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PRIMER NOTE

# Molecular identification of three sympatric species of wood mice (*Apodemus sylvaticus, A. flavicollis, A. alpicola*) in western Europe (Muridae: Rodentia)

J. R. MICHAUX,*† S. KINET,† M.-G. FILIPPUCCI,‡ R. LIBOIS,§ A. BESNARD† and F. CATZEFLIS†
**Unité de recherches zoogéographiques, ULg, Quai Van Beneden, 22; 4020 Liège, Belgique, †Labo. Paléontologie, cc064, ISEM, 34095 Montpellier cedex 05, France, ‡Dipartimento di Biologia, Universita di Roma 'Tor Vergata', Rome 00173, Italy, §Museu Nacional de Historial Natural, rua da Escola politecnica, 58, 1269–102 Lisboa, Portugal*

## Abstract

**The woodmouse (*Apodemus sylvaticus*) and yellow-necked fieldmouse (*Apodemus flavicollis*) are sympatric and even syntopic in many regions throughout their European range. Their field discrimination on the basis of external characters is a real challenge for many fields of research. The problem is even more complicated in the Alpine chain where they live sympatrically with a third similar species: *A. alpicola*. A rapid and simple method is proposed to discriminate the three species in processing field-collected biopsies as well as ethanol-preserved museum samples.**

*Keywords*: *Apodemus*, cytochrome *b*, molecular typing, species-specific primers

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The woodmouse (*Apodemus sylvaticus*) and yellow-necked fieldmouse (*Apodemus flavicollis*) are very common mammals in Europe. They are widespread in many western and central European countries although *A. flavicollis* is not found in the southern Iberian peninsula, western France, northern Belgium and the Netherlands (Montgomery 1999). As their ecological preferences overlap, they are sympatric and even syntopic (living in the same collecting locality) in many regions throughout their range. In central Europe, most adults can be easily discriminated, whereas elsewhere their phenotypes are often quite similar (Niethammer 1978), rendering problematic field discrimination. This is particularly true in the southern regions (northern Spain and continental Italy) (Niethammer 1978; Filippucci *et al*. 1984). The difficulty in discriminating the two species on the basis of external characters is a real challenge for ecological, behavioural, epidemiological and population management research. The problem is even more complicated in the Alpine chain where these two mice live sympatrically with a third similar species: *A. alpicola*, the Alpine mouse. *A. alpicola* resembles *A. flavicollis* to such a high degree that it was previously considered to be a subspecies of the latter (Musser *et al*. 1996).

Although the three *Apodemus* species can be distinguished by skull morphometry (Niethammer 1978; Storch & Lütt 1989) or by biochemical analyses (Gemmeke 1981; Vogel *et al*. 1991; Filippucci 1992), these techniques require either for the specimens be killed or the use of heavy field equipment (i.e. special freezer) for tissue sample processing and analysis. New methods are therefore needed for processing field-collected biopsies as well as ethanol-preserved museum samples. This paper reports the design of species-specific primers from the mitochondrial cytochrome *b* gene that allows these three *Apodemus* species to be rapidly and simply distinguished.

Tissue samples were obtained from four collections, J.R. Michaux collection (JRM-numbers; deposited at the University of Liège, Liege, Belgium), the tissue collection of mammals of Montpellier (Catzeflis 1991; T-numbers); the Natural History Museum of Nancy, France (MHNN-numbers) and the tissue collection of M.-G. Filippucci (other codes; deposited in Rome, Italy). DNA extraction from 95% ethanol-preserved tissues of four *A. sylvaticus* and four *A. flavicollis* (Table 1) was performed according to Sambrook *et al*. (1989). These animals were previously identified by

Correspondence: J. Michaux. †Labo. Paléontologie, cc064, ISEM, 34095 Montpellier cedex 05, France. Fax: 00 33 4 67 36 10; E-mail: johan@isem.univ-montp2.fr

