Molecular Ecology (1999) **8**, 1957–1969

PRIMER NOTES

Characterization of microsatellite loci in the queenless Ponerine ant *Diacamma cyaneiventre*

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Keywords: microsatellites, cross-amplification, social insects, queenless ants, *Diacamma*

Received 15 May 1999; revision accepted 5 July 1999

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A full understanding of colony organization and life histories in social insects can only be achieved by investigating the genetic structure of colonies and populations (Pamilo *et al*. 1997). Although most genetic studies on ants have been performed on species with complex colony organization (but see Tay *et al*. 1997), the study of morphologically primitive ants, characterized by limited caste dimorphism and small colony size, could provide new insights into the evolution of sociality (Peeters 1997). In some morphologically primitive species of Ponerine ants, the queen caste has been lost and one worker mates (the gamergate) and produces diploid offspring (Peeters 1991). In these queenless species the foundation of new colonies occurs only by fission. Even in such a relatively simple social organization, polymorphic genetic markers will be required to estimate the rate of gamergate turnover and colony fission, the extent of population viscosity and their effects on the evolution of sociality.

In this note, we characterize eight polymorphic microsatellite markers from the queenless species *Diacamma cyaneiventre*. Microsatellite markers have been characterized in a number of groups of ants (e.g. Chapuisat 1996; Herbers & Mouser 1998), but none are available for ponerine ants. We also report results of cross-amplification on 11 Ponerine species (seven *Diacamma* species and four belonging to other genera of Ponerinae).

Genomic DNA from nine *Diacamma cyaneiventre* larvae was extracted by a high-salt procedure using NaCl and digested with the BSP 143I restriction enzyme. Fragments between 300 and 600 bp were selected and ligated to a *Bam*HI-digested pBluescript II KS+ vector (Stratagene) and cloned in *Escherichia coli* SL-1 Blue cells (Stratagene). Synthetic oligonucleotides $(TC)_{10}$ and $(TG)_{10}$, labelled with the DIG system (Boehringer Mannheim) were used to screen about 1200 recombinant colonies. Out of 146 positive clones, 78 were purified with Qiaprep (Qiagen) and 24 were sequenced either manually using the T7-sequencing kit (Pharmacia) or an ALF express automatic sequencer (Pharmacia).

Primers flanking microsatellite repeats were designed for 10 loci using Primer 3 software (Rozen & Skaletsky 1996). Genomic DNA was prepared following either a classic phenol/chloroform or high-salt extraction and diluted to 1/5 before amplification. PCR reaction mixtures (10 μL final volume) contained 50 ng of template DNA, 75 μM dCTP, GTP and dTTP, 7.5 μM dATP, 0.025 μCi ^{33}P-dATP (Amersham), 4 pmoles each primer, 1× *Taq* buffer (containing 1.5 mM $MgCl_2$) and 0.25 U *Taq* DNA polymerase (Qiagen). For the D18 locus, the amount of dNTP, ^{33}P-dATP and *Taq* DNA polymerase was doubled to allow a better amplification of the longest allele in heterozygote individuals. Amplifications were performed in a PTC-100 (MJ Research) thermal cycler using the following parameters: 3 min at 94 °C followed by 30 cycles with 30 s at 94 °C, 30 s at the annealing temperature (see Table 1) and 1 min at 72 °C, and a final elongation step of 10 min at 72 °C. Amplification products were run on 6% polyacrylamide sequencing gels using an M13 sequence as a size marker.

All 10 microsatellite loci gave repeatable and scorable patterns. An estimate of the variability of these loci was determined using a sample of 45 unrelated workers. Eight loci were polymorphic (Table 1). The expected heterozygosity ranged from 0.39 to 0.95, the highest diversity being observed for an uninterrupted microsatellite showing the largest number of repeats (29). No significant deviations from Hardy–Weinberg proportions were detected using a subsample of 33 individuals collected in a locality near Kotigehara (Table 1).

Results of the cross-amplification are shown in Table 2. PCR conditions were as described above except that the annealing temperature was 52 °C for all primers. Where only one individual was extracted, DNA quality was confirmed using mitochondrial DNA primers known to amplify on these species (data not shown). Within the genus *Diacamma*, cross-species amplification was successful with an average of six pairs of primers amplifying per species. For *D. ceylonense* and *D.* sp. *(nilgiri)* from India, nine individuals were screened to check for polymorphism. Four microsatellites were polymorphic in *D. ceylonense* and three in *D.* sp. *(nilgiri)*. When used on different genera, the efficiency of amplification was low even though these genera belong to the same tribe as *Diacamma*. Our results may therefore indicate a recent common ancestor for the *Diacamma* species studied. In conclusion, given their level of polymorphism, the primers presented here should prove to be very useful for investigating population and colony genetic structure of different *Diacamma* species.

Acknowledgements

M. Cobb, C. Peeters, C. Tirard and M. Veuille are thanked for comments on the manuscript, S. Foitzig for providing samples of *Hypoponera opacior* and C. Peeters for other samples of the cross-amplification analysis. This work was supported by the Programme National Dynamique de la Biodiversité (grant

Table 1 Characteristics of microsatellite loci from *Diacamma cyaneiventre*. The number of alleles observed (Na), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated from a sample of 45 unrelated workers (one worker per colony) collected from two localities near Kotigehara and one near Kemmangundi (Karnataka state, India). The F_{IS} and the propability P of deviation from Hardy–Weinberg expectation were calculated on 33 workers originating from a single locality near Kotigehara using GENEPOP (Raymond & Rousset 1995)

Locus	AN	Repeat motif	Size (bp)	*Tm* (°C)	*Na*	H_O	H_E	F_{IS}	*p*	Primers (5′–3′)
DC5	AF147278	$(GA)_9$	141–149	57	4	0.51	0.51	+0.056	0.66	F: CGATAATAGGGCCGACTCAC R: TCAGTTCTGGTCATTGTCATAGG
DC8	AF147279	$(GA)_{12}$	134–144	55	6	0.58	0.52	−0.146	0.23	F: GGCCTTCTTTGACGTAATCG R: AATTTCTTGGAGCGAACCCG
DC11	AF147280	$(GA)_{12}$	215–251	52	11	0.69	0.60	−0.154	0.80	F: CGTCGTGGACTTGTGAAC R: ACGGTTGTTTGTACGATTGG
DC18	AF147281	$(GA)_{29}$	194–278	55	25	0.93	0.95	+0.039	0.21	F: GCAACATCGTGTGCTAGACTTC R: GTTTACGGTCGCGTGTCTTC
DC19	AF147282	$(GC)_2GG(GA)_{15}$	229–243	52	7	0.75	0.82	+0.086	0.49	F: ATTTGCAGAAACGGAACTCG R: GGTTCATTTGCATTCATCAGG
DC20	AF147283	$(GA)_2GT(GA)_{12}$	156–166	52	5	0.76	0.76	−0.041	0.95	F: CGATGTTTGTATCGTGATTCG R: ATTAAACGCCTCGGCTGTC
DC29	AF147284	$(CA)_8$–$(GC)_4$	208–214	52	4	0.42	0.39	−0.165	0.82	F: CGAAATCACGGAACTGTCG R: TAGAATTGATGCTTCGTC
DC52	AF147286	$(GA)_{16}$	161–189	55	7	0.58	0.68	+0.123	0.22	F: CGCCGTCAAAGACAAAGC R: TGCGCTAGAAACTCTCGTATTG

AN, accession number of the sequences available from GenBank.
Tm, annealing temperature.

