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TECHNICAL NOTE

Development of microsatellite markers for a diving duck, the common pochard (*Aythya ferina*)

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Abstract Ten polymorphic microsatellites for the common pochard were isolated from microsatellite enriched libraries. Seven microsatellites were obtained based on cross-species amplification. These 17 microsatellites exhibited polymorphism within a population sample, with numbers of alleles per locus ranging from 2 to 17 and expected heterozygosities from 0.053 to 0.916. These markers will be helpful for studying reproductive strategies in the pochard, as well as in addressing population genetic questions. Four of the microsatellite loci showed significant departures from Hardy–Weinberg equilibrium and/or high estimates of null allele frequencies in our study population. Hence, they should be used with caution.

Keywords Waterfowl · Conspecific brood parasitism · Extra pair paternity · Conservation genetic

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The common pochard, a palearctic species of diving duck, is widespread in Europe. As with other waterbirds, however, it deserves conservation management in light of the loss of its natural habitats (Amezaga et al. 2002). Effective conservation requires knowledge of the species' reproductive strategies as well as genetic diversity and population structure. Microsatellite genotyping of samples derived non-invasively from bird nests is effective in addressing these issues with minimal adverse effects on populations (e.g. Pearce et al. 1997; Kreisinger et al. 2010). This method allows estimations of renesting rates, breeding fidelity and research on alternative reproductive strategies such as conspecific brood parasitism (CBP) or extra pair paternity (EPP). Population genetic analyses in pochards can be of epidemiological importance, as this species is an important vector of avian influenza (Fereidouni et al. 2010). Since there are as yet no published microsatellite loci in the genus *Aythya*, our markers may also be of utility in studies of other endangered congeners such as Baer's pochard (*A. baeri*) and the Madagascar pochard (*A. innotata*).

To obtain primer sequences amplifying microsatellite loci (TG and ATCT repeats) in the pochard, we constructed two enriched genomic libraries according to Estoup and Martin (1996). In brief, genomic DNA was extracted from a blood sample (DNeasy kit, Qiagen) and digested with the *Rsa*I restriction enzyme (Promega). Restriction fragments were separated by gel electrophoresis, 500–900 bp long fragments were extracted (QIAquick Gel Extraction Kit, Qiagen) and ligated to RSA21 and RSA25 adaptors (Edwards et al. 1996). RSA21 oligos were used as PCR primers to amplify the fragments. Diluted PCR products were hybridized to biotinylated oligo probes $(TG)_{10}$ and $(ATCT)_5$, and streptavidin coated paramagnetic particles (Promega) were used to separate the fraction of DNA

enriched for these repetitive motifs. This DNA was then once more amplified using RSA21 primers, and PCR products were cloned using the pGEM-T Easy Vector system II (Promega). To identify clones containing microsatellite motifs, approximately 500 clones for each library were transferred onto Hybond-N+ membranes (Amersham), hybridized with digoxigeninend-labelled $(TG)_{10}$ and $(ATCT)_5$ probes (DIG Oligonucleotide Tailing Kit, Roche) and visualised using a DIG Nucleic Acid Detection Kit (Roche). In total, 59 positive clones were then sequenced by MACROGEN (South Korea).

Primers were designed for 24 loci with OLIGO software (Rychlik 2007). For each locus, the level of polymorphism was checked in 8 individuals by capillary electrophoresis. Markers that did not exhibit polymorphism (14 in total, GenBank accession numbers: HM562714–HM562726)

Table 1 Characteristics of 17 microsatellite loci in a sample of 37 putatively unrelated common pochards from a population in the Czech Republic

Locus	GeneBank acc. no	Repeat motif	Primer sequence	Size (bp)	A	H_O	H_E	F(Null)
AF01	HM365209	$(TG)_{68}$	F: TGATCGGTTTTGGTAAGTTTCT	156–190	16	0.784	0.88	0.0513
			R: TGACAGTTCAGGGTATCTTGTAAA					
AF07	HM365210	$(TG)_9$	F: TGGGTTTGGTCCTTATGTTTT	103–105	2	0.243	0.257	0.0199
			R: GAGATATTTCTGCCTACACATTCC					
AF08	HM365211	$(TG)_{51}$	F: CCCTCTCTTTAACCCGTTTC	195–315	17	0.622	0.916	0.1843
			R: TCCTAACACCCCTTCTTATTACC					
AF12	HM365212	$(TG)_{11}$	F: CCTGAGTGGTCAAACAGTGC	192–208	6	0.757	0.753	0
			R: CCTTCTGGAGCCTCAAACC					
AF14	HM365214	$(TG)_7$	F: CCTGTGCTAAAATGTAGTTGAGATT	97–105	4	0.486	0.51	0.006
			R: TTTATGAATGGTTTCCAGTAGCA					
AF17	HM365214	$(TG)_{20}$	F: CCCATGAGGTGTATAAGGATAGATG	137–150	6	0.486	0.435	0
			R: GGAGATGAGAAAGGCAACACTC					
AF18	HM365215	$(ATCT)_{12}(ATTT)_2$	F: ACCCAAACAACAGGCTAGG	173–179	4	0.459	0.518	0.0281
			R: AATTTAGGAGCAGAACCAGAGG					
AF19	HM365216	$(TG)_9TATCTA(TG)_3$	F: ACTGCTGGCAACACTTATCC	247–267	6	0.838	0.712	0
			R: AAATCTCACAAAGTTCAACAAGG					
AF22	HM365217	$(AT)_4TT(AT)_2(CT)_2(ATCT)_9$	F: TGGTTTAGTGGAGGGCTGTTA	192–208	5	0.459	0.713	0.2125
			R: CAACAGACAGAGATATGCTACGC					
AF23	HM365218	$(TCTA)_6TCTG(TCTA)_5TCCA$	F: ATGGCTTTGAAAATTACAGTAAAA	147–151	2	0.054	0.053	0
			R: CGTTAGGTTAGAGGTTGGACTCT					
Smo04[a]	AJ 427844	$(AG)18A(AAGG)_{10}$	F: ACTTTCCACAGCCTCTTTCACAA	157–193	3	0.054	0.054	0
			R: GACAGTGTTTGTCAATGGATTTT					
Smo11[a]	AJ 427851	$(TG)_{12}GA(G)_{13}(AG)_5$	F: AAATCAACCAAAGAGGCATAGCC	148–153	3	0.622	0.538	0
			R: GCAGTTGTTTTGGAGGACAGACA					
CMaat28[b]	AF509883	$(AAT)_{23}$	F: TTCCACATAAAAATTCATTCAGT	204–231	10	0.946	0.863	0
			R: TCCAGGTCACGTAGTTTTTAAGTA					
Caud013[c]	AY493258	$(AC)_{25}$	F: ACAATAGATTCCAGATGCTGAA	83–103	7	0.486	0.573	0.0842
			R: ATGTCTGAGTCCTCGGAGC					
Apl36[d]	AY498546	$(CA)_{13}GA(CA)_3(GA)_2$ $(CA)_2GA(CA)_{10}GA$	F: ATGCTTTGCTGTTGGAGAGC	127–199	3	0.351	0.302	0
		$(CA)_7GA(CA)_2TA(CA)_5$	R: TCCACTGGGTGCAAACAAG					
Apl12[d]	AY498542	$(GA)_{27}$	F: AGTTGACCCTAATGTCAGCATC	124–132	5	0.649	0.705	0.0394
			R: AAGAGACACTGAGAAGTGCTATTG					
Aph13[e]	AJ515889	$(GA)_{10}$	F: CAACGAGTGACAATGATAAAA	177–186	4	0.486	0.632	0.1373
			R: CAATGATCTCACTCCCAATAG					

