

PRIMER NOTE

Microsatellites from the vairone *Leuciscus souffia* (Pisces: Cyprinidae) and their application to closely related species

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Abstract

Eleven microsatellites were isolated from the vairone *Leuciscus souffia* (Risso 1826), an endangered fish that inhabits river systems in and around the Alps in Europe. The level of genetic diversity was assessed in 29 individuals of the subspecies *L. s. souffia*, and their variability was further estimated in seven individuals of a different subspecies, *L. s. muticellus*. Eight of these microsatellite loci were also applied to seven closely related cyprinid species. Availability of the reported microsatellite loci will facilitate the investigation of population genetic structure of these species with applications for the development of conservation strategies and phylogeographical approaches.

Keywords: conservation, freshwater fish, genetic diversity, *Leuciscus souffia*, microsatellites, population genetics

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Cyprinids are the most species-rich family of freshwater fishes in the Northern Hemisphere with a relatively uniform fauna in Central Europe. Since they are restricted to river and lake drainage systems, their distribution closely reflects their biogeographical history (Zardoya & Doadrio 1999). Therefore, cyprinids are appropriate model organisms to understand evolutionary mechanisms that drive the diversification and distribution of species. The vairone, *Leuciscus souffia* (Risso 1826), is a rather small cyprinid that inhabits the upper reaches of rivers in and around the Alps. Two subspecies are currently recognized: *L. s. souffia* in Alpine regions and north of the Alps and *L. s. muticellus* in Italy. Research on the vairone has so far focused on phylogeographical and taxonomic questions and was based on morphological characters, allozymes and mitochondrial DNA (Gilles *et al.* 1998; Salzburger *et al.* 2003; Salducci *et al.* 2004). Current research now focuses on the fine-scale genetic structure of the vairone on a smaller geographical scale to determine in more detail how genetic variability patterns relate to environmental processes. The faster evolving microsatellite markers will provide insights into

finer levels of population structure and, possibly hybridization, also in related species.

A partial genomic library enriched for microsatellites was constructed using the methods described by Tenzer *et al.* (1999) and later modified by Garner *et al.* (2000). Using standard phenol–chloroform methods (Sambrook *et al.* 1989), genomic DNA was extracted from one individual of *L. s. souffia*, and 30 µg was digested with the restriction enzyme *Mbo*I. Fragments ranging from 400 to 900 base pairs (bp) were ligated to specific linkers (Er1Bh1GATCSticky: 5'-GATCGGCAGGATCCACTGAATTCGC-3' and Er1Bh1-Blunt: 5'-GCCAATTCAGTGGATCCCTGCC-3'). Prehybridization polymerase chain reaction (PCR) amplification was performed for 15 cycles using one of the linker oligos. For enrichment, linked fragments were denatured and hybridized to 3' biotinylated (CA)₁₅ and (CT)₁₅ probes in 10 × SSC/0.2% SDS for 3 h at 55 °C. Using streptavidin magnetic beads (DynaBeads M-280 Streptavidin) with several washes (twice in 2 × SSC/0.2% SDS at room temperature, twice in 2 × SSC/0.1% SDS at 55 °C, and once in 1 M TE/50 mM NaCl at room temperature), the DNA that hybridized to the probe was separated. Microsatellite-enriched DNA was then PCR-amplified for 20 cycles, cloned into the Invitrogen pCRII TOPO vector and transformed into chemically competent *Escherichia coli* cells supplied with the TOPO TA Cloning Kit. Cells were grown on

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ampicillin-treated agar plates from which individual colonies were picked and regrown for 6–8 h in 96-well culture plates containing 150 µL of liquid 1 × LB/Amp solution with 20% glycerol. M13 forward (–20), and reverse primers were used directly on the bacterial cultures following the PCR protocol of Invitrogen to determine the insert size of clones. PCR products were sequenced using the BigDye version 3.1 cycle sequencing kit and fragments were visualized on an ABI 3100 automated sequencer (Applied Biosystems). Sequences with sufficient flanking regions were selected for primer development using the PRIMER 3 software (Rozen & Skaletsky 1998). PCR was performed in a total volume of 10 µL containing 0.25 µL of each primer (10 µM), 0.5 U Sigma RedTaq DNA polymerase, 1 µL each of MgCl₂ (10 mM), 1 × PCR buffer, and dNTPs (200 µM of each dNTP), 0.5 µL of DNA extract, and 5.5 µL double-distilled water. Thermocycling was performed on a GeneAmpPCR 9700 (Applied Biosystems) with the following two-stage protocol: a denaturation step (94 °C for 2 min), five cycles at an initial high annealing temperature (10 s at 94 °C, 10 s at the locus-specific annealing temperature, and 30 s at 72 °C) followed by 35 cycles at a lower annealing temperature (10 s at 94 °C, 10 s at the locus-specific annealing temperature, and 30 s at 72 °C). Forward primers were

labelled with fluorescent dyes FAM, HEX, and NED. Visualization of the fluorescently labelled PCR products was conducted on an ABI 3100 genetic analyser and allele sizes were scored against an internal GENESCAN-500 (Rox) size standard. Genotypes were obtained using GENESCAN analysis version 3.7 software from Applied Biosystems. Primer tests resulted in the identification of 11 polymorphic microsatellite loci. Using GENEPOP (web version 3.1; Raymond & Rousset 1995), none of the 45 pairwise comparisons showed significant linkage disequilibrium after applying Bonferroni corrections for multiple tests.

