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PRIMER NOTE

Multiplex panels of polymorphic microsatellite loci for two cryptic bat species of the genus *Pipistrellus*, developed by cross-species amplification within the family Vespertilionidae

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Abstract

The paper presents multiplex panels of polymorphic microsatellites for two closely related cryptic species *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*. We tested the cross-species amplification of 34 microsatellite loci, originally developed for five vespertilionid bat species. Ten and nine polymorphic loci in *P. pipistrellus* (mean number of alleles per locus = 10.5) and *P. pygmaeus* (8.1), respectively, in three multiplex polymerase chain reactions per species were amplified. All loci can be analysed in a single fragment analysis and can be used as markers to the study of evolution and the ecology of structured populations of socially living bats.

Keywords: Chiroptera, microsatellites, *Pipistrellus*, *pygmaeus*, sibling species

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The recent discovery of cryptic diversity in *Pipistrellus pipistrellus* species complex (Barratt *et al*. 1997) offers models for the studies of cryptic ecological variation in bats. Since socially living bats comprise structured populations influenced by the migration behaviour or geographical barriers (e.g. Pettit & Mayer 1999; Castella *et al*. 2001), study of their genetic structure using polymorphic microsatellite markers can give clues to their evolution and ecology (Burland & Worthington Wilmer 2001). Many microsatellite loci are versatile among bat species, and genotyping in multiplex sets has time and financial cost advantages (cf. Burland *et al*. 1998). Here we present multiplex panels of polymorphic microsatellites for two closely related cryptic species *P. pipistrellus* and *Pipistrellus pygmaeus* developed by the cross-species amplification within vespertilionid bats.

We tested the amplification of 34 microsatellite loci developed for five vespertilionid bat species (references in Table 1). Loci unsuccessfully tested for cross-amplification in *Pipistrellus* bats in the original studies were excluded.

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Single polymerase chain reactions (PCR) were performed in 10 μL reactions with *Taq* polymerase (Fermentas) (annealing temperature 50 °C, 3 mM $MgCl_2$), and forward primers of successfully amplified loci were 5′-end labelled with a fluorescent dye with the intent of simultaneous analyses of multiple loci (Table 1).

Finally, 10 and nine polymorphic loci in *P. pipistrellus* and *P. pygmaeus*, respectively, in three multiplex PCRs per species were amplified (Table 1). PCRs were performed on a Mastercycler ep gradient S (Eppendorf) by using either 1 × Multiplex PCR Master Mix (QIAGEN) or a mix of 0.2 mM of each dNTPs, 1 × *Taq* DNA buffer (Fermentas), 3 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Fermentas). The former was used for PCRs 1 and 2, while the latter for PCR 3 (Table 1). Each reaction contained forward and reverse primers in various concentrations (Table 1), 1.0 μL of extracted DNA and ddH_2O to the volume of 10 μL. Cycling parameters of PCRs 1 and 2 followed the manufacturer's protocol; PCR 3 had initial activation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 4 min. PCR

Table 1 Results of cross-species amplification of vespertilionid microsatellite markers in *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*

