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A rapid PCR-based test for species identification of two cryptic bats *Pipistrellus pipistrellus* and *P. pygmaeus* and its application on museum and dropping samples

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The identification of two cryptic bat species of the genus *Pipistrellus* using a non-destructive and quick method of multiplex PCR and agarose gel electrophoresis is described. Two primer combinations were able to produce species-specific bands that identified reliably individuals that were previously identified by mtDNA sequencing. Robustness of the method was subsequently successfully tested on 16 randomly selected free-living animals from central Europe (tissue samples obtained from a 3 mm punch of wing-membrane) identified to species on the basis of echolocation calls. Nine out of 15 museum specimens and 100% of fresh faecal samples from seven individuals were also successfully identified by this method. The described method thus provides a good way to routinely distinguish two *Pipistrellus* species by using non-destructive sampling of living individuals or droppings, and will be used in field studies of their ecology.

Key words: Chiroptera, cytochrome *b*, mtDNA, sibling species, non-invasive sampling

INTRODUCTION

The common pipistrelle (*Pipistrellus pipistrellus* sensu lato) has been considered a very common bat species in Europe (e.g., Jones, 1999). The use of bat detectors during last decades indicated the existence of two distinct phonic types (45 and 55 kHz) within this taxon (Ahlén, 1981; Miller and Degn, 1981; Weid and von Helversen, 1987; Zingg, 1990). Subsequent genetic differences found in the nucleotide sequences of the cytochrome *b* gene (cyt*b*) revealed that the common pipistrelle in fact comprised two distinct species, *Pipistrellus pipistrellus* (Schreber, 1774) and *Pipistrellus pygmaeus* (Leach, 1825) (Barratt *et al*., 1995, 1997). Nowadays this species-complex has come to represent one of the most comprehensive models for the study of cryptic variation in Palearctic bats (Hulva *et al*., 2004). The colonization history of the European continent and the current allopatric or sympatric distribution of these two taxa (Barratt *et al*., 1997; Mayer and von Helversen, 2001; Benda *et al*., 2003; Hulva *et al*., 2004) suggest species-specific differences in ecological requirements of both species (e.g., Barlow and Jones, 1999; Bartonička and Řehák, 2004; Davidson-Watts

and Jones, 2006; Nicholls and Racey, 2006). These two cryptic species of pipistrelles are almost indistinguishable morphologically and the only reliable field method to distinguish between species remains the analysis of echolocation calls (cf. Barlow *et al*., 1997; Häussler *et al*., 2000; von Helversen and Holderied, 2003). However, for common field methods such as investigation of day roosts, mist-netting, museum samples, and dead animals, records of echolocation calls are impossible to obtain. Therefore, there is a need for alternative methods that allow clear identification of these two genetically well-defined species.

The aims of the presented note were: 1) to describe a non-destructive and quick polymerase chain reaction (PCR)-based technique providing easy and reliable identification of two cryptic species of the genus *Pipistrellus*; and 2) to test the efficacy of the method for species identification of ethanol-fixed and dry museum material as well as dropping samples.

Materials and Methods

Identification of two cryptic *Pipistrellus* species was performed using multiplex PCRs and agarose gel electrophoresis. Genomic DNA was extracted from the 96% ethanol-preserved tissue samples (one toe or 3 mm punch of dry wing membrane from museum specimens or a 3 mm punch of wing-membrane from living individuals). Tissues were digested by proteinase K and the DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, GmbH & Co.) according to the manufacturer's instructions. Primers PIP-F and PIP-R (Table 1) designed on the basis of known sequences from Hulva *et al*. (2004) were used for the amplification and sequencing of a 320 bp fragment of the cyt*b* gene in all individuals. The PCR reaction contained 0.5 mM of forward and reverse primers (PIP-F and PIP-R), 0.2 mM of each dNTPs, 1 × Taq DNA buffer (Fermentas), 3 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Fermentas), 2 µl of extracted DNA and ddH_2O to the volume of 25 µl. Purified PCR products were directly sequenced by using BigDye Terminators Sequencing Kit version 1.1 (Applied Biosystems). Sequencing reactions were analysed on an ABI Prism 3130 Genetic Analyser (Applied Biosystems). The sequences were aligned using the software ClustalW incorporated in the BioEdit package version 7.0.0 (Hall, 1999) and bat species were identified on the basis of homology with known haplotypes of both taxa described from central Europe (Hulva *et al*., 2004).

