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Molecular identification of an endemic Alpine mammal, *Apodemus alpicola*, using a PCR-based RFLP method

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Molecular identification of an endemic Alpine mammal, *Apodemus alpicola*, using a PCR-based RFLP method. - The ability of a PCR-based restriction fragment length polymorphism (RFLP) analysis of the cytochrome *b* (mtDNA) to distinguish *Apodemus alpicola* from two other *Apodemus* species was investigated. The partial sequencing of the cytochrome *b* allowed the identification of one enzyme as being potentially diagnostic. This was supported by an analysis of 131 specimens previously identified using morphometric and/or allozymic data, indicating that the PCR-based RFLP method provides a rapid and reliable tool for distinguishing *A. alpicola* from its two co-occurring congenerics. The method is applicable to samples taken in the field for ecological studies, and could easily be adapted to the identification of museum samples.

Key-words: *Apodemus* - mtDNA - PCR - RFLP - identification.

INTRODUCTION

The species *Apodemus alpicola* Heinrich, 1952 is endemic to the Alps, often being found in sympatry with *A. sylvaticus* (Linnaeus, 1758) and *A. flavicollis* (Melchior, 1834). *A. alpicola* was originally considered as a high-altitude subspecies of *A. flavicollis* (Heinrich, 1951, 1952) and later described as a new species by Storch and Lütt (1989) based on morphological criteria. A biochemical confirmation was given by Vogel *et al.* (1991) and Filippucci (1992). In certain regions the overlap of the phenotypes is important (Yoccoz, 1992). Thus, the recognition of *A. alpicola* as a new species with some intermediate characteristics does not facilitate the identification problem, particularly when juvenile individuals are concerned.

Multivariate skull morphometrics separates 97 % of adult specimens (Reutter *et al.*, 1999). While this technique is indeed a good tool to identify museum material, it does not solve the problem of identifying juveniles and living individuals during

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field studies. Protein electrophoresis has proved to be more useful to distinguish the three *Apodemus* species without the need to sacrifice individuals and is applicable to young specimens (Reutter *et al.*, 2001). However, it requires to use fresh or frozen blood samples.

Although morphologic (Storch & Lütt, 1989; Spitzenberger & Englisch, 1996; Reutter *et al.*, 1999), karyotypic (Reutter *et al.*, in press) and allozymic studies (Vogel *et al.*, 1991; Filippucci, 1992; Filippucci *et al.*, 1996) have provided a new perspective on the systematics of *A. alpicola*, it does not solve the problem of the identification of living young animals in the field, a point that is important within the frame of population monitoring or ecological studies. Therefore, a technique is needed which is based on non-destructive sampling, small amounts of biological material, and which leads to a reliable identification of adult as well as young animals. A suitable candidate is the PCR-based restriction fragment length polymorphism technique (RFLP). This technique consists of three steps. First, a suitable part of the genome is amplified through polymerase chain reaction (PCR). Second, the PCR product is digested using endonucleases, which, for the purpose of species identification, should cut the amplified DNA fragment at different sites in the different species. Third, the digested DNA fragments are separated by electrophoresis and visualised (various staining protocols are available), revealing the restriction patterns. It is worth noting here that this technique requires only minute amount of DNA.

Previous surveys of total mitochondrial DNA variability have revealed that *A. sylvaticus* and *A. flavicollis* show much higher inter- than intraspecific variation (Tegelström & Jaarola, 1989; Michaux *et al.*, 1996, Michaux *et al.*, 1998). Furthermore, a recent analysis of cytochrome *b* sequences indicated that the divergence between *A. alpicola* and each of the two other species equals the divergence between *A. sylvaticus* and *A. flavicollis*, which is approximately 10 % (Martin *et al.*, 2000). Therefore, owing to the availability of cytochtome *b* sequences for various *Apodemus* species (Martin *et al.*, 2000), this gene was chosen to investigate the ability of mitochondrial DNA polymorphisms to discriminate *A. alpicola* from the other sympatric species. Such a technique could not only be used in the identification of living animals in the field, but also for museum specimens preserved in ethanol.

MATERIAL AND METHODS

Specimens Examined

Tissues samples (frozen liver or toe-clips) were obtained from a total of 131 specimens (46 *A. sylvaticus*, 45 *A. flavicollis*, and 40 *A. alpicola*) from 15 localities in Switzerland, France, Italy, Germany and Austria. The localities sampled (Fig. 1) cover more or less the range of the three *Apodemus* species in the alpine region. Sample sizes and locality names are indicated in Tab. 1. All specimens were assigned to species using skull morphology (Reutter *et al.*, 1999) and/or protein electrophoresis (Vogel *et al.*, 1991; Reutter *et al.*, 2001).