Table 2 Cross-species amplification of eight polymorphic loci developped for *Diacamma cyaneiventre*. For each species, the locality of collection and the number of individuals analysed is given in parentheses. The size of the PCR product is given except where no amplification was detected

	DC5	DC8	DC11	DC18	DC19	DC20	DC29	DC52
Diacamma ceylonense (9) (India, Bangalore)	145	151–153	221–251	177	219	166–172	217	196–222
D. sp. (*nilgiri*) (9) (India, Masinugodi)	145	151	223	177	223–225	164–172	217	172–220
D. indicum (2) (India, Kotti)	143–151	125–147	225	195	—	167	212–214	—
D. sp. (2) (Japan, Okinawa)	141	125–147	223–225	195	—	167	212	—
D. australe (1) (Australia, Darwin)	—	151	211	174	220	162	210	—
D. sp. (2) (Malaysia, Ulu Gombak)	153	143–145	227	184	—	152–158	224	150
D. pallidum (2) (HongKong, News Territories)	—	139	—	186	—	169	—	150
Streblognathus aethiopicus (2) (South Africa, Grahanstown)	—	125	—	—	—	—	—	—
Dinoponera quadriceps (1) (Brasil, Bahia state)	—	125	—	206	—	—	—	—
Harpegnathos saltator (1) (India, Jog Falls)	—	—	—	208	—	122–144	—	—
Hypoponera opacior (2) (USA, Arizona, Portal)	—	131	—	—	—	—	—	—

No. 2T0059) and is a part of a collaborative study (R. Gadagkar and C. Peeters) on indian *Diacamma* ants financed by CEFIPRA/IFCPAR (grant No. 1709–1).

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Characterization of microsatellite loci in the greater horseshoe bat *Rhinolophus ferrumequinum*

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Keywords: Chiroptera, microsatellite, PCR, primer, *Rhinolophus*

Received 5 June 1999; revision accepted 30 June 1999

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The greater horseshoe bat *Rhinolophus ferrumequinum* is distributed throughout the southern Palaearctic region from Britain to Japan (Schober 1998). Within Europe it is considered endangered over most of its range, having undergone a well-documented decline during the 1900s (Stebbings 1988). Since the 1950s, this species has been studied extensively, and is consequently one of the most well understood of all chiropterans (for reviews see Ransome 1990, 1991; Duvergé & Jones 1994). In recent years, the application of molecular techniques to ecological studies has provided new insights into population and social structure of mammals (Sugg *et al*. 1996; Hughes 1998). Therefore, to compliment ongoing field investigations of *R. ferrumequinum*, microsatellite studies were undertaken to investigate population structure and parentage. Here we report on the development and characterization of the primers.

A microsatellite library was constructed using a nonenriched method based on Rassman *et al*. (1991). *R. ferrumequinum* DNA was restricted with *Hae*III, *Alu*I and *Rsa*I, and ligated into pUC18. Transformation was carried out by electroporation, and transformants were screened with CA and GA dinucleotide repeats. Two rounds of screening were performed, and 34 positive clones were identified and sequenced. PCR primer pairs were designed for 12 microsatellite loci.

Wing biopsies were obtained under licence and genomic DNA extracted following the method described by Worthington Wilmer & Barratt (1996). Prior to amplification, one primer of the pair was end-labelled with [γ^{32}P]-ATP, by incubating with T4 polynucleotide kinase at 37 °C for 1 h. Separate 10-μL PCR reactions, carried out for each individual, contained 10–50 ng of DNA, 1.5 pmol each primer, 1 μL of DMSO, 0.15 mM dNTPs, 1 μL of 10× *Taq* buffer (IGI), 0.5–1 U of *Taq* polymerase (IGI) and 1.5–2.5 mM $MgCl_2$. Reactions were carried out in a Hybaid Omnigene thermal cycler with the following amplification conditions: 3 min at 94 °C; 30 cycles of 1 min at 94 °C, 2 min at primer-specific annealing temperature, 2 min at 72 °C; 10 min at 94 °C. PCR products were separated on a 6% polyacrylamide gel and alleles were sized using a M13 bacteriophage sequencing ladder.

The primers were tested with DNA from bats originating from seven localities in Britain and two in continental Europe. Of the 12 sets of primers, four amplified monomorphic loci. The primer pair Rferr04 amplified two independent polymorphic loci of overlapping allelic size range, which could not be separated by altering PCR conditions. In an attempt to redesign the primers for the two loci, several PCR products were cloned and sequenced. However, no sequence differences were found. This suggests that a gene duplication event has occurred in the past, and that while the numbers of repeat units may have evolved independently in the two copies of the gene, the flanking regions have been conserved. All other polymorphic loci (5–18 alleles) showed clear banding patterns, and observed heterozygosity (calculated across samples) ranged from 0.35 to 0.75 (Table 1). When tested with over 100 mother–young pairs, no mismatches were found, indicating that null alleles do not occur at high frequency.

To date, cross-species amplification has not been investigated with these primers. However, phylogenetic analyses of the genus *Rhinolophus* have reported levels of sequence divergence typical of those identified within other mammalian genera. In a comparison of cytochrome *b* sequences from *R. ferrumequinum* and three other species, Thomas (1997) found that sequence divergence ranged from 7% to 21%. Similarly, Cooper *et al*. (1998) reported close genetic similarities between two species of Australian rhinolophid, based on both allozyme analysis and mtDNA control region sequences (< 7.5% divergence). Therefore, as many previous studies of cross-species amplification in mammalian taxa, including Chiroptera (Burland *et al*. 1998), have reported success both within and among genera, it is highly probable that these

Table 1 Primer sequences (5′ to 3′) and some other characteristics of eight polymorphic microsatellite loci. Marker Rferr04 amplified two loci with overlapping alleles, and therefore heterozygosity values and allele number could not be resolved. Accession numbers (GenBank) refer to clone sequences from which the primers were designed