**Table 1** References of *Apodemus* tissues used for the experiments

| Species | Tissue sample | Geographic origin | Accession numbers |
|---|---|---|---|
| *Apodemus sylvaticus* | For cytochrome *b* sequence comparisons | | |
| | JMR-101 | Belgium (Ardennes) | AJ298598 |
| | JMR-103 | Belgium (Ardennes) | AJ298599 |
| | JMR-269 | France (Pyrenées-Orientales) | AJ298600 |
| | ROM-1 | Italy (Rome) | AJ298601 |
| | | Holland (Leiden) | AB033695 |
| | For typing tests | | |
| | T-1684, T-2141, T-2142, T-2143, T-2144 | Spain (Navarre) | — |
| | MGF-231, MGF-171, MGF-181, DG, DL | Italy (Abruzzes) | — |
| | Sem12 | Italy (Calabria) | — |
| | JMR-172 to JRM-176 | Italy (Tarquinia) | — |
| | ROM-1 | Italy (Rome) | — |
| | JRM-383 | Austria (Innsbrück) | — |
| | BIE-22, BIE-23, BIE-31 | Germany (Bielefeld) | — |
| | JRM-515 to JRM-519 | Germany (Dresde) | — |
| | JRM-101 to JRM-109 | Belgium (Ardennes) | — |
| | JRM-142, JRM-143 | France (Cap Lardier) | — |
| | JRM-269 to JRM-272, JRM-277, JRM-278, JRM-297, JRM-298, JRM-299 | France (Pyrénées-Orientales) | — |
| | T-665 | France (Allier) | — |
| | JRM-396 | France (Correze) | — |
| *Apodemus flavicollis* | For cytochrome *b* sequence comparisons | | |
| | JMR-332 | Belgium (Gembes) | AJ298602 |
| | T-666 | France (Allier) | AJ298603 |
| | BIE-26 | Germany (Bielefeld) | AJ298604 |
| | ASP-23 | Italy (Aspromonte) | AJ298605 |
| | | Switzerland (Champery) | AB032853 |
| | | Konstanz (Germany) | AF159392 |
| | For typing tests | | |
| | T-1685, T-2137 to T-2140 | Spain (Navarre) | — |
| | PEN-340, PEN-53, PEN-br, PEN-3, PEN-B7 | Italy (Abruzzes) | — |
| | ASP-12, ASP-21 to ASP-24 | Italy (Calabria) | — |
| | VOR-3 to VOR-5 | Austria (Vorarlberg) | — |
| | JRM-332, JRM-385, JRM-386 | Belgium (Ardennes) | — |
| | T-566, T-567 | Switzerland (Genève) | — |
| | UPS-29, UPS-16, UPS-23, JRM-447 to JRM-449, | Sweden (Uppsala) | — |
| | JRM-421 to JRM-424, | Slovenia (Kosjave) | — |
| | JRM-391, JRM-392 | France (Aude) | — |
| | T-666, T-667 | France (Allier) | — |
| | JRM-393 to JRM-395, JRM-397 | France (Correze) | — |
| | MHNN-9616 | France (Bellefontaine, Nancy) | — |
| | MHNN-9581K | France (Bure, Nancy) | — |
| | JRM-399 | France (Vercors) | — |
| | BIE-26 | Germany (Bielefeld) | — |
| | JRM-508 to JRM-510 | Germany (Leipzig) | — |
| | JRM-511, JRM-512 | Germany (Dresde) | — |
| *Apodemus alpicola* | For cytochrome *b* sequence comparisons | | |
| | — | Switzerland | AF159391 |
| | — | (Suzuki *et al*. 2000) | AB032854 |
| | For typing tests | | |
| | VOR-1, VOR-6, JRM-400 | Austria (Vorarlberg) | — |
| | JMR-136, JRM-385 | Suisse (Valais) | — |
| | JRM-398, JRM-399 | France (Savoie) | — |
| *Apodemus uralensis* | For typing tests | | |
| | JRM-257 | Czech Republic (Boheme) | — |
| | JRM-289, JRM-290, JRM-283, JRM-284 | Russia (Moscow region) | — |

enzymatic electrophoresis or morphological characters (Michaux and Filippucci, unpublished data).

A cytochrome *b* fragment (1000 bp) was amplified using the Universal polymerase chain reaction (PCR) primers L7 (5′-ACCAATGACATGAAAAATCATCGTT-3′) and H16 (5′-ACATGAATYGGAGGYCAACCWG-3′) (Kocher *et al*. 1989). Amplification reactions were carried out in 100 µL volumes including 25 µL of each 2 µM primer, 20 µL of 1 mM dNTP, 10 µL of 10× reaction buffer, 10 µL of purified water and 0.02 µL of 5 U/µL Promega taqDNA polymerase. Ten µL of a 1/40 dilution (200 ng) of DNA extract was used for a PCR amplification. All PCRs were performed for 33 cycles (20 s at 94 °C, 30 s at 50 °C and 1 min 30 s at 68 °C) plus 10 min at 68 °C, in a Labover PTC100 thermal cycler. PCR products were purified using the Ultra-free DNA Amicon kit (Millipore) and directly sequenced. Sequencing on both strands was carried out using a Big dye terminator sequencing kit (Perkin Elmer) and an ABI 310 automatic sequencer (Perkin Elmer).

