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L. Gigliarelli [a] , M. E. Puletti [a] , D. Giannetto [a] , E. Franchi [a] , L. Lanfaloni [a] , F. Panara [a] , M. Lorenzoni [a] & L. Lucentini [a]

[a] Università degli Studi di Perugia, Biologia Cellulare e Ambientale, Perugia, Italy

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Isolation of microsatellite markers in *Squalius lucumonis* (Bianco, 1983) and cross-species amplification within the family Cyprinidae and other freshwater fish species

L. GIGLIARELLI*, M. E. PULETTI, D. GIANNETTO, E. FRANCHI, L. LANFALONI, F. PANARA, M. LORENZONI, & L. LUCENTINI

Università degli Studi di Perugia, Biologia Cellulare e Ambientale, Perugia, Italy

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Abstract

The Etruscan chub *Squalius lucumonis* (Bianco 1983) is an endemic endangered fish inhabiting the lakes and rivers of Central Italy. A microsatellite-enriched genomic library was constructed in order to develop microsatellite loci and eight polymorphic loci were isolated from this species to assess the level of genetic diversity in fifty individuals of *S. lucumonis* collected in the Umbria Region. The number of alleles ranged from four (Sluc12) to 18 (Sluc4). Observed and expected heterozygosity per locus ranged from 0.05 (Sluc12) to 0.80 (Sluc4; Sluc7 and Sluc11) and from 0.22 (Sluc12) to 0.90 (Sluc4), respectively. Furthermore, these loci were tested on ten other Cyprinidae species and four freshwater fish species belonging to other families. Successful cross-priming amplifications were obtained for several species of Cyprinidae, whereas no positive results were obtained for other species. The availability of the reported microsatellite loci will facilitate population structure investigations of these species aiming to phylogeographical approaches and conservation strategies.

Keywords: *Cyprinidae*, *etruscan chub*, *endangered species*, *microsatellites*, *crosspriming*

Introduction

Cyprinidae is one of the most species-rich families of the European freshwater fish fauna and has a wide distribution range, though restricted to river and lake drainage systems (Zardoya & Doadrio 1999; Sanjur et al. 2003). Within the sub-family Leuciscinae, the genus *Squalius* was previously considered a subgenus of *Leuciscus,* but recent phylogenetic studies demonstrated that the *Squalius* species are not closely related to *Leuciscus* (Briolay et al. 1998).

The Etruscan chub *Squalius lucumonis* (Bianco, 1983) is an Italian endemic species restricted to Central Italy; it is present in the Arno, Tiber, Ombrone and Serchio river basins (Bianco 1983; Kottelat & Freyhof 2007). It inhabits small to medium-size premontane streams with Mediterranean water regime, and spawns in shallow riffle habitats in fast-flowing water (Kottelat & Freyhof 2007). Several characters distinguish *S. lucumonis* from other species of *Squalius* in Italy: 37–40 lateral line scales, 81/2 branched anal rays, 81/2 branched dorsal rays, convex or almost straight posterior margin of anus, black pigments along free margin of scales (Kottelat & Freyhof 2007).

Squalius lucumonis appears in the IUNC Red List of Threatened Species (IUNC 2011: www.redlist.org/) as an endangered species B2ab (i, ii, iii, iv); it is listed in the Annex II of the European Union Habitats Directive and in the Appendix III of the Bern Convention (Crivelli 2006; Kottelat & Freyhof 2007). To date, the population genetic structure of *S. lucumonis* has never been investigated, although a deeper knowledge would be required to evaluate how patterns of genetic variability relate to environmental processes in a phylogeographic and species conservation framework.