Table 1. Primers used in this study. PIP-F and PIP-R were designed on the basis of homology of known *Pipistrellus* sequences of the cyt*b* (F – forward, R – reverse) and they amplify a 320 bp fragment of this gene. The nucleotide position of the 5'-end of each internal forward primers specific to *P. pipistrellus* (Ppip-F and Ppip-F2) or to *P. pygmaeus* (Ppyg-F) is indicated in parentheses

Primer name	Sequence 5'-3'
PIP-F	CTCATTCATTGAYCTACCAGC
PIP-R	CAGCRAATAGTAAAATAACTCC
Ppip-F (114)	CATCTGTTTGGGACTACAGATCC
Ppip-F2 (210)	TCGAGATGTGAATTACGGATG
Ppyg-F (117)	CTGTCTAGGGCTGCAAAT

To test the specificity and efficacy of the multiplex PCR-based discriminating method we chose four museum ethanol-preserved specimens of *P. pipistrellus* and four of *P. pygmaeus* (Table 2). The strategy used to design the primers distinguishing both species was based on the search for regions with several species-specific substitutions. The fragment of 320 bp amplified with PIP-F and PIP-R was screened for sequences of 21–23 nucleotides characterised by zero, one or a maximum of two substitutions within species but differing by at least three mutations from the other species. The primers designed in those regions were then tested for species-specific amplification of the partial cyt*b* region. Primers PIP-F and PIP-R were added to the same pre-PCR mix and used as a control for successful PCR amplification. Once the species-specific primers were designed, we optimized the multiplex PCR reaction by testing various concentrations of primers and $MgCl_2$ and changing the annealing temperature. To verify the robustness of the species-identification method, the test was applied on biopsies from 16 randomly selected living individuals sampled in different localities across the Czech and Slovak Republics. From these bats, the characteristic terminal frequencies of echolocation calls (45 kHz for *P. pipistrellus*, 55 kHz for *P. pygmaeus*) were recorded using an ultrasound bat-detector D980 or a D240x (Pettersson Elektronik AB). We also used biopsies taken on older material, i.e., from 15 museum specimens of an age of 25–40 years (Table 2). Finally, we analysed fresh droppings obtained from seven trapped individuals identified by

TABLE 2. List of examined specimens (n = 46 individuals). M — male, F — female, ad — adult, juv — juvenile, OC — collection of the Regional Museum in Olomouc, IVB — collection of the Institute of Vertebrate Biology of the Academy of Sciences of the Czech Republic, * — PCR was not successful