Locus	Primer sequences (5′–3′)	Motif	Annealing temp. (°C)	Allele size range (bp)	No. of bats typed	No. of alleles	H_O	H_E	Accession no.
Rferr01	F: CCATCTCCATTACCACTCTG R: TGTCTACCCAGAAACAGCATC	$(GT)_{18}$	55	118–126	340	6	0.57	0.68	AF160200
Rferr03	F: CTAACCTGGTGAACCTCCCATTC R: CTATCACCTGCATCACCTGTGAAG	$(GT)_{18}$	57	211–241	340	11	0.67	0.77	AF160202
Rferr04	F: CTCGCTGGTTCACTAACTAGCAGAG R: GAGACTCTTTGGGAAATGAAGCC	$(GT)_{19}AT(GT)_7$	54	150–210	60	—	—	—	AF160203
Rferr06	F: GGATTTGCTGATGCTTCAGAGG R: TTGGCACACAGTAACTCCTCAGTG	$(TC)_{24}$	55	198–226	340	18	0.72	0.77	AF160205
Rferr08	F: TCTTTCCTCACAGCCTCTCAGTG R: TGGAGTTTTAGGTCCACATCCTCC	$(GT)_{22}$	55	140–150	340	6	0.71	0.68	AF160207
Rferr09	F: GGTCCTGGAAAAACACACACACTG R: TCCTCCCTTTGCGTTCTCTAGG	$(GT)_{18}$	65	122–140	207	9	0.73	0.74	AF160208
Rferr11	F: AGGGGAGTGACAGAGAGGGAATC R: TGGAGGTGGGAGGACAGAATCTG	$(GT)_{21}$	57	182–204	340	10	0.75	0.78	AF160210
Rferr12	F: CCAGCACTTCCATACAGAAACCTG R: ACATCAATATGCCCACTGACCC	$(GT)_{16}$	55	212–220	340	5	0.35	0.37	AF160211

F, forward primer; R, reverse primer.

primers will amplify in other members of this widespread genus (69 species throughout Europe, Africa, Asia and Australasia), and possibly other genera of the closely related family Hipposideridae.

Acknowledgements

Bats were caught and sampled under licences from English Nature, the Countryside Council for Wales and the Home Office. We thank Nancy Irwin for help in the laboratory. Roger Ransome, David Priddis, Tom McOwat, François Leboulenger and Danilo Russo provided or helped to collect samples. S.J.R. was supported by a NERC studentship, with additional support from Bat Conservation International and English Nature.

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Isolation and characterization of microsatellite loci in the walleye (*Stizostedion vitreum*), and cross-species amplification within the family Percidae

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Keywords: cross-species amplification, microsatellites, Percidae, *Stizostedion vitreum*, Teleostei, walleye

Received 9 May 1999; revision received 19 June 1999; accepted 22 June 1999

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The Percidae family comprises five species of particular economic interest in North America and Europe. *Stizostdion vitreum*, *S. canadense* and *Perca flavescens* in North America are commercially important species and new genetic tools are needed to improve their management and enhance selective breeding programmes. The same situation exists for the European species, *S. lucioperca* and *Perca fluviatilis*. Previous studies used allozyme and mitochondrial DNA as genetic markers to infer population genetic structure in walleye (Billington *et al*. 1992; Stepien & Faber 1998) and perch (Refseth *et al*. 1998). Although of interest for large-scale population structure and phylogeography, these markers may have limited usefulness for fine-scale studies, and parental analyses. Microsatellite loci have proven to be powerful markers and may be more useful in such situations (O'Reilly & Wright 1995; De García Leon *et al*. 1998). Six microsatellite markers were recently developed for the walleye (Borrer *et al*. 1999). Nevertheless, there is still a need for additional polymorphic markers for this species and the usefulness of walleye microsatellite in cross-amplification has not been investigated.

We developed walleye microsatellite markers for the purpose of selective breeding programmes and fine-scale population structure. Here, we present 11 new loci and examine cross-species amplification of these loci in sauger (*S. canadense*), zander (*S. lucioperca*), yellow perch (*P. flavescens*) and European perch (*P. fluviatilis*).

Genomic DNA was extracted from a walleye liver using standard proteinase K phenol–chloroform techniques (Sambrook *et al*. 1989) and pooled in a partial *Sau*3A1 digest. Fragments ranging from 400 to 900 bp were purified from a 1.7% agarose gel using DEAE paper (Sambrook *et al*. 1989; Rassmann *et al*. 1991). These fragments were then ligated into the *Bam*HI site of phosphatase-treated pUC18 ('Ready to go' kit, Pharmacia). Competent DH5α *Escherichia coli* cells (Gibco BRL) were transformed with the ligation products and grown on agar plates. Colonies were blotted on Hybond N+ nylon membranes (Amersham) which were hybridized with synthetic $(TC)_{10}$ $(TG)_{10}$ $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$ and $(TGTA)_6TG$ probes labelled with the DIG oligonucleotide kit (Boehringer Mannheim). The DIG nucleic acid detection kit (Boehringer Mannheim) was used for detection. Among the 2000 clones of the partial library screened, 138 (7%) were identified as positive. DNA from 21 positives was extracted using the QIAprep plasmid DNA prep kit (Qiagen) and sequenced after cycle sequencing using AmpliTaq (DNA) polymerase FS (Perkin-Elmer) using an ABI 377 automated sequencer. All these clones contained microsatellites and 11 primer sets amplified polymorphic and scorable products in *Stizostedion vitreum*. Characteristics of these primers pairs are described in Table 1.

To examine microsatellite variability, genomic DNA was extracted from fin tissues using a standard phenol–chloroform procedure. PCR was performed in a 12.5-μL volume with 10–50 ng of DNA, 300 pmol each primer, 75 μM each nucleotide, 1.2 mM $MgCl_2$, 1× *Taq* buffer (10 mM Tris-HCl pH 9, 50 mM KCl) and 0.25 units of *Taq* polymerase (Perkin-Elmer). PCR reactions were carried out in a Perkin-Elmer 480 DNA thermal cycler as follows: an initial denaturation of 3 min at 96 °C, followed by 30 cycles at 96 °C for 30 s, 30 s at the locus-specific annealing temperature (see Table 1) and 1 min at 72°, 5 min of final elongation at 72 °C. PCR products were separated by electrophoresis in 6% denaturing polyacrylamide gels and bands were visualized using autoradiography.

Variation at each locus was assessed in two samples of *S. vitreum* belonging, respectively, to the Tapani lake from St Anne du Lac, Québec, Canada and from the St Lawrence river, St Romuald, Québec, Canada (Table 1). The river sample shows the largest number of alleles per locus (7–11) and high observed heterozygosity (mean value 0.72, range 0.46–1, 95% confidence interval: $0.61 \leq H_O \leq 0.84$) whereas the Tapani lake sample shows less genetic variability with 3–9 alleles per locus and lower heterozygosity (mean value 0.50, range 0.13–0.71, 95% confidence interval: $0.40 \leq H_O \leq 0.62$).

The results of cross-species amplification are shown in Table 2. As expected, more successful amplification was obtained in the two additional *Stizostedion* species relative to *Perca*, with, respectively, 10 amplified loci for *S. canadense* and eight amplified loci for *S. lucioperca*. These results confirm the complementary usefulness of *S. vitreum* primers for the genetic characterization of closely related species of ecological and economical interest.