Microsatellites consist of short repeat DNA sequences reiterated to give short arrays at each locus and behave as codominant markers (Goldstein & Schlötterer 1999; Finger & Klank 2010). The high

*Correspondence: Lilia Gigliarelli, Università degli Studi di Perugia, Biologia Cellulare e Ambientale, via Pascoli 1, 06123 Perugia, Italy. Tel: +39 0755855617 Fax: +39 0755855736. Email: liliagigliarelli@hotmail.com

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variability of these loci makes them very powerful genetic markers, whose applications span over different areas, such as ancient and forensic DNA studies, population genetics, and conservation/management and genome mapping (Frankham et al. 2002; Ellegren 2004). Microsatellite flanking zones are constituted by nucleotide sequences highly conserved during evolution, allowing a microsatellite molecular analysis through specific primers application (Finger & Klank 2010).

To date, such markers have never been developed for *S. lucumonis*, and this species has never been included in microsatellite cross-priming testing within the Cyprinidae family. In this perspective, a microsatellite-enriched genomic library was constructed to isolate polymorphic microsatellites loci, following the biotin-enrichment protocol from Glenn and Shable (2005), modified by Gigliarelli et al. (2010).

Materials and methods

This research is consistent with National regulations. Individuals of *Squalius lucumonis* were caught by means of electrofishing (direct pulsating current, 1.5–4 kW) and non-lethally sampled: fresh scales specimens or a small piece of caudal fin (10mg) were removed and stored in 70% ethanol at −20 °C until processing. Fishes were immediately released after sampling in the collection site (Table I).

DNA was extracted using Wizard Genomic DNA Purification Kit (Promega) following a modified protocol (Lucentini et al. 2006a,b) and DNA concentration was estimated in a 1.0% agarose gel electrophoresis in presence of Mass Ruler DNA Ladder Mix (Fermentas). Ten micrograms of DNA were digested with *Rsa*I and *Xmn*I (NEB) and ligated to SuperSNX24 linkers. A mix of seven 3' biotinylated probes [(AG)12; (TG)12; (AAC)6; (AAG)8; (AAT)12; (ACT)12; (ATC)8, (MWG Biotech)] was used to hybridise with the linker ligated fragments. Hybridised probe DNA was captured using Streptavidin M-280 Dynabeads (Invitrogen). Enriched DNA was obtained and amplified using SuperSNX24Forward primer, then PCR products were cloned into pGem T Easy Vector, using JM109 competent cells (all from Promega). Positive (white) colonies were amplified using SuperSNX24Forward primer, then plasmidic DNAs (plDNAs) were extracted from the colonies showing PCR products of 300–1000 bp. PlDNAs were sequenced with Sp6 Universal Primer (Promega), through Big Dye Terminator procedure on an ABI PRISM 3730XL Sequencer (Applera). For clones containing microsatellites, primers were designed using Primer3 (http://frodo.wi.mit.edu/) and each forward primer was labelled with a fluorescent dye for fluorescent detection (Table II). Single locus PCRs were performed in 25 μl volumes containing 50 ng of genomic DNA, 10x Reaction Buffer (Euroclone), 50 mM $MgCl_2$ (Euroclone), 2.5 mM each dNTP (Euroclone), 10pmol of each primer (Applera), and 0.5 U of Taq polymerase (Euroclone). The amplification consisted of an initial denaturation (3 min at 95 °C) followed by 30 cycles of 45 s at 94 °C, 45 s at the primer-specific annealing

Table I. Species, sampling sites and relating coordinates.

