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TECHNICAL NOTE

Identification of novel microsatellite loci in the sand martin, *Riparia riparia*, and cross-amplification of loci from other bird species

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Abstract We isolated and characterised six novel microsatellite loci for paternity analysis in the sand martin *Riparia riparia*, by screening an enriched genomic library. In addition, we tested 16 already published microsatellite markers, five of which were also polymorphic in the sand martin. Only one of these 11 loci exhibited evidence of null alleles, and all were polymorphic (mean $H_o = 0.68$, range of number of alleles per locus = 4–24), making them suitable for individual heterozygosity quantification and paternity assessment in this species (exclusion probability of 11 unlinked loci = 0.999997).

Keywords Sand Martin · *Riparia riparia* · Paternity · Microsatellite · Cross-amplification

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Introduction

The sand martin, *Riparia riparia*, is a small migratory passerine bird that breeds colonially in burrows dug into river banks in Eurasia and North America (where their common name is bank swallow). Genetic parentage analyses using multilocus DNA fingerprinting have revealed substantial frequencies of extra-pair paternity and intraspecific brood parasitism in this species (Alves and Bryant 1998; Augustin et al. 2007), while additional genetic analyses has revealed that telomere length, corrected for age, predicts longevity (Pauliny et al. 2006). Further, by being able to assign parentage of offspring within an isolated colony, we can test a prediction of the hidden lek hypothesis, namely that females acquire extra-pair fertilization from neighbouring males (Wagner 1993, 1998). We report here the development of novel microsatellite markers for the sand martin that will allow further investigation of these interesting patterns by (1) enabling the assignment of parentage to extra-pair and brood parasite offspring, thus allowing researchers to measure the fitness of alternative mating strategies in this system, and (2) allowing the calculation of heterozygosity to further examine fitness in tandem with telomere length and other variables. In addition, we describe several additional markers developed for other bird species that amplify sand martin microsatellite loci, thus augmenting the robustness of microsatellite analyses in this species.

Methods

Blood samples were taken from sand martins captured at colonies near the village of Gávavencsellő on the Tisza River, Hungary (48°11′ N 21°35′ E) in 2004 and stored in

Queen's lysis buffer. DNA was then extracted with phenol–chloroform–isoamyl alcohol. To isolate and identify microsatellite loci, we followed the method described by Farias et al. (2003). Briefly, mixed genomic DNA from four sand martins (two male and two female) was digested with MboI restriction enzyme and enriched for CT and CA microsatellite-containing fragments in polymerase chain reactions (PCRs). Fragments were then ligated into a PCR 2.1 TOPO vector. Clones that produced a clean, single, product after PCR were sequenced. We sequenced 26 clones that potentially contained CA repeats and 66 clones that potential contained CT repeats, using a Beckman Coulter CEQ 8000 automated sequencer. In total, six CA microsatellite loci and 12 CT loci were identified. However, due to a problem with some microsatellite-containing fragments inserting into the vector with little or no flanking region, we could only design primers for two CA loci and seven CT loci. Primers were designed using Primer3 (Rozen and Skaletsky 2000).

Primer pairs that gave consistent, specific, products were tested for polymorphism. One primer in each pair was labelled with a fluorescent dye (D2-PA, D3-PA or D4-PA). The primer sequences and optimum annealing temperatures are listed in Table 1. We assessed polymorphism by typing at least 20 putatively unrelated individuals at each microsatellite locus. PCR was performed in 12.5 µl reaction volumes containing a forward primer (labelled with a Beckman Coulter dye: D2, D3 or D4) and reverse primer

Table 1 Characterisation of novel microsatellite loci isolated in the sand martin *Riparia riparia* and cross-amplification of loci from other bird species

