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Variable microsatellite markers amplify across divergent lineages of cyprinid fishes (subfamily Leusicinae)

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Cyprinidae is arguably the most species-rich, primary fish family in the world with over 2000 species distributed on four continents (Helfman et al. 1997). The family includes economically and scientifically important freshwater species such as carps, minnows, barbs, and zebrafishes. Thirty-nine of the 270 or so cyprinid species that occur in North America are threatened or endangered, and 29 of those occupy arid river basins in the southwestern US (US Fish and Wildlife Service 2002). Conservation efforts for threatened and endangered cyprinids are increasingly dependent on genetic data for guidance in defining conservation units and designing captive propagation plans for endangered taxa (Hedrick et al. 2000). In this study, we present primer sequences and PCR conditions for eight polymorphic DNA microsatellite loci developed for cyprinids. We tested each primer pair for its ability to amplify representatives of three genetically divergent North American cyprinid lineages (all in the subfamily Leuciscinae – Cavender and Coburn 1992), and summarize previously published cross-amplification results and microsatellite allele frequency data from one European cyprinid fish lineage (Salguiero et al. 2003). Two of the cyprinid fish species examined in this study are in danger of extinction.

Microsatellite loci were isolated from a partial genomic library constructed for the common shiner, *Luxilus cornutus*, which is an abundant cyprinid found in the northeastern US. Nucleic acids were obtained through density gradient ultracentrifugation purification (Dowling et al. 1996) and then digested with the restriction endonuclease *Dpn*II (New England Biolabs). Resulting fragments were size-selected in the range of approximately 200–800 base pairs (bp) by electrophoresis through a low-melting point agarose gel and purified using Prep-A-Gene (Bio-Rad) DNA purification kits. Size-selected fragments were ligated into pUC18, and heat-shock transformed into *E. coli* strain DH5α following Sambrook et al. (1989). The resulting partial genomic library consisted of 1920 clones that were transferred to nylon membranes. Membranes were screened for three classes of synthetic oligonucleotide probes: di-($[CA]_{15}$, $[GA]_{15}$); tri-($[CCT]_7$, $[ATT]_7$); and tetranucleotide ($[GATA]_5$, $[GAGC]_5$, $[GTCA]_5$, $[CTCA]_5$, $[GACT]_5$, $[CTAG]_5$, $[GCAT]_5$, $[GCAC]_5$) repeats (Sigma-GenoSys). The tri- and tetranucleotide screening identified ten positive clones; subsequent nucleotide sequencing of nine clones revealed a diversity of tri- and tetranucleotide repeats. Dinucleotide screening yielded a minimum of 38 positive clones. Nucleotide sequencing of 14 positive colonies indicated dinucleotide repeats.

Polymerase chain reaction (PCR) primers were designed from unique nucleotide sequence regions flanking microsatellites using the computer progam OLIGO^{TM} (Macintosh vers. 4.0, National Biosciences). Annealing temperatures and $MgCl_2$ conditions were optimized for eight primer pairs (Table 1), using genomic DNAs isolated from one *L. cornutus* and one *L. chrysocephalus* individual as templates for PCR. A locus was considered optimized when PCR produced one or two strongly amplified bands with minimal stutter bands and other ancillary products. PCR amplification was carried out in 10 μl volumes containing 1 μl (50–200 ng) sample DNA, 1 μl 10X reaction buffer

Table 1. Microsatellite DNA loci obtained from the common shiner *Luxilus cornutus*. Product lengths in base pairs (bp) were determined from clones obtained from a partial genomic library. Microsatellite loci were originally developed for hybridization study of *L. cornutus* and *L. chrysocephalas*, but preliminary genetic screening indicated shared alleles even among geographically distant samples. Allele range sizes are not available. Repeat sequence indicates the repeat motif [in brackets] and the number of uninterrupted copies observed in the cloned allele. DNA sequences of clones are listed in GenBank under sequential accession numbers AY318777–AY318784

Locus	Primer sequences (5′-3′)	Product size (bp)	Repeat sequence	Annealing temp. (°C)	$[MgCl_2]$
Lco1	CAC GGG ACA ATT TGG ATG TTT TAT AGG GGG CAG CAT ACA AGA GAC AAC	159	$[GATA]_8GGC$ $TA[GATA]_2$	60	1.5
Lco2	ATT TTT AGG AGT GAT GTT CAG CAT CAA GTG TGT CAT TGA GGA AGT GAG	190	$[TGTC]_6[TATC]_3$ $TATA\ [TG(A)TC]_{16}$	53	2.25
Lco3	GCA GGA GCG AAA CCA TAA AT AAA CAG GCA GGA CAC AAA GG	225	$[TG]_9$	60	2.5
Lco4	ATC AGG TCA GGG GTG TCA CG TGT TTA TTT GGG GTC TGT GT	197	$[GT]_5ATTTT$ $[GT]_5[GA]_{11}$	60	2.25
Lco5	TTA CAC AGC CAA GAC TAT GT CAA GTG ATT TTG CTT ACT GC	118	$[CAGA]_2[CA]_{14}$	57	2.5
Lco6	CCA CAC AGA GCA TTT GTA TT GTG GCT GAT TTC ATT ATT CA	130	$[CA]_{12}$	57	2.5
Lco7	AGA AGG TAA GAG GAA TGT GT TGA GGT AGA TAG CCA AGT TT	129	$[GT]_2GTTT[GT]_8$ $GA[GT]_3$	57	2.5
Lco8	GCT TTG AAC ACT TGG CTT AT AGG CTG GAC TGA ATC ACT TC	255	$[TC]_{25}[TG]_5$	63	2.25