Species	River/Lake	Coordinates
S. lucumonis (Bianco, 1983)	Assino	43° 17′ 20″ N 12° 22′ 08″ E
S. lucumonis (Bianco, 1983)	Paglia	42° 43′ 43″ N 12° 07′ 38″ E
S. lucumonis (Bianco, 1983)	Lama	43° 32′ 25″ N 12° 14′ 50″ E
S. lucumonis (Bianco, 1983)	Passano	43° 32′ 35″ N 12° 14′ 60″ E
Alburnus alburnus alborella (De Filippi, 1844)	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Barbus tyberinus Bonaparte, 1839	Fersinone	42° 55′ 31″ N 12° 12′ 18″ E
Chondrostoma genei (Bonaparte, 1839)	Soara	43° 27′ 27″ N 12° 18′ 21″ E
Gobio gobio (Linnaeus, 1758)	Nestore	43° 22′ 33″ N 12° 11′ 10″E
Pseudorasbora parva (Temminck et Schlegel, 1846)	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Rutilus rutilus (Linnaeus, 1758)	Piediluco	42° 31′ 01″ N 12° 45′ 00″ E
Scardinius erythrophthalmus (Linnaeus, 1758)	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Squalius cephalus (Linnaeus, 1758)	Tevere	43° 17′ 02″ N 12° 21′ 37″ E
Telestes souffia (Risso, 1827)	Tevere	43° 38′ 89″ N 12° 03′ 22″ E
Tinca tinca (Linnaeus, 1758)	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Anguilla anguilla (Linnaeus, 1758)	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Esox flaviae Lucentini, 2011	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Perca fluviatilis Linnaeus, 1758	Trasimeno	43° 09′ 11″ N 12° 05′ 21″ E
Salmo trutta Linnaeus, 1758	Nera	42° 51′ 42″ N 12° 58′ 47″ E

Table II. Characteristics of *Squalius lucumonis* microsatellite markers. Locus name, microsatellite motif, primers sequence and GenBank codes are reported. F: forward primer; R: reverse primer; Ta: optimal annealing temperature; bp: range of scored alleles; Na: number of scored alleles; *H*o and *H*e: observed and expected heterozygosity; null: null alleles. *P*-value: probability.

Locus name, motif sequence, primer sequence	Ta	bp	Parameters
Sluc4 (AG)$_{26}$	63°C	136 – 198	Na/ null: 18/no
F: 6Fam-AAGCATTACCCATGCAGAGC			*H*o: 0.80
R: AGCTGCAACACAACCTCCAT			*H*e: 0.90
GenBank: HQ585925			*P*-value: 0.62
Sluc5 (TTC)$_{10}$(TTA)$_5$	63°C	242 – 358	Na/ null: 11/no
F: Vic-GAGAAAGAGAGACCAATCCATAGTT			*H*o: 0.70
R: CAAAGCAAGCATCAAACCTG			*H*e: 0.79
GenBank: HQ585926			*P*-value: 0.37
Sluc7 (CTTT)$_{16}$	63°C	200 – 298	Na/ null: 16/no
F: Ned-GGAAACTGACACATCGCTTG			*H*o: 0.80
R: CGAAGGACTGGACTGGAAAG			*H*e: 0.81
GenBank: HQ585927			*P*-value: 0.74
Sluc10 (CTATT)$_{13}$	61°C	202 – 247	Na/ null: 10/yes
F: Pet-AACACAAAGCCCAACAGTCC			*H*o: 0.70
R: CACAACAACAATATTCAACACATCA			*H*e: 0.89
GenBank: HQ585929			*P*-value: 0.00
Sluc11 (CA)$_{14}$	63°C	236 – 268	Na/ null: 5/no
F: Pet-CACACTGGCACCTCTGACAT			*H*o: 0.80
R: AGCCCCGTCAACAAACTGT			*H*e: 0.63
GenBank: HQ585930			*P*-value: 0.00
Sluc12 (TC)$_{11}$TGA(CA)$_7$C	63°C	212 – 302	Na/ null: 4/yes
F: Vic-AACAAAAGCGCAATGTTTTACA			*H*o: 0.05
R: GCTCTATTCGCGTTTGGTGT			*H*e: 0.22
GenBank: HQ585931			*P*-value: 0.00
Sluc13 (CA)$_{35}$	61°C	228 – 236	Na/ null: 7/no
F: 6Fam-CACCCAGGCAATAAACAAGG			*H*o: 0.35
R: GGGTTAAGGGTCGGTTTAGG			*H*e: 0.36
GenBank: HQ585932			*P*-value: 0.01
Sluc16 (AG)$_{29}$	63°C	184 – 254	Na/ null: 9/no
F: 6Fam-AAAAGCCCTCATCAAGCAGA			*H*o: 0.48
R: ATGGAAAGACACCCCCTTCT			*H*e: 0.76
GenBank: JF417968			*P*-value: 0.00

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temperature (Table II), and 45 s at 72 °C; then a final 40 min elongation step at 72 °C was performed to push addition of extra adenosine nucleotides at 3’-end and minimise the plus-A artefacts.