Acknowledgements

We thank Sylvain Martin for technical assistance, A. Paquette (Pisciculture Ferme-Neuve), Christoph Jäggi, Neil Billington for providing tissues, and M. Parent for her assistance in microsatellite typing. This project was supported by the FCAR-SORDAC grant, program Actions concertées, Québec. Contribution to the program of GIROQ.

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Table 1 *Stizostedion vitreum* microsatellite primers sequences, amplification conditions, allelic diversity (A) and observed (H_O) and expected (H_E) allelic diversity in samples of the St Lawrence river ($N = 13$) and the Tapani lake ($N = 38$). Characteristics of the predominant continuous repeat region in each cloned allele are also given, the clone sequences from which the primers were designed have GenBank Accession nos AF144734–AF144744

Locus	Primer sequence (5′–3′)	T (°C)	Size range (bp)	Repeat type	Population	A	A (tot.)	H_O	H_E
Svi L1	CTACAGGCTACAAACAAG	53	199–237	$(GT)_{27}GA(GT)_3TT(GT)_{32}$	Tapani lake	9	13	0.68	0.78
	CACAAACATTTTGGGCAG				St-Lawrence river	7		0.69	0.78
Svi L2	TAAGACATAAACATACTCTG	53	175–203	$(TAA)_{14}$	Tapani lake	6	11	0.38	0.36
	TATACTGAGAGTGGAGACAT				St-Lawrence river	9		0.62	0.85
Svi L3	GAGTTTATGAGTTGGAACGC	53	187–285	$(TG)_4(TA)_4TTTA(CA)_{14}$	Tapani lake	8	13	0.59	0.77
	GATGTAAATGATGGAACAGG				St-Lawrence river	8		0.92	0.86
Svi L4	CGTGGCCAGAAAATTTTGATG	55	205–237	$(CA)_6AA(CA)_{14}$	Tapani lake	6	9	0.71	0.75
	GCTATATGTAGTAGGGGGTC				St-Lawrence river	7		0.46	0.87
Svi L5	CATATCCTACTGTAGTATGG	55	188–224	$(CA)_{18}$	Tapani lake	6	11	0.13	0.13
	CAAATCCCATTTACACCCAC				St-Lawrence river	9		1.00	0.90
Svi L6	AGAGGAAGAAGAGGTATC	53	111–137	$(AC)_{17}$	Tapani lake	4	9	0.58	0.62
	TTAAAGGGTAAGTCCACTG				St-Lawrence river	7		0.92	0.89
Svi L7	GATGTGCATACATTTACTCC	53	195–235	$(TG)_{22}$	Tapani lake	6	11	0.39	0.42
	GCTTTAATCTGCTGAGAAC				St-Lawrence river	8		0.69	0.89
Svi L8	GCTTATACGTCGTTCTTATG	53	105–145	$(TG)_{22}$	Tapani lake	4	9	0.42	0.53
	ATGGAGAAGCAAGTTGAG				St-Lawrence river	7		0.46	0.88
Svi L9	TACTGTTCACTTATCTATCC	53	243–297	$(CA)_{18}AA(CA)_3A(AC)_4$	Tapani lake	8	13	0.66	0.72
	TGTATGTGTGTGTTCATGT				St-Lawrence river	7		0.77	0.78
Svi L10	GGTAATGTATTTTCAGTTATTGC	55	201–251	$(CA)_{33}$	Tapani lake	7	13	0.53	0.46
	GCTGTTCTCCAAGTAAAGCC				St-Lawrence river	9		0.75	0.89
Svi L11	AGGGTATGGCATGATAAG	53	123–209	$(TG)_{26}G(TG)_8$	Tapani lake	3	11	0.47	0.48
	CTCTACATTTCATCAGACAG				St-Lawrence river	11		0.69	0.92

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Table 2 Number of alleles and observed heterozygosity values from cross-species amplification within the family Percidae using *Stizostedion vitreum* microsatellite primers. Amplification was attempted on N individuals. Numbers in parentheses are the number of individuals that amplified for each species/locus combination

		Locus										
Species	N	*Svi* L1	*Svi* L2	*Svi* L3	*Svi* L4	*Svi* L5	*Svi* L6	*Svi* L7	*Svi* L8	*Svi* L9	*Svi* L10	*Svi* L11
Stizostedion canadense	9	—	7 (9)	8 (9)	2 (9)	4 (9)	2 (9)	2 (9)	6 (9)	7 (9)	6 (9)	4 (9)
			$H = 0.78$	$H = 0.78$	$H = 0.11$	$H = 0.44$	$H = 0.11$	$H = 0.33$	$H = 0.78$	$H = 0.78$	$H = 0.56$	$H = 0.56$
Stizostedion lucioperca	8	—	2 (8)	—	1 (8)	—	1 (8)	4 (8)	4 (8)	2 (5)	4 (8)	4 (8)
			$H = 0.37$		$H = 0$		$H = 0$	$H = 0.50$	$H = 0.50$	$H = 0$	$H = 0.87$	$H = 0.75$
Perca flavescens	16	1 (16)	—	—	—	—	3 (15)	—	2 (16)	—	7 (16)	1 (16)
		$H = 0$					$H = 0.20$		$H = 0.25$		$H = 0.87$	$H = 0$
Perca fluviatilis	18	5 (18)	—	—	1 (18)	—	4 (18)	8 (18)	—	—	2 (18)	1 (18)
		$H = 0.28$			$H = 0$		$H = 0.06$	$H = 0.28$			$H = 0.11$	$H = 0$

—, indicates no amplification.

Table 1 Characterization of six microsatellite loci in the emu (*Dromarius novaehollandiae*), n = 14 in screeening for polymorphism

Locus	Primer (5′ to 3′)	H_E	H_O	Annealing temperature*	Repeat structure	Size (bp)
emu5	F: ACTTCCTCAAGGCTCACAAATCTG R: CATGGCAGCAGCACATAAAACTG	0.87	0.67	67 °C, 60 °C	$(CA)_{15}$	202–257
emu18	F: GATCGTGGCTGAACTCTGTGG R: CCTTCAGGTCACTTCAGTGC	0.68	0.78	54 °C, 52 °C	$(CA)_{36}$	180–232
emu33	F: AAAGGTATGGCGTAGGGTTTGG R: TACATTTGGCAGCTATGCACTTC	0.97	0.93	65 °C, 54 °C	$(CA)_{26}$	168–200
emu49	F: CCAAGCGAGTTCTTTTGCTG R: GATCTGTAGAACCGGCCATGTGC	0.76	0.58	67 °C, 54 °C	$(CA)_{18}$	190–220
emu50	F: CACACTGCAATTCTCACTGGAGTC R: TCCCCACAAGCGTTTGCATTGTC	0.86	1	56 °C, 52 °C	$(CA)_{18}$	297–327
emu63	F: TGTGGGGATACAGAAATCAGTGG R: TAGCTCAAACAAGGGGCAGCAC	0.89	1	58 °C, 52 °C	$(CA)_{35}$	189–285

*The different annealing temperatures refer to that used in the first and second phase of PCR, respectively.
H_O, observed heterozygosity; H_E, expected heterozgosity.