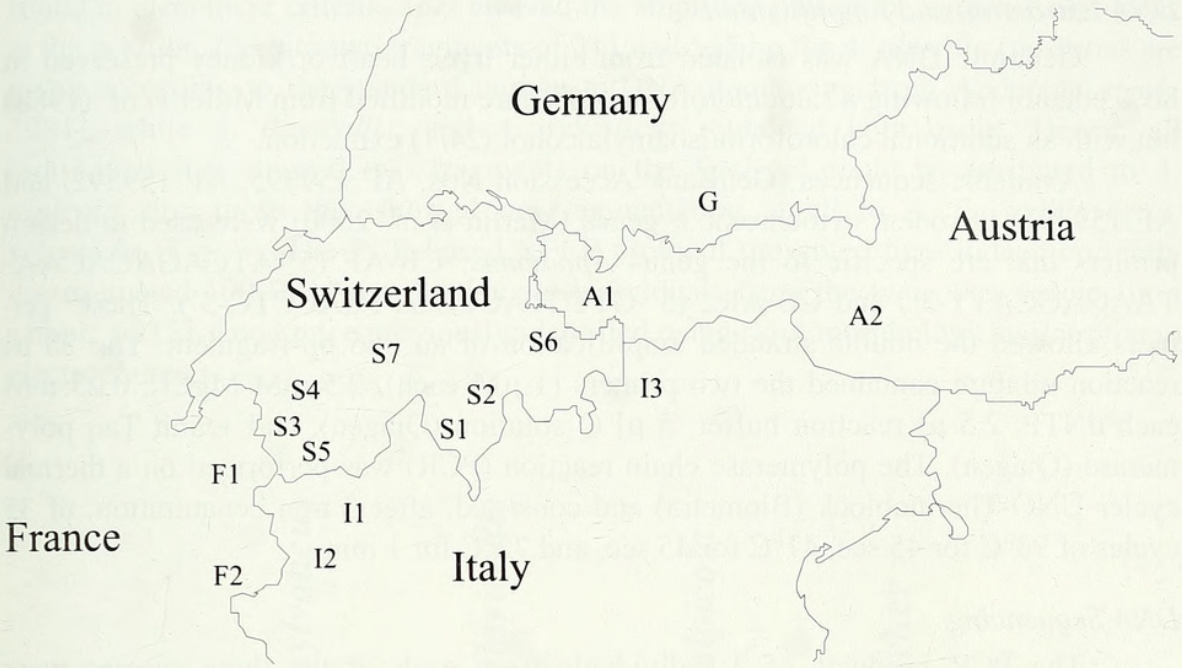

FIG. 1
Geographic distribution of the sampled localities.

TABLE 1
A survey of material examined (sampling localities and number of individuals). All specimens were previously identified using morphometric [(m)] and/or allozymic [(a)] methods.

Locality (map symbol)	*A. sylvaticus*	*A. flavicollis*	*A. alpicola*
Switzerland			
Gordevio TI (S1)		4 (a)	
Prosito TI (S2)	3 (a)		
Martigny VS (S3)		4 (a)	
Sanetsch VS (S4)	1 (a)	1 (a)	6 (a)
Bourg St. Bernard VS (S5)	1 (m)	2 (m)	2 (m)
Chur GR (S6)	10 (m)	10 (m)	
Haslital BE (S7)	3 (a)	1 (a)	2 (m)
Germany			
Garmisch (G)		6 (a)	
Austria			
Silbertal (A1)	1 (a,m)	2 (a,m)	9 (m)
Hohe Tauern (A2)		5 (m)	7 (m)
Italy			
Valle d'Aosta (I1)	7 (a,m)	1 (a)	3 (a,m)
Gran Paradiso NP (I2)	7 (m)	7 (m)	2 (m)
Vinschgau (I3)	8 (m)	1 (m)	5 (m)
France			
Morzine (F1)		1 (m)	
Mt. Cenis (F2)	5 (m)		4 (m)

DNA Extraction and Amplification

Genomic DNA was isolated from either liver, heart or kidney preserved in 80% ethanol following a salt/chloroform procedure modified from Miller *et al.* (1988) but with an additional chloroform/isoamylalcohol (24/1) extraction.

