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TECHNICAL NOTE

Development of microsatellite loci in the European Dipper, *Cinclus cinclus*

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Abstract Eighteen polymorphic microsatellite DNA loci were isolated in the Central European subspecies of the European Dipper (*Cinclus cinclus aquaticus*). The loci were tested for polymorphism using a test panel of 24 breeding birds. Numbers of alleles ranged from 2 to 21 per locus and expected heterozygosities varied between 0.47 and 0.83. Two loci (Cici10 and Cici12) proved to be Z-linked. Some pairs of loci exhibited significant linkage disequilibrium but not the two loci that are located on the Z-chromosome. This pattern suggests that demographic effects rather than physical linkage are likely responsible for the observed levels of linkage disequilibrium. These loci will be useful for applied conservation projects and for investigations of the dispersal and mating patterns of European and other dippers.

Keywords Dipper · Cinclidae · *Cinclus mexicanus* · Multiplex PCR · Z-linked loci

The only passerine birds able to dive in fast-flowing rivers are the five species of dippers (Cinclidae; Aves). Not surprisingly, perhaps, given the adaptations that are required to inhabit such a habitat, this genus with its five species is monophyletic (Voelker 2002). As extreme habitat specialists, dippers are sensitive to environmental change, e.g. to changes in water quality (Tyler and Ormerod 1994) or to climate change (Hegelbach 2001; Moreno-Rueda and Rivas 2007). Consequently, some populations in Europe (Henderson et al. 2004) and North America (Anderson et al. 2008) have been declining substantially and are, therefore, of conservation concern.

The palaeartic representative, the European or White-throated Dipper *Cinclus cinclus*, has the widest geographical distribution. Its populations are distributed over Europe, Asia and a small strip of Northern Africa and several subspecies have been described based on morphology (Tyler and Ormerod 1994). The subspecies status of Central Europe's *Cinclus cinclus aquaticus* has been confirmed recently using *cytochrome b* sequence variation (Lauga et al. 2005).

Here, we describe the isolation and characterization of 18 variable di- and tetranucleotide microsatellite loci from a population of *Cinclus cinclus aquaticus* near Zurich, Switzerland. Blood samples were preserved in APS-buffer pH 8.0 (Sibley and Ahlquist 1981), containing 10% EDTA, 1% sodium fluoride and a small amount of thymol sufficient to ensure saturation. DNA was extracted using the QIAmp DNA mini kit (QIAGEN).

An enriched DNA library was made by ECOGENICS GmbH (Zürich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer et al. 1999) and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$ and $(ACAG)_7$ oligonucleotide repeats (Gautschi et al. 2000a, b). Out of the 864 recombinant colonies screened, 310 gave a positive signal after screening with fluorescently labelled CA or ACAG repeats. Plasmids from 140 positive clones were sequenced as described in Gautschi et al. (2000a) and primers were designed for 29 CA and six ACAG microsatellite inserts. Of these, 17 and 6, respectively turned out to be polymorphic in a panel of 24 unrelated individuals screened for polymorphism on ELCHROM gels (Table 1). Five loci were not developed further.

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Table 1 Characteristics and summary statistics of 20 polymorphic microsatellite loci isolated from a *Cinclus cinclus aquaticus* population in Switzerland and genotyped in all breeding birds born in 1999 in one river system (Küsnacht)

Locus name/GenBank accession no.	Repeat motif	Primer sequence (5′–3′) and fluorescent label	Multiplex panel no.	Primer concentration (μM)	T_a (°C)	Size range (bp)	N	N_a	H_O	H_E	HW (exact)
Cici 01	(CA)21	GTGGAGCAGGAATCTAAACTGG	1	2.00	63	100–150	24	16	0.2917	0.7839	**0.0001**
FJ177034		FAM-CCTGCCCAGAGTTTTTAATGC									
Cici 02	(GT)17	AGCATCACAGGAGCAAAGTG	1	1.00	63	90–140	24	10	0.5417	0.6658	0.1987
FJ177035		VIC-CTACGGCTACATCTTCACTTCC									
Cici 03	(CA)20 AA (CA)4	GGGGCCTCATGATGGATAG	2	2.00	61	140–190	23	14	0.8261	0.6947	0.5032
FJ177036		VIC-CTGTGAAGTTATCAGAGGATGTTTG									
Cici 04	(GT)22	GGATGATCTGGGCTATGTGC	1	1.00	63	155–210	24	14	1	0.8229	0.2669
FJ177037		FAM-CAGCTCTGGTGGTCAGGTG									
Cici 05	(CA)21	FAM-CTCCGGTCACTCCCATACC	1	6.00	63	220–270	24	16	0.875	0.7752	0.5196
FJ177038		GGTGCTGGTGATGTTGCAG									
Cici 06	(TGGA)3 TGGG (TGGA)12	PET-CAGCAAAAGCATACCCTGACT	1	1.00	63	95–150	24	10	0.875	0.7752	0.0788
FJ177039		GCTTTATTACCCCTGCTGGAT									
Cici 07	(GT)23	NED-TAAGTCCCGAGCACCATCTG	1	2.00	63	190–250	24	13	0.8333	0.7292	0.1842
FJ177040		TTTCGTTCTCCCAGAGCAGT									
Cici 08	(GT)14	FAM-CCCCAAATATCCTACCACACC	2	2.00	61	210–250	24	9	0.6667	0.7465	0.6924
FJ177041		TTTGGGTTCAAACAGGATGG									
Cici 09	(CTGT)6 (CT)3	GAAGGCCAGATGACATTGCT	1	0.35	63	100–130	24	2	0.4167	0.4688	0.6644
FJ177042		NED-CCCTTGCCTTGCAGACTGT									
Cici 10[a]	(ATCT)14 (GTCT)5 (ATCT)2 (ATCC)11	VIC-TGTGGATAAAGGATCTCAATGC	1	1.00	63	145–202	24	10	0.2917	0.651	**<0.0001**
FJ177043		CCCAGAAAAGGTACAGGATGG									
Cici 11	(GT)23	PET-GAAGCGCAAGGAGTTTTCAC	2	2.00	61	150–260	24	21	0.8333	0.7344	0.7129
FJ177044		GCTGGACTCCTGTGTTTGACT									
Cici 12[a]	(TCAT)17 TATGGA (TCAT)4	VIC-CAAACCTGCACGTGGAATG	1	0.50	63	205–280	24	15	0.375	0.6892	**0.0001**
FJ177045		GTCTGGCCCCACAGAGTTAC									
Cici 13	(GTCT)11 GTACCCC (CA)5	NED-GTGTCCAGTCAGCTCAAAAGC	1	0.70	63	140–185	24	7	0.6667	0.5799	1.0000
FJ177046		TAGAGATTCATGAGCACCTTGG									
Cici 14	(GTCT)9	ACCGCCAACAAACAAAAGG	1	2.00	63	155–230	24	14	0.9583	0.7873	0.4615
FJ177047		PET-TCTCCTGTTAGCCCCGAAG									
Cici 16	(CA)12	FAM-TTAGTGGGGCTTCTGAGCAC	2	2.00	61	253–300	24	7	0.4167	0.48	0.1602
FJ177049		CAGGCAAGTTCAGCCACAG									
Cici 17	(CA)20	ACTTGGGATGGGAACCACT	2	2.50	61	211–310	24	14	0.7083	0.6623	0.2907
FJ177050		VIC-TGGAATCTACTTATGGCTGCAT									