Isolation and characterization of microsatellite loci in the emu, *Dromaius novaehollandiae*, and cross-species amplification within Ratitae

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Keywords: emu, microsatellite, cross-species amplification, ratite

Received 9 May 1999; revision accepted 8 July 1999

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The emu (*Dromaius novaehollandiae*) is endemic to Australia and found extensively across a variety of habitats. These birds are highly mobile and believed to consist of homogeneous, largely nomadic populations, which exhibit little social structure. Emus have become commercially important as a result of farming, both in Australia and overseas. Genetic studies have not as yet been undertaken and may offer a useful approach for determining social structure and gene flow, as well as assisting in the design of breeding programs aimed at maintaining genetic diversity. Markers would be useful in identifying individuals and determining parentage. Cross-species amplification of emu markers means that they can also be used for studying the relationships between the emu and other ratite birds. Here, we describe the first development of microsatellite loci from the emu, and investigate their suitability for use in other ratite birds.

DNA was isolated from emu blood by the standard phenol–chloroform methods (Sambrook *et al*. 1982), digested with *Sau*3A1, run on a 1.5% low-melting-point agarose gel, and the 350–500 bp region excised and purified. These fragments were ligated into the pZERO™-plasmid cut with *Bam*HI (Invitrogen, CA, USA) and transformed into competent cells (Top10F, Invitrogen). Transformed colonies were subsequently transferred to Hybond N+ membranes and screened with radiolabelled $(dA–dC)_n$–$(dG–dT)_n$ probe. Positive clones were identified by exposure to X-ray film and selected from the original filters to establish the initial microsatellite library. This library was subjected to a second round of hybridization with the $(dA–dC)_n$–$(dG–dT)_n$ probe to confirm positive colonies. Sixty-seven positive colonies were identified from approximately 4800 colonies. Ligated emu DNA was isolated from the positive plasmid clones and screened for microsatellites by cycling sequencing with infra-red dye labelled primers on a Li-Cor gene sequencer. Thirty-nine sequenced clones resulted in 24 different microsatellites. Seven primer pairs were designed on the basis of sequence flanking the dinucleotide microsatellites that contained > 8 uninterrupted repeats. Six of these loci were used in this study.

Amplifications were performed in 10-μL reaction volumes using 50 ng of genomic DNA, 4 pmoles of forward primer end-labelled with an infra-red dye (Li-Cor Inc., USA), 1 pmole of unlabelled forward primer and 5 pmoles of unlabelled reverse primer, 200 μM of dNTPs (Perkin-Elmer, USA), 0.1 U of Amplitaq Gold (Perkin-Elmer, USA), 0.1 M Tris-HCl, pH 8.3, 0.5 M KCl, and 1.5 mM $MgCl_2$. The thermal cycling (FTS-4000 thermal sequencer; MJ Research, USA) comprised 10 min at 95 °C, followed by five cycles of 30 s at 94 °C, 30 s at the selected higher annealing temperature, and 45 s at 72 °C, followed by 28 cycles at 94 °C, 30 s at the selected lower annealing temperature, and 72 °C for 45 s (see Table 1). A final extension was carried out at 72 °C for 2 min. Amplified fragments (2 μL) were resolved on a 6% denaturing polyacrylamide gel for 2 h using a Li-Cor DNA analyser. Fragment sizes were determined by reference to a standard base pair ladder. Emu microsatellites showed Mendelian codominance in established

Table 2 Number of alleles observed in ratites using six primer pairs designed for emu microsatellite loci

Microsatellite locus	Emu (14)	Cassowary (6)	Kiwi (6)	Ostrich (5)	Rhea (6)
emu5	8	1	1	—	—
emu18	6	2	—	3	2
emu33	12	3	1	2	3
emu49	10	2	2	1	1
emu50	12	2	1	4	—
emu63	17	2	2	—	—

The number of individuals tested is shown in brackets.
—, no amplification.

family groups. The extent of the polymorphism of these markers was tested in emus and also other ratite species (see Table 2) and heterozygosities for emus were determined and reported in Table 1.

This report identifies a panel of highly polymorphic microsatellite loci in emus which will permit study of parentage and population heterogeneity in emus. Further, it is shown that emu primers can amplify all extant birds of the ratite group, with up to four alleles being demonstrated in the ostrich (Table 2). This is consistent with the report by Kimwele *et al*. (1998), who found that five out of six ostrich primers amplified up to four alleles in the emu. Given that the emu is estimated to have diverged from the ostrich and rhea at approximately 80 Ma corresponding to a $T_{50}M$ value of 17 (Sibley & Ahlquist 1990), the emu primers performed better than expected, based on calculations from the relationship of Primmer *et al*. (1996) of decreasing microsatellite performance with evolutionary distance.

These microsatellites have been designated emu5, emu18, emu49, emu50 and emu63 and submitted to GenBank database (Accession numbers AF147058, AF147059, AF147060, AF147061, AF147062, AF147063).

Acknowledgements

We sincerely thank Raquel Perez Clariget (Department of Animal Production, Faculty of Agriculture, Garzon, Montevideo) for providing rhea samples, Currumbin Sanctuary (Currumbin, Queensland, Australia) for providing cassowary samples and John F. Cockrem and his students Murray A. Potter and Dominic C. Adams (Conservation Endocrinology Research Group, Massey University, New Zealand) for the provision of kiwi samples. This study was supported in part by an ARC grant through the University of Western Australia.

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Isolation of microsatellite loci in European catfish, *Silurus glanis*

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Keywords: catfish, microsatellites, PCR, primers, *Silurus glanis*

Received 15 May 1999; revision accepted 4 July 1999

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The Siluriformes order consists of species with high worldwide economical importance, with the most important belonging to the Clariidae and Ictaluridae families. In Europe, during the past few years there has been increased interest in the aquaculture of Silurus glanis. Allozyme and mitochondrial DNA studies revealed little variation in this species (Triantafyllidis *et al*. 1999a, 1999b) and stressed the need for the development of microsatellite markers to accurately assess and monitor genetic variation in natural and fish-farmed populations.

We report here the isolation and characterization of microsatellite loci in *Silurus glanis*. We also investigate whether *S. glanis* primers amplify microsatellite loci in *S. aristotelis*, endemic to Greece, and *S. triostegus*, found in the Tigre and Euphrates rivers.