Code	Species	Sex	Age	Locality	Date of collection
Specimens used to optimize PCR conditions of identification test					
PIP10	*P. pipistrellus*	M	ad	Hranická propast, CZ	June 9, 2001
PIP11	*P. pipistrellus*	M	ad	Hranická propast, CZ	June 27, 2004
PIP18	*P. pygmaeus*	F	ad	Křivé jezero, CZ	April 29, 2002
PIP133	*P. pipistrellus*	M	ad	Brno, CZ	August 20, 2004
PIP134	*P. pipistrellus*	F	ad	Brno, Královo pole, CZ	March 28, 2001
PIP138	*P. pygmaeus*	F	ad	Vranovice, CZ	May 19, 2004
PIP139	*P. pygmaeus*	F	ad	Vranovice, CZ	August 16, 2004
PIP141	*P. pygmaeus*	F	ad	Křivé jezero, CZ	May 16, 2005
Live specimens used to verify the robustness of the test					
DOL1	*P. pipistrellus*	F	ad	Slanská Huta, SK	May 30, 2006
NOV22	*P. pygmaeus*	F	ad	Novosedly, CZ	May 18, 2006
PIP102	*P. pipistrellus*	F	juv	Kramolín, CZ	July 6, 2003
PIP105	*P. pipistrellus*	F	juv	Bučovice, CZ	July 3, 2003
PIP15	*P. pygmaeus*	F	ad	Břeclav, CZ	July 8, 2003
PIP16	*P. pygmaeus*	F	juv	Hlohovec, CZ	July 8, 2003
PIP22	*P. pygmaeus*	F	ad	Výškovice, CZ	July 11, 2004
PIP36	*P. pygmaeus*	F	ad	Lednice, CZ	July 16, 2004
PIP55	*P. pipistrellus*	F	ad	Žižkov, CZ	July 7, 2003
PIP78	*P. pipistrellus*	M	juv	Ruské, SK	August 24, 2004
PIP82	*P. pipistrellus*	F	juv	Adamov, CZ	August 19, 2003
PIP92	*P. pipistrellus*	F	ad	Ledce, CZ	June 13, 2004
SEČ7	*P. pipistrellus*	F	ad	Sečovská Polianka, SK	May 29, 2006
SNM23	*P. pipistrellus*	F	ad	Slanské Nové Mesto, SK	May 30, 2006
SOL25	*P. pipistrellus*	F	ad	Doľany, SK	May 24, 2006
TYN30	*P. pygmaeus*	F	ad	Týnec, CZ	May 11, 2006
Museum specimens used to verify the applicability of the test					
OC Zo-3518	*P. pipistrellus*	M	ad	Šternberk, CZ	February 16, 1966
OC Zo-3519	**Pipistrellus* sp.	F	ad	Šternberk, CZ	February 16, 1966
OC Zo-3625	*P. pipistrellus*	F	ad	Šternberk, CZ	December 29, 1966
OC Zo-4528	**Pipistrellus* sp.	M	ad	Šternberk, CZ	January 30, 1978
OC Zo-4529	**Pipistrellus* sp.	M	ad	Šternberk, CZ	January 30, 1978
OC Zo-4533	**Pipistrellus* sp.	F	ad	Přerov, CZ	June, 26, 1978
OC Zo-4534	**Pipistrellus* sp.	F	ad	Přerov, CZ	June, 26, 1978
OC Zo-4577	**Pipistrellus* sp.	F	ad	Rájec, CZ	July 21, 1977
OC Zo-4620	*P. pipistrellus*	M	ad	Přerov, CZ	July 30, 1978
OC Zo-4644	*P. pipistrellus*	M	ad	Šternberk, CZ	February 2, 1980
OC Zo-5059	*P. pipistrellus*	M	ad	Šternberk, CZ	February 8, 1984
IVB 1.1.47	*P. pipistrellus*	M	ad	Brno, CZ	January 6, 1970
IVB 1.1.98	*P. pipistrellus*	M	ad	Brno, CZ	May 17, 1971
IVB 1.3.16	*P. pipistrellus*	M	ad	Brno, CZ	April 2, 1976
IVB 1.4.1	*P. pipistrellus*	M	ad	Brno, CZ	February 5, 1979
Samples used to test DNA extraction from droppings					
SOL39	*P. pipistrellus*	F	ad	Doľany, SK	June 28, 2006
SOL41	*P. pipistrellus*	F	ad	Doľany, SK	June 28, 2006
SOL43	*P. pipistrellus*	F	ad	Doľany, SK	June 28, 2006
SEČ9	*P. pipistrellus*	F	ad	Sečovská Polianka, SK	July 3, 2006
RUS12	*P. pipistrellus*	F	ad	Ruské, SK	July 4, 2006
BLA16	*P. pygmaeus*	M	juv	Bratislava, SK	July 27, 2006
BLA21	*P. pygmaeus*	F	juv	Bratislava, SK	July 27, 2006

their ultrasound recordings (Table 2). One to four droppings from each individual were collected into tubes containing silica gel and stored at room temperature. Faecal DNA extraction was performed within three weeks of collection using the QIAamp DNA Stool Mini Kit (Qiagen), following the manufacturer's protocols with slight modifications, i.e. time of incubation at final elution step was increased from 1 to 5 min, and elution performed in two steps, each with 100 µl EA buffer (this can increase the amount of DNA eluted from column membrane).