(500 mM KCl, 100 mM Tris [pH 9.0], 10% Triton-X 100), 200 μM of each dNTP, 1–2 mM $MgCl_2$, 5 pmols of each PCR primer, and 0.1 μl *Taq* DNA polymerase (isolated from a clone, Pluthero 1993). Initially, PCR products were labeled by incorporating [α^{32} P]-dATP. In subsequent PCR experiments with other cyprinid taxa, primers were end-labelled with [γ^{32} P]-dATP. PCR amplification consisted of 25–30 cycles of denaturation at 94 °C for 30 s, annealing at 56–62 °C for 30 s, and extension at 72 °C for 30 s, preceded by an initial denaturation step at 94 °C for 2 min. PCR products were analysed on 6% polyacrylamide gels (Sequagel, National Diagnostics) and visualized by autoradiography.

Eight pairs of microsatellite primers (Table 1) developed from the *L. cornutus* library were tested for their ability to amplify microsatellites in cyprinid fishes. Three species from North American were screened; *Platygobio gracilis* (flathead chub), *Rhinichthys cataractae* (longnose dace), and the federally endangered *Hybognathus amarus* (Rio Grande silvery minnow). DNA isolation, PCR, and scoring were conducted as above. However, annealing temperature was held constant at 48 °C and $MgCl_2$ held constant at 2.0 mM per reaction across all species. Genotype data were used to compute allele frequencies and observed and Hardy-Weinberg expected heterozygosities. For comparison, we included data on amplification and variability of these eight primers in the endangered *Anaecypris hispanica* on the Iberian Peninsula (Salguiero et al. 2003).

Despite some variability in expected product lengths and heterozygosity, microsatellite primer pairs generated from *Luxilis* produced well-resolved, polymorphic, and scorable products for five of eight loci, on average, for the other cyprinids examined (Table 2). Identical PCR conditions were employed but size ranges of alleles often did not overlap across loci (Table 2), which indicates that subsets of these microsatellite loci are potentially good candidates for multiplex PCR. Allelic diversity (i.e., the number of alleles, 0 = no amplification) was tested for association among taxa by Pearson correlation analysis. Significant correlation (r = 0.84; $P < 0.05$) of allelic diversity was observed for *Rhinichthys* and *Platygobio*, which may reflect their rela-

Table 2. Number of alleles, size range of alleles in base pairs (bp), observed (direct count) heterozygosity (H_O), and Hardy-Weinberg expected heterozygosities (H_e), scored in cross-species amplification experiments using eight loci developed from *Luxilus cornutus*. Number of individuals examined (n) is indicated for each species. Allele frequency data and PCR conditions for *Anaecypris hispanica* are described in Salguiero et al. (2003)

Locus	*Hybognathus amarus* (*n* = 12)				*Rhinichthys cataractae* (*n* = 12)				*Platygobio gracilis* (*n* = 12)				*Anaecypris hispanica* (*n* = 310)			
	Alleles	Size range (bp)	H_o	H_e	Alleles	Size range (bp)	H_o	H_e	Alleles	Size range (bp)	H_o	H_e	Alleles	Size range (bp)	H_o	H_e
Lco1	10	200–348	1.00	0.94	12	258–306	1.00	0.94	12	236–270	1.00	0.93	38	300–444	0.79	0.94
Lco2	–	–	–	–	1	254	0.00	0.00	–	–	–	–	–	–	–	–
Lco3	6	235–265	1.00	0.84	3	242–244	0.58	0.55	–	–	–	–	22	241–303	0.72	0.76
Lco4	2	228–237	0.36	0.42	3	233–239	0.42	0.04	3	230–240	0.58	0.54	33	250–320	0.80	0.90
Lco5	2	130–153	0.08	0.08	11	146–178	1.00	0.89	14	150–180	1.00	0.93	–	–	–	–
Lco6	5	160–189	0.58	0.59	–	–	–	–	5	180–182	0.75	0.79	1	N/A	0.00	0.00
Lco7	8	137–163	1.00	0.80	3	220–224	0.10	0.10	3	154–160	0.33	0.49	–	–	–	–
Lco8	5	278–302	0.83	0.80	4	288–294	0.75	0.73	–	–	–	–	–	–	–	–

–: scorable products were not obtained.
N/A: data not available.

tively recent common ancestry (Simons and Mayden 1998). Patterns of allelic diversity in *Hybognathus* and *Anaecypris* were not significantly correlated with other study species. Thus, allelic variability in one species is probably not a good predictor of variability in other cyprinid taxa. However, microsatellite loci developed in this work appear to be sufficiently variable and reliable for population and conservation genetic applications for a diverse group of leuciscin and phoxinin cyprinid fishes.

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