Cloning and characterization of *S. glanis* microsatellite sequences was performed following Rassman *et al*. (1991). Genomic DNA was purified from the blood of a *S. glanis* individual (Clouzioux fish farm, France) and digested to completion with *Sau*IIIA. A partial genomic library was established in a pUC 18 dephosphorylated vector (Pharmacia). The ligated fragments ranged in size from 0.6 to 0.9 kb. Sublibraries constructed using *Escherichia coli* XL1 Blue competent cells (Stratagene) were screened using digoxigenin-labelled synthetic oligonucleotides. A total of 1200 clones containing

Table 1 Characteristics of 10 *Silurus glanis* microsatellite loci. Repeat motif and PCR product size (in bp) of the sequenced allele, optimal annealing temperature for radioactive PCR for *S. glanis* (T_a), primer sequence and GenBank Accession no. Sequences for four additional loci are also accessible: Sgl1154bINRA (Accession no: AF146411), Sgl140bINRA (AF146412 and AF146413), Sgl381INRA (AF146417), Sgl7164INRA (AF146422)

Locus	Core Repeat	Size	T_a (°C)	Primer sequence (5′–3′)	Accession number
Sgl1154aINRA*	$(AC)_{13}$. $(AC)_4$. $(AC)_7$	300	54	GAGTAAATTTGTGTTGGGAAC	AF146410
				GTCTTTACAAGTTTTGGCAGG	AF146411
Sgl140aINRA*	$(AC)_{31}$	126	58	CCCTCCTGCTGGCATCC	AF146412
				GCAAAACCTAGAGGTGCTTTAC	
Sgl310INRA	$(TG)_{21}TC(TG)_7$	121	57	CTACTTGCGACCTACTTGAAC	AF146414
				GTAACCCACACAACTTCAGG	
Sgl325INRA	$(GT)_{37}$ … $(GT)_4$	226	59	CTGGCCGGAGAATAGCCTG	AF146415
				GGGCTCATAACATACTCATTTCAC	
Sgl33INRA	$(AC)_{24}$	153	58	CCACTTATGCACCTGAAGG	AF146416
				GGCCAATTAAACAGGTACAG	
Sgl5fINRA	$(CA)_{14}CT(CA)_3CT(CA)_7$	138	55	CCAATTTACCTCAGACTACTTCTG	AF146418
				GCACGTGCAAAGTCCTG	
Sgl695INRA	$(ATAG)_{18}$	188	56	CTTTGGTGAGTCAGAAACACG	AF146419
				GCACTACTGGTAGATGCT	
Sgl7159INRA	$(AG)_5TG(TGAGAG)_5$ $(TG)_{25}AG(TG)_{17}$	253	54	CTGCTCAATCAAAGTTGGTTC	AF146420
				CAAACTAAGTTCAGCCAGGC	AF146421
Sgl7eINRA	$(TG)_{40–50}$	235	59	GTGAATGTGCTTTAAGGGC	AF146423
				GTTCATGGTGTCACTGCG	AF146424
Sgl7fINRA	$(CA)_{21}$	226	58	GGCTGTATGTTAAGTTATTTTCAG	AF146425
				CTGAGCAGTGGCCAGAATG	

*Clones Sgl140INRA and Sgl1154INRA contained two microsatellite loci each, which were denoted a and b.

catfish DNA inserts were screened. Seventeen clones harbouring a strong hybridization signal were sequenced and microsatellite motives, with repeat numbers ranging from 11 to more than 40, were found in all but one. Polymerase chain reaction (PCR) conditions were optimized for 10 loci (Table 1) using primers radiolabelled with γ^{33}P. PCR products were electrophoresed in 6% acrylamide sequencing gels.

PCR amplification and polymorphism assessment were performed on 20 *S. glanis* (River Kizilirmak, Sariyar dam, Turkey), 15 *S. aristotelis* (Lake Trichonida, Greece) and 15 *S. triostegus* (River Euphrate, Ataturk dam, Turkey) individuals. Ten polymorphic loci were amplified in *S. glanis* (Table 2). Eight and nine out of 10 gave a PCR product in *S. aristotelis* and *S. triostegus*, respectively. A 50% success in cross-priming has been reported for mammal (Moore *et al*. 1992) and avian (Primmer *et al*. 1996) species that have diverged approximately 20 million years ago. The high proportion of *S. glanis* primers which successfully amplified and revealed polymorphism in the other two species suggests a divergence of less than 20 million years ago between the three species and/or high levels of genomic conservation among these species.

Significant departures from Hardy–Weinberg equilibrium were tested using GENEPOP (Raymond & Rousset 1995). Heterozygote deficits were found at locus S7–159 in *S. triostegus* and S7-E in *S. aristotelis*. Several explanations can account for these deviations, the most probable being the occurrence of null alleles which is frequent in cross-priming experiments (Paetkau & Strobeck 1995).

Allele numbers, unbiased expected (H_E, Nei 1978) and observed (H_O, direct-count) heterozygosity values for *S. glanis* were in the range of 2–7, 0.41–0.85 and 0.35–1.00, respectively. The three species showed similar mean H_E and H_O values. However, the mean number of alleles per locus was more than double in *S. aristotelis* and *S. triostegus*. This is not a consequence of sample size as the difference is even bigger when the mean number of alleles per locus is corrected for sample size (not shown).

The amount of variability is much higher at microsatellite than at protein loci (Table 2). As expected, microsatellites will be powerful tools for studying population genetic structure. The success in cross-priming indicates that these loci have the potential to work in other *Silurus* species that belong to economically important genera in Asia (such as *Wallago* and *Ompok*). Similar cross-priming success has been achieved for different genera of the Clariidae and Ictaluridae families (Galbusera *et al*. 1996; Liu *et al*. 1999).

Acknowledgements

The authors acknowledge the help of Prof. Inci Togan (Middle East Technology University, Ankara, Turkey), Seyit Ahmet Oymak (Haran University, Sanliurfa, Turkey) and Prof. C. Triantaphyllidis (Aristotle University of Thessaloniki, Greece). This work was funded by a Protagoras-INRA contract on 'Microsatellite locus variation in *Silurus glanis*' and a European contract (ERC CIPA-CT93–0274) on 'Genetic and reproduction studies of European silurid fishes: application to conservation and fish-farming'. Detailed cloning and sequencing gel electrophoresis procedures are available at HTTP://www.inapg.inra.fr/dsa/microsat/microsat.htm.