Microsatellite polymorphisms were screened on fifty individuals sampled in different sites of Umbria (Table I), using an ABI PRISM 310 Sequencer with GeneScan-500 (LIZ-500) as an internal size standard, then the fragments obtained were analysed using GeneMapper 5.0 (all from Applera).

Null alleles presence was evaluated by means of Micro-Checker 2.2.3 (Van Oosterhout et al. 2004); linkage disequilibrium among loci was evaluated by means of Genepop 4.0 (Raymond & Rousset 2005). The number of alleles per locus, and observed and expected heterozygosities were calculated using Arlequin 3.5 (Excoffier & Lischer 2010).

In addition, to determine whether these primers might be used to amplify microsatellites loci in both closely- and less-closely related species, the entire procedure was performed on ten Cyprinidae species and other four species not belonging to this family but inhabiting freshwater environments (Table I). A particular attention was focused on *S. cephalus*, a species inhabiting the same habitats and characterised by high bio-ecologic plasticity, consistent intra-specific variability and capability of hybridization with other Leuciscinae species (Zerunian 2004).

Results and Discussion

Two hundred and eighty positive colonies were screened and 114 colony-PCR products were sequenced revealing that 36 (31.5%) contained microsatellites; of these, 47.2% were simple dinucleotides, 19.4% interrupted dinucleotides, 5.55% simple trinucleotides, 8.3% interrupted trinucleotides, 5.55% tetranucleotides, 2.77% pentanucleotides and 11.1% compound microsatellites. The small size of the flanking regions limited the design of primers and only 27 primer pairs could

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be designed and optimised. Amplification products revealed that 17 pairs were monomorphic, eight were polymorphic and two pairs did not amplify. Only polymorphic loci were registered with the GenBank database under the accession numbers HQ585926-HQ585932 and JF417968 (Table II).

After Bonferroni correction, there was no indication of linkage disequilibrium among loci and null alleles presence was suggested for Sluc10 and Sluc12. The number of alleles, repeat motifs, product size, annealing temperatures and heterozygosities at each of the eight polymorphic microsatellite loci are reported (Table II). Alleles number ranged from four (Sluc12) to 18 (Sluc4). Observed heterozygosity per locus ranged from 0.05 (Sluc12) to 0.80 (Sluc4; Sluc7 and Sluc11); and expected heterozygosity ranged from 0.22 (Sluc12) to 0.90 (Sluc4) (Table II). Three loci (Sluc4, Sluc5, Sluc7) were out of Hardy–Weinberg equilibrium.

The multiple primer pairs were also tested on individuals (from one to thirty-six) of 14 species belonging to Cyprinidae, Anguillidae, Esocidae, Percidae, Salmonidae families (Table III). Amplifications were successful in 68.7% of Cyprinidae, although a lack of variability was observed in some related species at certain loci (Table III). In particular, all loci were amplifiable in *Alburnus alburnus alborella*, and, with the exception of Sluc4, in *Squalius cephalus* and *Gobio gobio* (Table III). Conversely, *Barbus tyberinus* and *Tinca tinca* showed positive results only for Sluc11, whereas all the other investigated Cyprinidae failed amplifications at one or two loci (Table III). No amplicons were obtained in species from the other families (Table III).

Conclusions

Microsatellites markers have been developed for several Cyprinidae species, including *Squalius aradensis*, *S. cephalus*, *Leuciscus souffia*, *L. idus*, and *L. leuciscus* (Mesquita et al. 2003; Barinova et al. 2004; Larno et al. 2005; Vyskocilova et al. 2007; Muenzel et al. 2008; Dubut et al. 2009; Boto et al. 2011). Furthermore previous studies pointed out the usefulness of cross-species amplification of microsatellites in Cyprinidae to establish markers for population genetics studies (Muenzel et al. 2008; Dubut et al. 2010). Since *S. lucumonis* has never been included in cross-testing, no data were available for this endemic species of Central Italy with regard to such molecular markers, and the population genetics was never investigated.