We selected two reliable combinations of primers (Table 1) that produced species-specific bands in the set of individuals. The first combination (Fig. 1A) used 0.3 µM of external forward primer (PIP-F), 0.6 µM of external reverse primer (PIP-R), 0.4 µM of internal forward primer Ppip-F, 0.2 mM of each dNTPs, 1 × Taq DNA buffer (Fermentas), 3 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Fermentas), 1.0 µl of extracted DNA and ddH_2O to the volume of 10 µl. The second combination (Fig. 1B) contained 0.1 µM of PIP-F, 0.8 µM of PIP-R, 0.5 µM of Ppyg-F, 0.2 µM of Ppip-F2 and the same amount of other chemicals as the first combination. All amplifications were performed in a thermal cycler (Mastercycler, Eppendorf, AG) using the following thermal cycling parameters: predenaturation (94°C, 3 min), 35 cycles of denaturation (94°C, 30 s), annealing (61°C, 30 s) and extension (72°C, 30 s), with a final extension (72°C, 4 min). PCR conditions for amplification of DNA from dry museum specimens were the same, with 3.0 µl of extracted DNA in the total reaction volume of 10 µl. For DNA extracted from droppings, the PCR mix contained 2.5 mM $MgCl_2$ and 0.3 U of HotMaster Taq DNA polymerase (Eppendorf) plus 3.2 ng of BSA and we used 3.0 µl of extracted DNA in 10 µl reaction. Cycling parameters were used following Hájková *et al*. (2006), i.e., predenaturation (94°C, 2 min), 35 cycles of denaturation (94°C, 30 s), annealing (61°C, 30 s) and extension (65°C, 60 s) and a final extension (65°C, 10 min). To exclude amplifications in morphologically closely related species (*Pipistrellus kuhlii* and *P. nathusii*) we also examined sequences of cyt*b* of these species accessible in GenBank and applied the PCR test on three dry museum individuals of each species.

Results

Each of two multiplex PCRs allowed clear non-destructive and even non-invasive identification of both species. In the first combination of primers (A), one specific band and one band of positive control (320 bp) were visible in *P. pipistrellus* and only the positive control band in *P. pygmaeus* (Fig. 1A). In the second combination (B), the positive control (320 bp) was visible in both species, but the species differed by the length of the specific fragment (approx. 160 bp in *P. pipistrellus* and 230 bp in *P. pygmaeus* — Fig. 1B). The same results were achieved with the optimised protocols on biopsies from all 16 living animals which were identified by their echolocation calls and on nine of the 15 museum specimens (Table 2). In the remaining six museum specimens, the PCR did not work probably due to degradation of DNA prior to the experiment. PCR amplification of faecal DNA from all seven individuals was successful and results were again consistent with species identification by ultrasound criteria. Both primer combinations gave identical results in all analysed individuals. Cross-amplification of both primer combinations in *P. nathusii* and *P. kuhlii* museum specimens did not produce any band, suggesting exclusive applicability of these tests to discriminate only *P. pipistrellus* and *P. pygmaeus*. Similarly, when we aligned sequences of these congeneric species, there were 3–9 mutations inside the each primer sequence. It corroborates negative amplification of species-specific primers between *P. pipistrellus* and *P. pygmaeus* (there are 3–5 mutations only — Table 3).