For the variation screen, 29 individuals of *L. s. souffia* from a wild population in the Schwarzach river in Austria were used. Levels of variability of these loci were moderate to high with three to 14 alleles per locus and expected heterozygosities ranging from 0.38 to 0.90 (Table 1). Two microsatellite loci were found to deviate from Hardy–Weinberg expectations based on exact tests using GENEPOP. While the positive F_{IS} for *Lsou19* indicates the occurrence of null alleles at this locus, homozygote deficiency was detected at *Lsou09*. *Lsou10* was fixed in *L. s. souffia* (279 bp), whereas variability was observed in the Italian population 'Parrono' (*L.S. muticellus*) and four closely related species (Table 2). Testing eight microsatellite primer pairs in closely

Table 1 Eleven microsatellites for *Leuciscus souffia* based on a sample of 29 individuals from one population in the river Schwarzach in Austria

Locus	Fluorescent dye	Primer sequence (5'–3')	Core motif	Size (bp)	T_a (°C)	N_A	H_O	H_E	F_{IS}	P
<i>Lsou05</i>	HEX	F: CTGAAGAAGACCTGGTTTCG R: CCCACATCTGCTGACTCTGAC	(CA) ₁₇	178–200	60°C/55°C	6	0.759	0.745	–0.018	0.259
<i>Lsou08</i>	HEX	F: GCGGTGAACAGGCTTAACTC R: TAGGAACGAAGAGCCCTGTGG	(GT) ₁₇	187–195	62°C/58°C	5	0.552	0.640	+0.140	0.619
<i>Lsou09</i>	FAM	F: TCGTCAACGAAATTAACACTGG R: GTCACATGGTCAGGGAAAGG	(GA) ₃₄	110–158	60°C/55°C	14	0.920	0.904	–0.018*	< 0.001
<i>Lsou10</i>	FAM	F: ATGAGGGTGATGAGGAGCAG R: CTCGTCCTGTCTGTCTGTCTCG	(GT) ₂₇	279	60°C/55°C	1	–	–	–	–
<i>Lsou11</i>	FAM	F: TCGCAGAAGTTCCTCTGACC R: GCCGATCAGCATTACCAAAC	(GT) ₁₃	262–270	62°C/58°C	5	1.000	0.745	–0.351	0.020
<i>Lsou14</i>	HEX	F: AGTCGCCCATCTACTGTTTC R: CGATCAGCAGCTCATTTGC	(GT) ₆ GC(GT) ₈ GC(GT) ₁₀	232–266	60°C/55°C	12	0.889	0.901	+0.013	0.064
<i>Lsou19</i>	FAM	F: TCCCGTGGAGAAACTACAGG R: TTCTTCGGTGAGTGTTCGATG	(GT) ₃₂	178–218	60°C/55°C	7	0.321	0.456	+0.300*	0.001
<i>Lsou21</i>	FAM	F: GGCAGGAGGACGTCTATGAG R: TCATGAAGTCGCTGTGGTTC	(GT) ₁₃ N ₃₄ (GT) ₈ N ₂₈ (GT) ₁₀	281–291	60°C/55°C	4	0.483	0.511	+0.057	0.834
<i>Lsou27</i>	FAM	F: CGCCTAATGCGTATCGTTG R: CCTTCCAGCTGATTCAAACG	(GT) ₆ GC(GT) ₇ GC(GT) ₁₀	197–313	60°C/55°C	5	0.414	0.494	+0.165	0.204
<i>Lsou29</i>	NED	F: AAAATGATGCTGTGCAATGG R: CCATCTTTGTCCCCATAACG	(GT) ₁₀	311–327	60°C/55°C	3	0.345	0.379	+0.092	0.241
<i>Lsou34</i>	NED	F: CCAGACAGGGTGATGATTTCC R: GTAGCGACGTTTCAGGTTCTCG	(GT) ₁₅	228–278	62°C/58°C	10	0.724	0.826	+0.126	0.009

T_a , annealing temperature; bp, base pairs; N_A , number of alleles; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , fixation index; P , P value for HW test ($\alpha = 0.05$); *, significant at the Bonferroni corrected significance level (0.006). GenBank Accession nos EF209002–EF209012.