Locus	Repeat motif in clone	Primer sequence (5′–3′)	N	T_a	[$MgCl_2$]	N_a	bp	H_o	H_e	Ex	Reference
Rri2	$(CA)_7GGTA(CA)_4$	F: CTAGGGTGAGTTTCACTTAAGC	21	50	3	6	343–365	0.67	0.68	0.40	This study
		R: CCGGTGAGTTTTGGAAAAGG									
Rri3	$(CA)_{10}$	F: CCTTTAAACTGCTCCTGTACC	20	50	3	4	198–202	0.40	0.48	0.25	This study
		R: CAGAAAGTGTAGCGAAAAGG									
Rri4	$(CT)_8$	F: TTTACTCCCTGTCTCAGTCC	21	60	3	5	154–162	0.38	0.38	0.21	This study
		R: GGCTGAAAAGAGCATTCC									
Rri5	$(CA)_{23}$	F: AACAGCACTGGAATTACTGG	20	60	4	24	231–559	0.50	0.97	0.89	This study
		R: GAGGAATCTGTGAACACACC									
Rri8	$(CT)_{15}$	F: CAGAGTTCATGGAGCTTGTC	21	55	3	13	236–260	0.81	0.92	0.79	This study
		R: GATGAAACTTGGGATTGAGATG									
Rri9	$(CT)_{15}$	F: CCAATGTGTTTCCACAGG	23	55	3	12	324–346	0.83	0.91	0.78	This study
		R: AGCTAAGAGCCAAGAAAACC									
Hru6	$(AAAG)_{17}$ $(AG)_2(AAAG)_2$	F: GCTGTGTCATTTCTACATGAG	20	60	3	18	151–331	0.90	0.95	0.85	Primmer et al. (1995)
		R: ACAGGGCAGTGITACTCTGC									
Hru7	$(AAACC)_2$ $(AAAC)_3$	F: GCATTCACAGTGTAGACAATG	20	60	3	5	132–144	0.50	0.60	0.36	Primmer et al. (1995)
		R: GATCACTATGAGTCCCGAA									
Escμ6	$(CA)_{15}CG(CA)_{10}$ $GATA(CA)_3$	F: CATAGTGATGCCCTG	20	60	3	21	129–205	0.95	0.96	0.87	Hanotte et al. (1994)
		R: CCAAGTGCTCCTTAA									
Pdoμ5	$(CA)_3G(CA)_{21}$	F: GATGTTGCAGTGACCTCTCTTG	22	55	3	13	222–276	0.82	0.80	0.62	Griffith et al. (1999)
		R: GCTGTGTTAATGCTATGAGG									
Aar4	$(CA)_{13}$	F: GATGACTAAGGTCTCTGGTGTG	22	60	3	8	109–129	0.73	0.81	0.60	Hansson et al. (2000)
		R: GTTTGTGCATCAATTAGTCATG									

The number of individuals tested (N), primer annealing temperature in °C (T_a), the $MgCl_2$ concentration in mM ([$MgCl_2$]), number of alleles (N_a), allele size range in base pairs (*bp*), observed (H_o) and expected (H_e) heterozygosity, and the probability of excluding a father (Ex) are listed for each locus. Cloned Rri sequences have been deposited with GenBank under Accession numbers GQ372862–GQ372867

(0.4 mM each), 0.625 units of FIREPol DNA polymerase, 1× reaction buffer, the optimal concentration of magnesium chloride ($MgCl_2$) (see Table 1), 0.1 mM deoxyribonucleotide triphosphates (dNTPs) and 25 ng of genomic DNA. PCRs were run on a Biometra T1 thermocycler. An initial denaturation step (94°C, 5 min) was followed by 30 cycles of 30 s at 94°C, 30 s at the locus-specific annealing temperature (Table 1), 60 s at 72°C, and a final extension step for 10 min at 72°C. The PCR products were subjected to electrophoresis on a Beckman Coulter CEQ 8000 automated sequencer, and fragment sizes were estimated with the Beckman Coulter CEQ 8000 fragment analysis software.

In addition to isolating novel microsatellite loci for the sand martin, we tested 16 existing microsatellite markers designed for other bird species for cross-amplification with sand martin DNA. Specifically, the primers tested were Hru1, 2, 3, 4, 5, 6, 7 and 8 (Primmer et al. 1995), Hru10 (Primmer et al. 1996), Pocc6 (Bensch et al. 1997), Escμ6 (Hanotte et al. 1994), Mcyμ6 (Double et al. 1997), Ppi2 (Martinez et al. 1999), Ltr6 (McDonald and Potts 1994), Aar4 (Hansson et al. 2000) and Pdoμ5 (Griffith et al. 1999). We tested the polymorphism of six of these loci that consistently produced strong single PCR products.

Results and discussion

Of the nine novel loci isolated in the sand martin, one did not produce clean, scorable, alleles, and two were monomorphic. The remaining six were all polymorphic to a variable degree (N_a = 4–24, mean H_o = 0.60; Table 1). In addition, five published loci were polymorphic in this species (N_a = 5–21, mean H_o = 0.78; Table 1). Together, the novel and published loci exhibited a combined second-parent exclusion probability of 0.999997 (calculated using Cervus 3.0; Kalinowski et al. 2007). When additional tests implemented in Cervus 3.0 (Kalinowski et al. 2007) were used, only one locus (Rri5) was found to deviate significantly from Hardy–Weinberg equilibrium ($P < 0.001$). This locus showed evidence of heterozygote deficiency, possibly due to the presence of null alleles. However, the polymorphism of this locus (24 alleles from 20 typed individuals) and allele size range (231–559 bp) suggested that at least some of those individuals typed as homozygotes might, in fact, have had alleles larger than the maximum read range of the Beckman Coulter fragment analysis software (600 bp). We were unfortunately unable to sequence the entire length of the Rri5 locus and so have little information on the exact characteristics of this locus. The omission of this locus from parentage analysis still resulted in a very high second-parent exclusion probability of 0.99997. Using GENEPOP (Raymond and Rousset 1995), we found no evidence of linkage disequilibrium between the loci (all $P > 0.05$). This set of 11 markers will, therefore, be a valuable tool for documenting patterns of paternity and heterozygosity levels in the sand martin.

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