Table 2 Unbiased expected (H_E) and observed (H_O) heterozygosity values and number of alleles (A) in the three species using 10 *Siluris glanis* primers

	S. glanis			*S. triostegus*			*S. aristotelis*		
Locus	H_E	H_O	A	H_E	H_O	A	H_E	H_O	A
Sgl140aINRA	0.650	0.737	3	0.862	0.733	7	0.067	0.064	2
Sgl1154aINRA	0.649	0.737	5		NP	NP	0.899	0.929	12
Sgl310INRA	0.456	0.550	5	0.905	0.727	10	0.901	0.867	10
Sgl325INRA	0.849	1.000	7	0.860	0.800	9	0.917	0.867	17
Sgl33INRA	0.631	0.850	3	0.905	1.000	11	0.876	0.667	11
Sgl5fINRA	0.409	0.350	2	0.738	0.786	9		NP	NP
Sgl695INRA	0.750	0.850	5	0.920	0.800	12	0.924	0.933	13
Sgl7eINRA	0.649	0.550	6	0.000	0.000	1	0.248	0.133*	3
Sgl7fINRA	0.488	0.600	3	0.782	0.667	6		NP	NP
Sgl7159INRA	0.553	0.550	3	0.922	0.727*	14	0.906	0.867	12
Mean values	0.608	0.677	4.20	0.766	0.693	8.78	0.717	0.666	10.00
SE	(0.043)	(0.061)	(0.51)	(0.098)	(0.092)	(1.27)	(0.123)	(0.127)	(1.79)
Allozyme study†		0.039	1.16		—	—		0.037	1.41

*Indicates significant departure from Hardy–Weinberg equilibrium.
†Mean values from Triantafyllidis *et al*. (1999a).
NP, no PCR product.

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Isolation and characterization of polymorphic microsatellite loci in the European bullhead *Cottus gobio* L. (Osteichthyes) and their applicability to related taxa

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Keywords: Cottidae, *Cottus gobio*, microsatellites

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The bullhead, *Cottus gobio*, is widely distributed in European rivers and lakes and generally inhabits cold oxygen-rich water bodies. In contrast to many other native freshwater species, *C. gobio* populations are probably not influenced by stocking and dispersal abilities are low. Recent allozyme studies of *C. gobio* in southern Germany (Riffel & Schreiber 1995; Hänfling & Brandl 1998a, b) have shown very high levels of genetic differentiation among local populations. These known features of *C. gobio* make it a well-suited species to investigate the origins and the maintenance of the genetic population structure. Furthermore, the bullhead is an important indicator species for conservation management as it is highly sensitive to human impacts in the environment.

Table 1 Primer sequences and characteristics of 12 bullhead (*Cottus gobio*) microsatellite loci

Locus	Repeat in clone	Primer sequences (5′–3′)	Sequenced allele	Cycling profiles
Cgo310MEHU†	$(GA)_{25}$	CACTGTCATGTAGCGGCTC *AGAACCAGTGTTTGACTCTGC	198	2 min 95 °C
Cgo56MEHU†	$(GT)_{20}$	*ACAGTGAACCACGGTATCGG ATGACACTTTCCTGCAGAACC	235	45 s 92 °C 45 s X °C 30× 45 s 72 °C
Cgo91MEHU†	$(CT)_{12}$	TTTTCAGTTTTGTGTACTCCTG *TGACTTTATTGGTTTGCTGC	227	10 min 72 °C
Cgo1016PBBE‡	$(GT)_2(GC)_3(GT)_2$ GCGTGC$(GT)_8$	*AATCGCAGCCTCCACCTTCC ACGAGTCCCCCAGCAGAACAC	130	5 min 95 °C 30 s 95 °C
Cgo1033PBBE‡	$(CA)_{16}$	*GCTAATTTACTCCTGGGTCA TGGCACTTGATTTGTGAG	108	30 s X °C 5× 75 s 72 °C 30 s 94 °C
Cgo1114PBBE‡	$(GT)_7TT(GT)_9$	*GTGACTGAGCCTTGAGATTC GAACCAACGGAAATGAAAC	127	30 s X °C 25× 75 s 72 °C 5 min 72 °C
Cgo05ZIM§	$(GT)_{14}$	*CCTGCTCTCACAAGGACTTC CCCGCAATCTATTTATTTCT	180	
Cgo18ZIM§	$(GT)_{19}$	*GCAGTGGGTGTGTCATAAAT TTGCCAGCACTGACAGTAAA	240	2 min 95 °C
Cgo34ZIM§	$(AT)_6GT(AT)_3$ $N_{114}(GT)_8GCCG$ $TAC(GT)_{12}$	*GAAGTCTGTCTTTGATATG CATGATTTGAAACATACTG	299	45 s 92 °C 45 s X °C 30× 45 s 72 °C
Cgo22ZIM§	$(GT)_{19}$	*TGGAGGTGTGAGAGATGATT GTCACGGGCAATTCCAACAG	242	10 min 72 °C
Cgo33ZIM§	$(CA)_9CC(CA)_7$	*CAAAAGACAGACCTGTTGAC TTAACAGTGAAGGATGTGAG	163	
Cgo42ZIM§	$(CA)_{23}$	*CCTGAAGAAAGAAATGACAA GAGAGCACTGTCAACATACA	234	

*Fluorescent-labelled primer.
Sequences of cloned fragments have GenBank Accession nos: †AF17923–AF179233; ‡AJ245506–AJ245508; §AF177157–AF177162.

The allozyme markers employed so far have provided only limited resolution at the population level. Microsatellite loci, which can be more polymorphic than allozyme markers, have not been developed for any cottid fish. Here we report 12 new microsatellite loci of the bullhead *C. gobio* and their amplification in other related taxa. Subsets of the loci have been developed independently in three different laboratories, three loci in the Molecular Ecology and Fisheries Genetics Laboratory, Hull, UK (MEHU), three loci in the Division of Population Biology, Berne, Switzerland (PBBE) and six loci in the Zoological Institute, Munich, Germany (ZIM) (Tables 1 and 2). The procedures for screening and cloning of microsatellites and fragment size detection varied among laboratories and are detailed in separate protocols. The loci CgoMEHU were developed following the protocol of D'Amato *et al*. (1999). Prior to microsatellite screening a random anplified polymorphic DNA (RAPD)-enrichment technique similar to Cifarelli *et al*. (1995) was applied. Overall, 1300 colonies were screened with radioactively labelled probes. Forty-five clones were size selected (inserts < 1000 bp) and sequenced on an ALFexpress™ automatic sequencer (Pharmacia). PCR was carried out in a 15-μL volume, using 10–30 ng of genomic DNA, 0.2 mM each dNTP, 3 pmol of each primer, 1× NH_4 buffer (Bio-Line) and 0.2 U of *Taq* polymerase (Bio-Line). A Mastercycler gradient (Eppendorf) was used for cycling reactions and products were run on an ALFexpress™ for size detection. The loci CgoPBBE were cloned as described in Estoup *et al*. (1993) and in detailed protocols by A. Estoup and J. Turgeon available at the internet site HTTP://www.inapg.inra.fr/dsa/microsat/microsat.htm.