Table 1 continued

Locus name/GenBank accession no.	Repeat motif	Primer sequence (5′–3′) and fluorescent label	Multiplex panel no.	Primer concentration (μM)	T_a (°C)	Size range (bp)	N	N_a	H_O	H_E	HW (exact)
Cici 22	(CA)19	NED-GCTGGACCTCCTTGGGTACT	2	8.00	61	260–330	24	16	0.6667	0.7118	0.6577
FJ177055		AGCAGCACCAGTGGAAGAGT									
Cici 23	(CA)16	FAM-ACCGTTTGAGGTGCCAGA	2	2.00	61	108–160	24	15	0.9167	0.8325	0.4559
FJ177056		AATGGCTGTGGTCTTGTTCA									

One primer of each pair was 5′ fluorescently labelled with 6-FAM, NED, PET or VIC (ABI)

T_a annealing temperature of primer pairs, N number of individuals genotyped at each locus, N_a observed number of alleles, H_O observed heterozygosity, H_E Nei's unbiased expected heterozygosity; HW (exact), exact P values of a test for deviations from Hardy–Weinberg equilibrium at each locus, with tests significant after Bonferroni correction in bold type

[a] Z-linked

The remaining 18 polymorphic microsatellites were split into two panels for multiplex PCR. Panel one included eleven and panel two seven *Cinclus* primer pairs. In addition, two loci designed for other bird species were also included in panel two: QmAAT31 (Hughes et al. 1998) and Ase64 (Richardson et al. 2000). Amplification was conducted in a final volume of 5 μl containing 2 μl of PCR multiplex kit (QIAGEN), between 0.1 and 2.2 μM of each primer, one of which was labeled with a fluorescent dye (Table 1), and 20–40 ng of template DNA, using the following cycling parameters: 15 min of denaturation at 95°C, followed by 28 cycles of 30 s at 94°C, 90 s at 61°C (panel 2) or 63°C (panel 1) and 60 s extension at 72°C and a final extension of 30 min at 60°C (GeneAmp® PCR System 9700, ABI). Fragment analyses were performed on an ABI Prism® 3100 Avant DNA analyzer and allele sizes scored against the size standard GS-500 LIZ™ using GeneMapper v3.7™ software (ABI).

All 18 loci were tested for polymorphism, deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using SAS/Genetics (SAS Institute Inc., version 9.1.3) and a test panel of 24 breeding birds that were all born in the same year and the same river. The loci showed between 2 and 21 alleles and expected heterozygosities ranged from 0.47 to 0.83 (Table 1). Significant deviations from HWE were detected at three loci (Cici01, Cici10, and Cici12). Sex-specific analyses revealed that two of these loci (Cici10 and Cici12) were Z-linked, i.e. only males were heterozygous. The heterozygote deficit at Cici01, however, seemed to be the result of null alleles.

Tests for linkage disequilibrium revealed that Cici11 and Cici17 were in significant LD after sequential Bonferroni correction (corrected $P = 0.038$). These loci could in principle be physically linked. However, these two loci did not exhibit significant LD in other cohorts (data not shown) and the two Z-linked loci that were known to be on the same chromosome did not exhibit significant LD. This pattern suggests that the levels of LD detected in our test panel were more likely caused by demographic factors such as bottlenecks and immigration rather than by physical linkage.

The high levels of genetic variation (number of alleles and heterozygosities) present at these 18 microsatellite loci render them ideal for the analysis of the dipper mating system. Moreover, they may also prove useful for the analysis of dispersal and population structure. Finally, ten loci (Cici02, Cici04, Cici05, Cici08, Cici10, Cici11, Cici12, Cici13, Cici15, and Cici16) also proved polymorphic in the American Dipper (*Cinclus mexicanus*; Anderson et al. 2008) suggesting that our loci will also work in other dipper species.

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