Available sequences (GenBank Accession Nos. AF 159395, AF 159392, and AF 159391) of rodent cytochrome *b* genes (Martin *et al.* 2000) were used to design primers that are specific to the genus *Apodemus*: CB-AF (5'-ATCAGACACAA-TAACAGCATT-3') and CB-AR2 (5'-GTTCTACTGGTTGACCTC-3'). These primers allowed the double stranded amplification of an 866 bp-fragment. The 25 µl reaction mixture contained the two primers (1 µM each), 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 2.5 µl reaction buffer, 5 µl Q solution (Qiagen), and 1 unit Taq polymerase (Qiagen). The polymerase chain reaction (PCR) was performed on a thermal cycler UNO-Thermoblock (Biometra) and consisted, after 3 min denaturation, of 35 cycles of 93°C for 45 sec, 47°C for 45 sec, and 72°C for 1 min.

DNA Sequencing

The PCR products of 3 individuals from each of the three species were sequenced in order to identify restriction enzymes that would be potentially diagnostic, i.e. with interspecific but no intraspecific variability. PCR products were first purified using the Qiaquick purification kit (Qiagen) according to the manufacturer's instructions. The purified products were then sequenced with the Dye Mix 2.0 sequencing kit (Perkin Elmer). The reactions were carried out in a 10 µl volume consisting of 0.5 µM primer, 4 µl Dye mix, and 5.5 µl PCR product (which corresponds to 30-70 ng DNA). The sequencing reaction consisted, after 3 min denaturation, of 25 cycles of 96°C for 20 sec, 50°C for 15 sec, and 60 °C for 4 min. Sequences were then precipitated and run on a 6% polyacrylamide gel on an ABI 373 sequencer (Perkin Elmer). Each PCR product was sequenced using the two primers CB-AF and CB-AR2 in order to sequence both strands. The alignment of sequences and search for restriction sites were carried out using Sequencher 3.0 (Gene Codes Corp.).

Digestion with Restriction Enzyme

The enzyme *SpeI* was identified as being potentially diagnostic to discriminate *A. alpicola* from the other two species (see Results), and tested on 131 individuals. Each of these samples were analysed with *SpeI* according to the following protocol: 10 µl of the amplified DNA were digested in a 25 µl reaction mixture comprising 2.5 units *SpeI* (A//CTAGT) (Life Technologies) and 2.5 µl REACT®4, the digestion buffer, according to the manufacturer's instructions. The digested samples were subsequently run on a 1% agarose (BioRad) gel (120x200 mm, 150 ml) during 1-2 h at 120 V, and stained with ethidium bromide.

RESULTS

For each of the three species, three individuals taken from alpine populations were sequenced in order to identify restriction sites that are fixed within species and variable between species in the area of sympatry. A site cut by the enzyme *SpeI* was

found to meet these criteria. *SpeI* cleaved the amplified part of the cytochrome *b* gene at the position 723 into two fragments of 311 and 555 bp for *A. alpicola* (positions are given according to the standard human mtDNA numbering from Anderson *et al.*, 1981), while *A. flavicollis*, and *A. sylvaticus* remained both uncut. Hence, all individuals that showed two fragments on the *SpeI*-gel could be attributed to *A. alpicola*, and those that showed no fragmentation at all to *A. flavicollis* or *A. sylvaticus* (Fig. 2). The PCR-based RFLP protocol presented here indeed correctly discriminated 100 % of the *A. alpicola* individuals from the two other species in a sample of 131 wood mice previously identified using skull morphology and/or protein electrophoresis.