Locus [a–e]: Results of cross-species amplifications of microsatellites originally described in [a] Paulus and Tiedemann (2003), [b] Stai and Hughes (2003), [c] Huang et al. (2005), [d] Denk et al. (2004), and [e] Maak et al. (2003); *A* number of alleles, H_E expected heterozygosity and H_O observed heterozygosity, $F_{(Null)}$ frequency of null alleles

Table 2 Characteristics of three multiplexes that were use for genotyping of the common pochard

Locus	Multiplex	Fluorescent dye	Primer concentration	Annealing temperature (°C)
AF07	1	PET	0.050	57
AF08	1	6-FAM	0.040	57
AF12	1	NED	0.020	57
AF14	1	6-FAM	0.040	57
AF17	1	NED	0.030	57
AF01	2	NED	0.040	57
AF18	2	PET	0.040	57
AF19	2	NED	0.030	57
AF22	2	6-FAM	0.035	57
AF23	2	6-FAM	0.030	57
Smo04	3	VIC	0.040	55
Smo11	3	6-FAM	0.040	55
CMaat28	3	6-FAM	0.060	55
Caud013	3	VIC	0.050	55
Apl36	3	NED	0.040	55
Apl12	3	PET	0.060	55
Aph13	3	PET	0.045	55

Locus Name of the locus, *Multiplex* number indicate identity of the locus to a particular multiplex, Fluorescent dye of forward primers according to NED, PET, VIC, 6FAM system, Concentration of individual primers in µmol/µl and annealing temperature in °C

were excluded from further analyses. In addition, 47 microsatellite markers originally described for related species were tested (Maak et al. 2003; Paulus and Tiedemann 2003; Stai and Hughes 2003; Denk et al. 2004; Huang et al. 2005) using PCR followed by agarose gel electrophoresis.

We optimized three PCR multiplexes consisting of 17 microsatellite loci that are potentially useful for pochard (10 obtained from our genomic libraries and 7 from cross-species amplification, Tables 1, 2). In total, 37 pochard individuals from one breeding locality (Doudlebia, Czech Republic, 49°6′27″N, 14°18′10″E) were screened for genetic polymorphism, using a PCR mixture consisting of a Qiagen multiplex kit (Qiagen) and fluorescently labeled primers. The PCR reaction consisted of a 15-min 95°C denaturation step, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 90 s at temperature specified in Table 2 and extension at 72°C for 60 s. The final extension step was 30 min at 60°C. Fragments were separated on an ABI 3100 sequencer (Applied Biosystems). Allele scoring was performed using Gene Marker (SoftGenetic).

Observed and expected heterozygosities, non-exclusion probabilities for individual loci and estimates of null allele frequencies were computed using CERVUS 3 (Kalinowski et al. 2007). Deviations from Hardy–Weinberg (HW) equilibrium and linkage disequilibrium between individual loci was tested using exact tests in GENEPOP (Raymond and Rousset 1995). We used the Bonferroni procedure to correct for Type I errors associated with multiple comparisons.

The probability that two individuals in the population will share an identical genotype was negligible ($PI = 6.26 \times 10^{-13}$). Our panel is thus suitable for analyses of reproductive strategies that require identification of sequential breeding attempts, i.e. breeding site fidelity and renesting and for analyses of alternative reproductive strategies such as CBP and EPP (non-exclusion probability of the first parent and second parent was 0.0051 and <0.0001 respectively).

Some of these loci need to be used with a caution, since we detected significant departure from HW equilibrium at AF08, and high estimates of null allele frequency were observed at locus Aph13, Caud013 and AF22 (Table 1). In addition, there was evidence for linkage disequilibrium between AF08 and Apl12 ($P < 0.001$).

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