bgh.e4.110F	22	ATCACTGAGAAGCTGGC	+	+	This study
Bgh-1.e5.32R	23	AGTGGCAGGGAGTCATTG	−	+	This study
Bgh-2.i3.226F	24	GTACTATAGTAAGCAGAAATGG	+	+	This study
Bgh-2.i3.438F	25	TGAAAATCTGGAATCTGAGGG	+	+	This study
Bgh-2.i3.669R	26	GTCTTAAGTCGCTGGGGTATA	+/−	+	This study
Bgh-2.i3.736R	27	AAGACCCAATTTATACCATGA	+/−	+	This study
Bgh-2.i3.774R	28	CATCCTACAATTTAAAAGGCAGC	+	+	This study
Bgh-2.i3.774F	29	GCTGCCTTTTAAATTGTAGGATG	+	+	This study
Bgh-2.e5.32R	30	AGTGGSAGGGAGTCGTTC	+	+	This study

No. corresponds to Figure 1. Number after dash refers to locus; i and e refer to intron and exon, respectively; F and R refer to forward and reverse, respectively. + refers to positive amplification, − refers to no amplification, and +/− refers to amplification in some but not all species tested.

19 specimens of 4 species of the subgenus *Barbus* (*B. barbus*, *B. carpathicus*, *B. haasi*, and *B. prespensis*). All reactions were done with Multiplex PCR kit (Qiagen), in 10 μl containing 0.5 μM of each primer and approximately 30 ng of template DNA. Cycling profiles were 15 min at 95 °C (1 cycle); 60 s at 94 °C, 90 s at 55 or 59 °C, and 90 s at 72 °C (30 cycles); and 10 min at 72 °C (1 cycle). Annealing temperature was 59 °C for primers no. 16 and 23, and 55 °C for the remaining. All sequences were obtained on an ABI 3730 DNA Analyzer using Big Dye 3.1 terminator (Applied Biosystems). Big Dye 3.1 terminator (Applied Biosystems) sequencing reactions of *GH-1* using primers no. 16 and 23 were done at 60 °C annealing temperature. Sequences were deposited in public nucleotide databases with the following accession no.: *GH-1* (FR872141–FR872195), *GH-2* (FR872196–FR872248), *S7-1* (FR872249–FR872303), and *S7-2* (FR872304–FR872376).

Estimates of sequence polymorphism (i.e., number of SNPs, average number of nucleotide differences, and nucleotide diversity) were obtained using DnaSP v5.10.01 (Librado and Rozas 2009). Potential cross-amplification of *S7* and *GH* in other genera was assessed by sequence similarity of each new primer to the nucleotide database at NCBI. We conducted a stringent search using BLASTn 2.2.24 (Altschul et al. 1997) against the Cypriniformes nucleotide database (excluding *Danio rerio* to reduce database redundancy) with a word size of 15 and high gap costs (12 to open and 8 to extend). PCR products can be generated using primers with up to 15–20% base-pair mismatches with the template, in particular in the 5′ end (Rychlik 2000). On the other end, the yield is substantially reduced for primers with 2 mismatches within the last four 3′-termini bases (Rychlik 2000). Primers with 3′-termini mismatches are extended 10^{-2}- to 10^{-6}-fold less efficiently than correctly matched 3′-termini primers (Huang et al. 1992), and amplification is also constrained due to mismatches in the penultimate nucleotide (Ayyadevara et al. 2000). Therefore, sequences with any mismatches within 3 bp of the 3′ end of the primer were discarded, and only sequences with more than 85% similarity were considered, as a requirement for potential positive amplification.

Table 2 Size range of amplicons in subgenera *Barbus* and *Luciobarbus* obtained with the newly designed paralog-specific primers

	Barbus					*Luciobarbus*					*Barbus* + *Luciobarbus*		
Locus	Primer no.	Length	S	*k*	π	Primer no.	Length	S	*k*	π	S	*k*	π
S7-1	1 + 8,9	745–757	13	5.400	0.00763	1 + 7	747–785	42	5.378	0.00760	67	7.063	0.00998
S7-2	10 + 14	741–788	33	9.128	0.01327	10 + 14,15	733–750	38	4.133	0.00601	76	6.459	0.00939
GH-1	16 + 30	660–896	17	6.913	0.01262	16 + 23	947–969	12	0.734	0.00134	33	2.174	0.00397
GH-2	24 + 30	932–1062	33	11.181	0.01271	24 + 30	1022–1098	39	6.643	0.00755	83	10.964	0.01246

Locus polymorphism data are given for each subgenus separately and both combined. Primer no. corresponds to Figure 1. For primer sequences, see Table 1. S = number of segregating sites, k = average number of nucleotide differences, and π = nucleotide diversity.

Results and Discussion

All 30 newly designed primers successfully amplify the locus of interest in either one or both subgenera (Table 1). Only one paralog-specific primer is necessary to amplify a specific paralog, either in combination with another paralog-specific IPEC primer or a universal EPIC primer. This latter finding is particularly relevant, as it allows a greater number of primer pair combinations to be used when amplifying a particular locus. The hybrid annealing strategy developed here avoids expensive and time-consuming cloning while guaranteeing both specificity and generality of amplification reactions. This property could be extended to members of multigene families and other organisms with duplicated genomes, both animals and plants, increasing specific paralog amplification success rate by combining IPEC and EPIC primers. Furthermore, different loci can be multiplexed as long as annealing temperatures and amplicon lengths are similar, also increasing efficiency of use. These factors are usually considered potential drawbacks in SNP discovery and genotyping in nonmodel organisms (Garvin et al. 2010).

The total amount of sequence obtained varied from approximately 3000–3500 bp (Table 2), substantially increasing the amount of nuclear sequence data, and therefore, potential SNPs available for *Barbus*. In addition, the high levels of sequence polymorphism found in subgenera *Barbus* and *Luciobarbus* (i.e., total number of segregating SNPs, average number of nucleotide differences, and nucleotide diversity) indicate these variable markers are sufficiently polymorphic for population and species level applications, such as phylogenetic and population genetic studies (Table 2). Furthermore, there are several indels that confer species-diagnostic size differences and can be used for rapid species identification by fragment length analysis of the amplicons (not shown, Gante 2009). These results are in agreement with introns being a prime source of SNP and indel data for use in molecular phylogenetics (Creer 2007).

A stringent BLASTn search against the Cypriniformes nucleotide database was conducted to test the general utility of these primers for studies of other cypriniform fishes. Results indicate that 10 of the 30 newly designed primers show a sequence similarity of more than 85% and are a perfect match in the last 3 bp of the 3′ termini in Balitoridae, Catostomidae, and several Cyprinidae (Supplementary Table 1). These properties indicate these primers are likely to be successfully used for PCR (Huang et al. 1992; Ayyadevara et al. 2000; Rychlik 2000) and potentially cross-amplify *S7* and *GH* in many genera besides *Barbus* and *Luciobarbus*, increasing their applicability across cypriniform fishes.

Our study shows that IPEC is a good strategy for selective amplification of nuclear loci in organisms with duplicated genomes, such as in the case of salmonids (Ryynänen and Primmer 2006). Additionally, paralog-specific amplification following a hybrid annealing strategy requires the use of only one paralog-specific IPEC primer, allowing the simultaneous use of EPIC primers of more general cross-amplification. Altogether, the nuclear primers made available here represent a valuable and reliable source of SNP and indel data in a diverse, taxonomically challenging group of fishes with duplicated genomes, for population and species level studies.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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