Table 3. Number of substitutions in sequences complementary to primer sequences used in this study in four morphologically similar species of the genus *Pipistrellus*. Cyt*b* sequences of *P. pipistrellus* and *P. pygmaeus* originated from Hulva *et al*. (2004) and *P. kuhlii* and *P. nathusii* from the GenBank (Accession Nos. AJ504444, AJ504446, DQ120848–49)

Primer	*pipistrellus*	*pygmaeus*	*kuhlii*	*nathusii*
PIP-F	0–1	0–2	3–4	2
PIP-R	0–2	0	4–5	4
Ppip-F	0–1	5	7–8	9
Ppip-F2	0–1	3–4	3–5	4
Ppyg-F	4–5	0	4–5	5

Discussion

The results indicate that living pipistrelles or samples obtained from bat droppings can be reliably identified. Our method requires only a small piece of wing-membrane taken from living animals, and with some limitations can be adapted also to determine old museum specimens. As Vege and McCracken (2001) already suggested, the use of faecal DNA in bats is promising, since this sampling technique does not harm or stress the animals. Droppings can be easily collected during trapping or other manipulation of captured animals. After further testing, the method can possibly also be extended to samples obtained without contact with the animal (e.g., fresh droppings from day roosts). In conclusion, the presented method is reliable and easy to perform for routine identification in various field studies. Thanks to negative PCR results (without any bands) in tested individuals of *P. kuhlii* and *P. nathusii*, the method can certainly be used in the field. The application of the tests can increase the quality of faunal inventories for this common species-complex in central Europe, especially in regions where studied species occur in sympatry. Theoretically, based on a low number of mutations in sequences where the primers are designed, the test can also be successfully used in other populations (Table 3). Hence, it could help in the investigation of the species-specific ecological requirements, and in general, it can be widely used in studies concerning the ecology and evolution of cryptic vertebrate species (cf. Barlow and Jones, 1999; Davidson-Watts and Jones, 2006; Nicholls and Racey, 2006; Racey *et al*., 2007).

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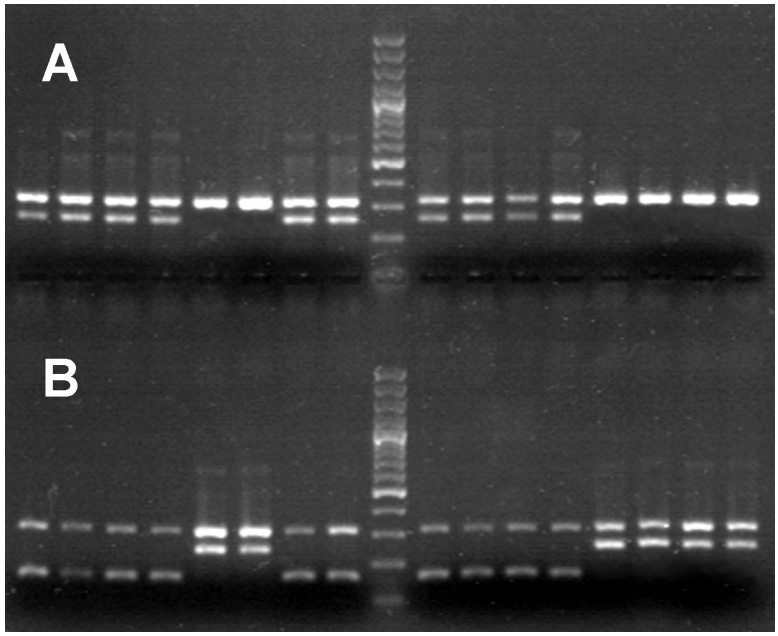

Fig. 1. Species-specific amplification of cyt*b* fragments of *P. pipistrellus* (lanes 1–4, 7–12) and *P. pygmaeus* (lanes 5–6, 13–16). DNA was amplified with external primers PIP-F, PIP-R and species-specific internal primers Ppip-F (A) or Ppip-F2 + Ppyg-F (B). PCR products were separated on 1.5% agarose gel and visualised by ethidium bromide

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