Locus	*n*	Size (bp)	*A*	H_E	H_O	NAF	Dye	PCR	FC (μM)
P. pipistrellus									
b22*	24	131	1	—	—	—	—	—	—
EF1†	24	144–164	10	0.786	0.792	0	NED	1	0.15
EF4†	19	224–251	11	0.903	1.000	0	PET	1	0.5
EF6†	24	161–189	11	0.860	0.750	0.063	PET	2	0.5
EF15†	24	244	1	—	—	—	—	—	—
D15‡	24	91	1	—	—	—	—	—	—
F19‡	24	185	1	—	—	—	—	—	—
H29‡	23	164–186	10	0.873	0.870	0	NED	3	0.2
NN8§	24	155–182	17	0.919	0.625‡	0.143	—	—	—
NN18§	23	282–296	8	0.853	0.739	0.043	NED	2	0.6
P13	23	112–140	12	0.860	0.870	0	VIC	3	0.1
P20	24	100–102	2	0.254	0.292	0	FAM	3	0.2
P217	24	181–212	13*	0.860	0.833†	0.008	VIC	1	0.15
P219	24	123–182	13*	0.836	0.958	0	FAM	2	0.1
Paur02††	24	207	1	—	—	—	—	—	—
Paur05††	23	239–255	8	0.874	0.565†	0.160	FAM	1	0.2
P. pygmaeus									
b22*	24	131	1	—	—	—	—	—	—
EF1†	23	168–178	6	0.819	0.652†	0.093	NED	2	0.15
EF4†	18	220–247	9	0.798	0.611	0.098	PET	2	0.6
EF6†	24	171–187	9	0.883	0.833	0.011	PET	2	0.4
D15‡	24	91	1	—	—	—	—	—	—
F19‡	24	185	1	—	—	—	—	—	—
NN18§	22	282–324	9	0.836	0.591‡	0.111	NED	3	0.3
P13¶	23	120–148	14*	0.901	0.696†	0.098	VIC	3	0.3
P20¶	24	98	1	—	—	—	–	—	—
P217¶	24	204–217	6*	0.720	0.667	0.017	VIC	1	0.08
P219¶	23	140–186	9	0.837	0.783	0.018	FAM	1	0.5
Paur02††	24	207–211	3	0.571	0.667	0	NED	1	0.03
Paur05††	24	243–257	8	0.777	0.708	0.037	FAM	3	0.7

Locus, *, *Myotis bechsteinii* (Kerth *et al*. 2002), †, *Eptesicus fuscus* (Vonhof *et al*. 2002), ‡, *Myotis myotis* (Castella & Ruedi 2000), §, ¶, *Nyctalus noctula* (Petri *et al*. 1997; Mayer *et al*. 2000), ††, *Plecotus auritus* (Burland *et al*. 1998); *n*, number of individuals successfully genotyped per locus; *A*, number of alleles, *, alleles differing by 1 bp (indels); H_E, expected heterozygosity, nonbiased estimate according to Nei (1978); H_O, observed heterozygosity, loci that significantly deviated from Hardy–Weinberg equilibrium are marked by †, $P < 0.05$ and ‡, $P < 0.01$; NAF, null allele frequency calculated by FREENA software; PCR, multiplex PCR in which the locus was amplified; FC, final concentration of primers in multiplex PCRs. Locus NN8 was not included in the optimization of multiplex panels because of high frequency of null alleles and frequent indels.

products were mixed together (1 μL of each) and analysed in single fragment analysis on ABI 3130 Genetic Analyser using LIZ500 Size standard (Applied Biosystems).

To assess the polymorphism of loci, 24 females from one colony of each species were genotyped. Nine microsatellites showed high level of polymorphism, whatever the species concerned. In both species, some loci exhibited 1-bp shifts in allelic size, suggesting the presence of insertion or deletion in the flanking region (Table 1). Mean number of alleles per locus was 10.5 in *P. pipistrellus* and 8.1 in *P. pygmaeus*. Exact tests performed using GENEPOP 3.4 (Raymond & Rousset 1995) revealed significant deviations from the Hardy–Weinberg equilibrium for some loci in both species due to a deficit of heterozygotes. The analysis in FREENA (Chapuis & Estoup 2006) revealed that it could be possibly caused by the presence of null alleles (Table 1). Genotypic linkage disequilibrium between 10 polymorphic loci in *P. pipistrellus* and nine loci in *P. pygmaeus* were tested for both populations by exact tests using the Markov chain methods in GENEPOP 3.4 (Raymond & Rousset 1995), and these tests reported no evidence of linkage disequilibrium between any of the analysed loci. There was a visible tendency to more successful cross-amplifications with primers derived from phylogenetically closer genera (*Nyctalus*, *Eptesicus*). In conclusion, sets of markers optimized for two *Pipistrellus* species will allow analysis of population genetics and relatedness structure of these model bats.

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