This research reports, for the first time, a pool of species-specific microsatellites that were isolated for *S. lucumonis* through the construction of a microsatellite-enriched genomic library. Eight of these loci were polymorphic and a standard analysis of their alleles underlined high levels of genetic variability in *S. lucumonis*. Heterozygosity values suggested that they constitute a valid tool for population genetic analyses in this species. In particular, considering the endemic nature of *S. lucumonis*, these loci might be employed in the assessment of population differentiation (Lucentini et al. 2006b), in phylogeographic approaches aiming to compare this species with other congeneric, as well as in studies aiming to the evaluation of the effective populations size (Lucentini et al. 2009). The recording of such information might be important not only as a basic knowledge of an endemic species, but also

Table III. Cross species amplification results. The number of alleles is reported in bold, the allelic range is reported in parentheses.

	Species	n	Sluc4	Sluc5	Sluc7	Sluc10	Sluc11	Sluc12	Sluc13	Sluc16
Cyprinidae	*Alburnus alburnus alborella*	2	**3**(140–146)	**2**(246–250)	**2**(290–320)	**1**(202)	3(228–238)	2(168–190)	1(230)	1(208)
	Barbus tyberinus	1	/	/	/	/	**1**(226)	/	/	/
	Chondrostoma genei	2	/	**2**(218–226)	**2**(278–286)	**3**(202–212)	**2**(234–236)	**2**(190–196)	/	**1**(206)
	Gobio gobio	2	/	**1**(290)	**1**(290)	**3**(202–220)	**3**(236–252)	**2**(220–224)	**2**(230–234)	**1**(210)
	Pseudorasbora parva	2	**1**(128)	**1**(232)	**3**(268–287)	**3**(202–240)	**2**(236–248)	**1**(190)	/	**1**(210)
	Rutilus rutilus	1	**2**(138–146)	**2**(142–254)	**2**(266–310)	/	**1**(244)	**1**(200)	/	**2**(214)
	Scardinius erythrophthalmus	2	/	**1**(194)	**1**(258)	**1**(202)	**1**(234)	**1**(190)	**1**(242)	/
	Squalius cephalus	36	/	**8**(212–278)	**6**(272–356)	**2**(178–202)	7(196–242)	**6**(190–228)	**4**(230–236)	**1**(208)
	Telestes souffia	1	/	**2**(248–254)	**1**(258)	**2**(202–216)	**1**(234)	**2**(160–212)	**2**(234–242)	/
	Tinca tinca	1	/	/	/	/	**1**(250)	/	/	/
Other families	*Anguilla anguilla*	2	/	/	/	/	/	/	/	/
	Esox flaviae	2	/	/	/	/	/	/	/	/
	Perca fluviatilis	2	/	/	/	/	/	/	/	/
	Salmo trutta	2	/	/	/	/	/	/	/	/

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to perform scientifically supported management and conservation strategies.

Finally, the cross-priming testing produced interesting information, highlighting the potential usefulness of these markers to investigate the genetic variability of other European Cyprinids. The scarce polymorphism observed in some species at different loci, while amplifications were successful in 68.7% of Cyprinidae, might be due to the small sample size investigated. To verify the polymorphism levels of these loci in such species, species-specific application of such microsatellite loci should be performed. It is interesting that one locus (Sluc4) did not amplify in *S. cephalus,* even though for these species a great number of samples (36) was investigated. Furthermore, for the other seven loci, the alleles size ranges were not completely superimposable and, in one case (Sluc10) this range is substantially disjointed. Because the differentiation between *S. lucumonis* and *S. cephalus* was debated (Gandolfi et al. 1991; Zerunian 2004), the lack of amplification of a species-specific locus in one of the two species and the individuation of species-specific alleles are important findings contributing to the distinction of these species.

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