Approximately 200 colonies were screened using a mixture of six probes $(TC)_{10}$ $(TG)_{10}$ $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$ and $(TGTA)_6TG$. Plasmid DNA of positive clones was purified (QIAprep Spin Miniprep Kit, Qiagen). Both strands of the insert were sequenced and detected on a Li-Cor automated sequencer (Li-Cor Inc.). PCR amplifications were performed in 10 μL volumes using a PTC100-machine (MJ Research). Each reaction contained 20 ng of genomic DNA, 2 pmol of each primer, 0.06 mM of each dNTP, 1× PCR buffer (Qiagen) and 0.25 U of *Taq* polymerase (Qiagen). The products were analysed on a Li-Cor automated sequencer. Cloning of the loci CgoZIM followed the protocol of Rassmann *et al*. (1991) using the pZErO-2™ plasmid vector (Invitrogen) instead of the phage M13. Approximately 1600 colonies were hybridized with radioactively labelled $(GT)_7G$ and $(AG)_7A$ probes. Positive-clones DNA was isolated following a protocol similar to the plasmid small-scale preparation protocol (lysis by boiling) in Sambrook *et al*. (1989). The inserts were sequenced

Table 2 Amplification results of 12 *Cottus gobio* microsatellite loci in *Cottus gobio* and related taxa

Species	T_A (°C)	[Mg] (mM)	*n*	N_A	Size range (bp)	T_A (°C)	[Mg] (mM)	*n*	N_A	Size range (bp)
	Cgo91MEHU*					Cgo05ZIM				
C. gobio	62	1.5	25	1	227	49	1.5	25	23	136–232
C. poecilopus	57	2.0	5	4	215–235	49	1.5	4	3	172–178
C. hangiongensis	57	2.0	3	4	213–229	49	1.5	3	2	202–206
C. amblystomopsis	57	2.0	3	3	233–259	49	1.5	3	4	222–240
C. pollux	62	1.5	3	1	215	49	1.5	3	5	194–242
C. bairdi	62	1.5	2	1	219	49	1.5	2	4	198–232
T. quadricornis	59	1.5	4	1	205	49	1.5	4	2	188–226
	Cgo310MEHU					Cgo18ZIM				
C. gobio	62	1.5	25	6	173–197	49	1.5	25	17	227–283
C. poecilopus	59	1.5	5	1	177	49	1.5	4	4	229–277
C. hangiongensis				—	—	49	1.5	3	2	243–247
C. amblystomopsis				—	—				—	—
C. pollux				—	—	49	1.5	3	1	203
C. bairdi	62	1.5	2	1	182	49	1.5	2	3	293–301
T. quadricornis	62	1.5	4	1	227	49	1.5	4	7	215–333
	Cgo56MEHU					Cgo34ZIM				
C. gobio	62	1.5	25	12	220–254	49	1.5	25	7	301–319
C. poecilopus	62	1.5	10	1	238	49	1.5	4	3	297–305
C. hangiongensis	62	1.5	3	1	230	49	1.5	3	2	319–323
C. amblystomopsis	62	1.5	3	1	244	49	1.5	3	6	321–365
C. pollux	62	1.5	3	2	228–232	49	1.5	3	3	321–333
C. bairdi	62	1.5	2	3	242–250	49	1.5	2	1	281
T. quadricornis	59	1.5	4	6	248–290	49	1.5	4	2	281–289
	Cgo1016PBBE					Cgo22ZIM				
C. gobio	61	0.9	25	10	124–140	53	1.5	25	23	176–316
C. poecilopus	61	0.9	3	1	≈ 165	53	1.5	4	3	200–206
C. hangiongensis	61	0.9	3	1	138	53	1.5	3	2	206–212
C. amblystomopsis	61	0.9	2	1	124	53	1.5	3	2	164–174
C. pollux	61	0.9	3	1	136	53	1.5	3	4	206–248
C. bairdi	61	0.9	2	2	125–127	53	1.5	2	3	202–222
T. quadricornis				—	—				—	—
	Cgo1033PBBE					Cgo33ZIM				
C. gobio	56	1.0	25	11	89–130	49	1.5	25	13	149–201
C. poecilopus	56	1.0	3	2	117–123	49	1.5	4	3	157–161
C. hangiongensis				—	—	49	1.5	3	3	163–169
C. amblystomopsis				—	—	49	1.5	3	1	159
C. pollux				—	—	49	1.5	3	2	171–177
C. bairdi	56	1.0	2	3	136–200	49	1.5	2	1	153
T. quadricornis				—	—	49	1.5	4	3	167–185
	Cgo1114PBBE					Cgo42ZIM				
C. gobio	56	1.0	25	10	121–141	49	1.5	21†	19	218–326
C. poecilopus	56	1.0	3	2	115–119	49	1.5	4	4	204–344
C. hangiongensis	56	1.0	3	3	127–153	49	1.5	3	3	230–236
C. amblystomopsis				—	—	49	1.5	3	3	260–278
C. pollux	56	1.0	3	1	129	49	1.5	3	4	216–262
C. bairdi	56	1.0	2	1	131	49	1.5	2	2	266–270
T. quadricornis	56	1.0	4	6	134–206	49	1.5	4	1	242

*Locus proved to be polymorphic for *C. gobio* in other than the reference populations.
†Four specimens of the Doubs sample did not amplify.
n, number of studied specimens; N_A, number of alleles; —, no alleles obtained.

employing the Big-Dye-Ready-Reaction-Kit (Perkin-Elmer) and products were detected on an ABI 377Prism™ automated sequencer (Perkin-Elmer). Microsatellite-PCR was carried out in a 10-μL volume using a PTC100-machine (MJ Research). For each reaction 20 ng of genomic DNA, 0.2 mM each dNTP, 10 pmol each primer, 1× *AmpliTaq* buffer (Perkin-Elmer) and 0.25 U of *Taq* polymerase (Perkin-Elmer) were employed. Fragment analysis was performed on the ABI 377Prism™

automatic sequencer. Loci, primer sequences, core sequences, length of sequenced alleles and different cycling profiles employed in each laboratory are listed in Table 1. Annealing temperature and $MgCl_2$ concentrations are shown in Table 2. Variability of the loci was tested in five populations of *C. gobio* from the rivers Rhine and Danube (Germany), Doubs (Switzerland), Po (Italy) and Derwent (UK). Five individuals from each population were analysed. Results of each population were pooled and are shown in Table 2. Additionally, we tested the amplification of these primers in five other species of the genus *Cottus*: *C. poecilopus* (River Vah, Danube, Slovakia), *C. hangiongensis, C. amblystomopsis, C. pollux* (Japan) and *C. bairdi* (North Toe river, NC, USA) and in one species of the genus *Triglopsis* (syn. *Myoxocephalus*), *Triglopsis quadricornis* (Povjoviken, Finland) (Table 2). The 12 loci characterized in this study should be applicable to analysis of the among-population variation and the genetic history of *C. gobio* and also in other species within the Cottidae family.

Acknowledgements

We would like to thank J. Holčik, T. Kontula and A. Nolte for providing samples. B.H. thanks B. Trigg for help in the field and M. E. D'Amato and G. R. Carvalho for advice in the process of microsatellite screening. The work of B.H. was supported by EU-TMR grant ERBFMBICT972792. C.R.L. thanks Alex Kohler for his laboratory assistance. The work of C.C.E. was funded by the Umweltbundesamt.

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