Table 2 Variability of microsatellite loci in *Leuciscus souffia* and closely related *Leuciscus*, *Chondrostoma* and *Phoxinellus* species

Taxon	N	Lsout05 Size [bp] (N _A)	Lsout08 Size [bp] (N _A)	Lsout09 Size [bp] (N _A)	Lsout10 Size [bp] (N _A)	Lsout11 Size [bp] (N _A)	Lsout14 Size [bp] (N _A)	Lsout19 Size [bp] (N _A)	Lsout21 Size [bp] (N _A)	Lsout27 Size [bp] (N _A)	Lsout29 Size [bp] (N _A)	Lsout34 Size [bp] (N _A)
<i>L. s. souffia</i>	29	178–200 (6)	187–195 (5)	110–158 (14)	279 (1)	262–270 (5)	232–266 (12)	178–218 (7)	281–291 (4)	197–313 (5)	311–327 (3)	228–278 (10)
<i>L. s. muticellus</i>	7	180–182 (2)	189–193 (2)	112–114 (2)	273–277 (2)	278–288 (3)	274 (1)	178 (1)	287–295 (4)	×	×	226–228 (2)
<i>L. illyricus</i>	7	186–194 (3)	183–197 (5)	–	247 (1)	–	274 (1)	–	277 (1)	189–197 (4)	×	224 (1)
<i>L. turskyi</i>	3	182–198 (3)	197–207 (5)	–	275 (1)	–	276 (1)	–	275–277 (2)	×	×	222–228 (2)
<i>L. polylepis</i>	3	188 (1)	191–197 (4)	–	275–289 (2)	–	282–284 (2)	–	277–291 (2)	189–195 (2)	311–321 (2)	224–228 (2)
<i>L. microlepis</i>	4	182–192 (4)	191–197 (2)	–	247–283 (2)	–	276–284 (4)	–	277 (1)	187–295 (3)	×	222–240 (5)
<i>C. phoxinus</i>	3	202–204 (2)	187–193 (3)	–	×	–	224 (1)	–	297–309 (3)	×	337–353 (3)	220–222 (2)
<i>P. metohiensis</i>	5	178–206 (3)	189–201 (4)	–	289–333 (3)	–	276 (1)	–	285–291 (3)	×	307–309 (2)	222–228 (3)
<i>P. dalmaticus</i>	6	180–204 (8)	187–211 (7)	–	273–289 (3)	–	276–278 (2)	–	277–343 (5)	×	309–325 (3)	210–232 (6)

N, number of individuals; N_A, number of alleles; bp, basepairs; ×, no amplification product with PCR; –, locus not genotyped for this species.

related species demonstrates amplification success with moderate to high levels of heterozygosity at each locus. Additional species tested were *Leuciscus illyricus*, *Leuciscus turskyi*, *Leuciscus polylepis*, *Leuciscus microlepis*, *Chondrostoma phoxinus*, *Phoxinellus metohiensis*, and *Phoxinellus dalmaticus*. The reason for lack of variability in some related species at some loci might be due to the small sample size investigated (see Table 2). However, the interspecific data indicate that these markers will be a valuable tool for population genetic studies for other cyprinid species as well. Therefore, the reported microsatellites have the potential to become an important tool in future population genetic analyses of these fishes with regard to phylogeographical approaches and conservation issues.

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References

- Garner TWJ, Gautschi B, Röthlisberger S, Reyer H-U (2000) A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology*, **9**, 2173–2175.
- Gilles A, Chappaz R, Cavalli L, Lörtscher M, Faure E (1998) Genetic differentiation and introgression between putative subspecies of *Leuciscus souffia* (Teleostei: Cyprinidae) of the region of the Mediterranean Alps. *Canadian Journal of Fisheries and Aquatic Sciences*, **55**, 2341–2354.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Rozen S, Skaletsky HJ (1998) PRIMER 3. Available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html (accessed on 17 December 2004).
- Salducci MD, Martin J-F, Pech N, Chappaz R *et al.* (2004) Deciphering the evolutionary biology of freshwater fish using multiple approaches-insights for the biological conservation of the Vairone (*Leuciscus souffia souffia*). *Conservation Genetics*, **5**, 63–77.
- Salzburger W, Brandstätter A, Gilles A *et al.* (2003) Phylogeography of the vairone (*Leuciscus souffia*, Risso 1826) in Central Europe. *Molecular Ecology*, **12**, 2371–2386.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Tenzer I, Ivanishevich SD, Morgante M, Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *Phytopathology*, **89**, 748–753.
- Zardoya R, Doadrio I (1999) Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *Journal of Molecular Evolution*, **49**, 227–237.