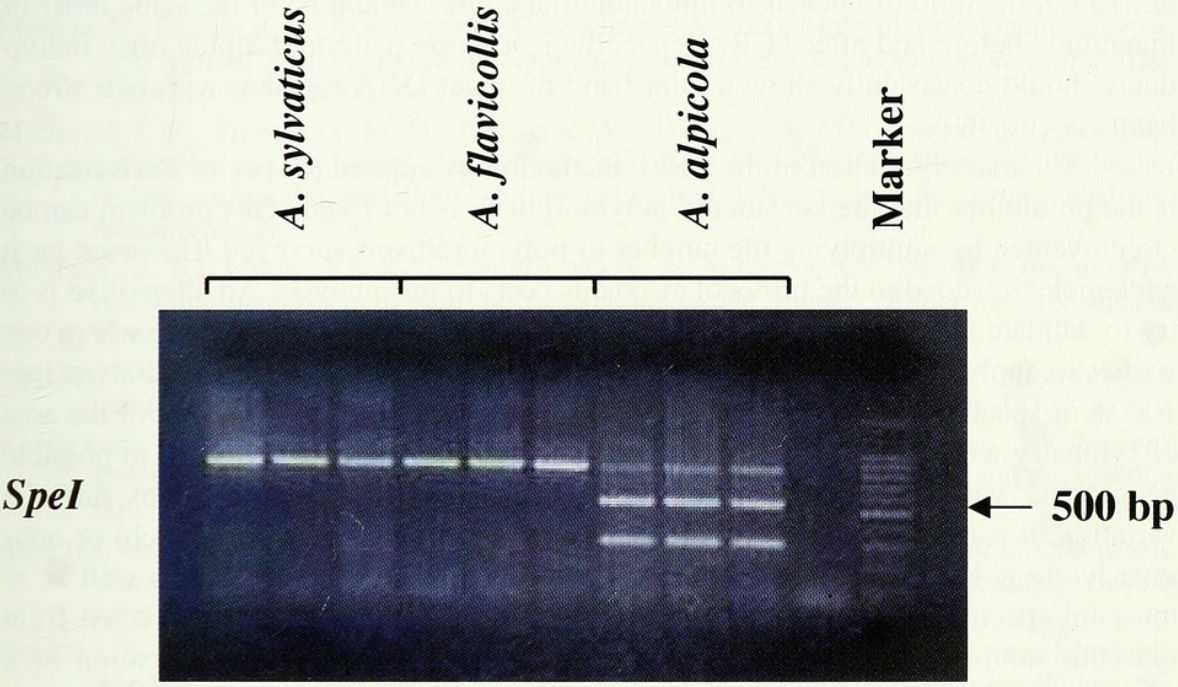

FIG. 2

Representative examples of fragment patterns after *SpeI* endonuclease digestion of an 866 bp fragment of the cytochrome *b* gene. *Apodemus alpicola* showed two fragments after *SpeI* digestions, *A. flavicollis*, and *A. sylvaticus* remained uncut.

DISCUSSION

To our current knowledge, the alpine mouse *Apodemus alpicola* is the only mammal endemic of the Alps. For promoting its conservation, a better knowledge of its status and ecology is needed. However, any progress is currently impeded by the problem of identification, which concerns the three sympatric species *A. sylvaticus*, *A. flavicollis* and *A. alpicola*. Our initial aim was therefore to provide mammalogists and ecologists with an identification technique based on non-destructive sampling that would permit to discriminate *A. alpicola* from the other two wood mouse species in

the field. The here presented PCR-based RFLP method fulfil this condition, allowing a reliable identification of *A. alpicola*.

One potential problem of the method is that, as seen on Fig. 2, a faint band of uncut DNA is visible in all cases. Rather than an excess of DNA, this probably results from the coamplification of a nuclear copy of the cytochrome *b*, which has lost the restriction site used in this study, together with the targeted original mitochondrial cytochrome *b* (Zhang & Hewitt, 1996). One may wonder whether such a pattern could be reversed in *A. alpicola* (a strong band of uncut DNA and hardly visible bands of digested DNA), leading to a pattern that would be difficult to interpret. First, this reversed pattern was never observed in our sample of 40 alpine mice. Second, each cell contains only two copies of each nuclear gene for several thousands copies of mitochondrial DNA. Because the PCR mechanism is actually linear (Rameckers *et al.*, 1997), the ratio of nuclear to mitochondrial copies should be of the same order of magnitude before and after PCR. Hence, the restriction pattern of alpine mice individuals should consistently show a faint band of uncut DNA together with two strong bands of cut DNA.

The main limitation of the RFLP methodology applied to species identification is the possibility that the considered polymorphism is not fixed. This problem can be circumvented by multiplying the number of polymorphisms surveyed. However, each endonuclease added to the protocol also adds costs to the analysis. An alternative is to try to validate the method using samples that are representative of the area where one wishes to apply it. We chose the second option, and the results are rather convincing: 100 % of specimens that originate from 15 localities covering about 75 % of the area of sympatry were correctly discriminated. The method is therefore robust to possible geographic variation, and, because it is based on PCR technology of mtDNA variation, it can be applied to slightly invasive (e.g. toe clipping, ear punch) or non-invasive (e.g. hair follicles) ecological samples (Taberlet *et al.*, 1999), as well as to museum specimens (Thomas *et al.*, 1990). However, because DNA extracted from museum samples is usually degraded, primers that lead to the amplification of a shorter piece of cytochrome *b* around the restriction site could be designed to increase the probability of successful amplification.

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