Cytochrome *b* fragments of 971 bp were obtained for all animals, and have been deposited in the EMBL GenBank under accession numbers AJ298598–AJ298605. These were aligned and compared with sequences available in GenBank: *A. sylvaticus* (AB033695), *A. alpicola* (AF159393, AB033695) and *A. flavicollis* (AB032853, AF159392) using the ED editor (MUST package, Philippe 1993).

Using all aligned sequences and taking site variations into account, a pair of specific primers was designed for each species. The three pairs of primers contain the nucleotidic characteristics (in bold type) of each targeted species. Numbers in parentheses indicate the position of the primers with regard to the complete cytochrome *b* gene sequence of *Mus musculus* (Bibb *et al*. 1981):

SylUP: 5′-GAGG**A**GGATTCTCAGTAGA**C**-3′ (539)
SylDN: 5′-TTAAT**A**TG**G**GGTGG**G**GT**G**TT**A**-3′ (834)
FlaUP: 5′-AGCTACA**CTA**ACACGTTT**C**-3′ (561)
FlaDN: 5′-GC**G**TA**T**GCAAATAGGAA**G**TA**C**-3′ (864)
AlpUP: 5′-TAACAGCATTCTCTTCAGT**C**CAC**A**-3′ (224)
AlpDN: 5′-TATGG**G**AT**A**GCTGATA**G**TA**AG**-3′ (489)

The efficiency of this molecular discrimination was tested on 48 *A. sylvaticus* specimens, 50 individuals of *A. flavicollis* all coming from across western Europe and seven *A. alpicola* specimens (Table 1). These samples were previously identified by morphological or enzymatic characters (Lymberakis, Filippucci, Macholan, Catzeflis and Michaux, unpublished data). Each individual was tested with the three pairs of specific primers. Amplification reactions and cycle profiles were the same as indicated above, with the exception of an annealing temperature of 58 °C. When amplification was positive, specific primers amplified 265–290 bp fragments. A complete concordance between the molecular typing and the previous morphological and biochemical identifications is observed (Fig. 1).

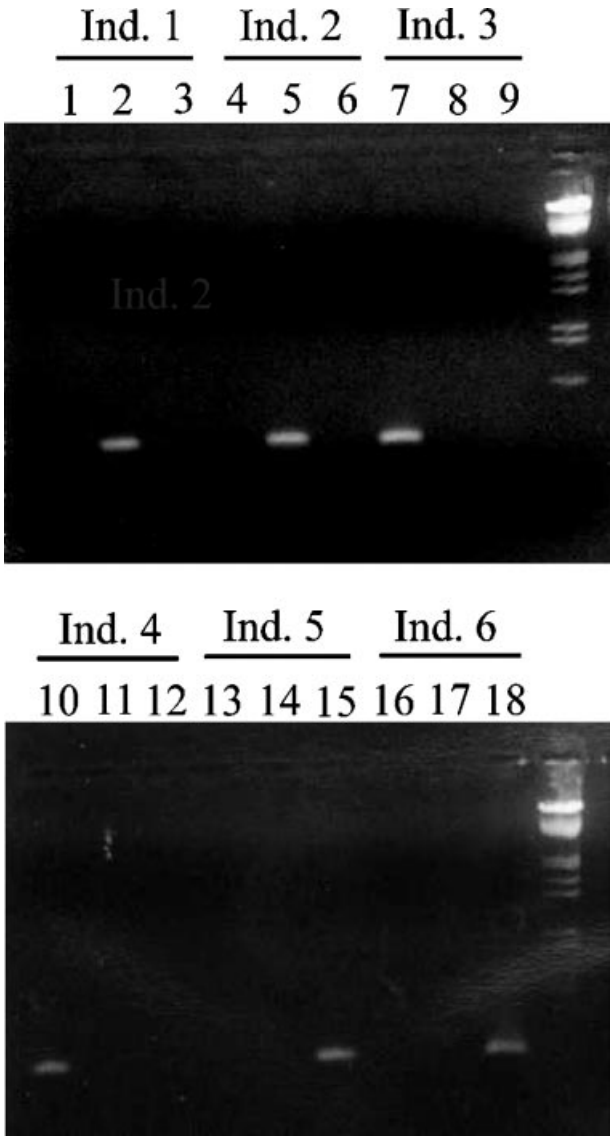


**Fig. 1** species-specific amplification of cytochrome *b* in *Apodemus* specimens. DNA was extracted from *Apodemus* individuals and amplified with primers specially designed for *A. flavicollis* (FLAUP and FLADN, lanes 2, 5, 8, 11, 14 and 17), *A. sylvaticus* (SYLUP and SYLDN, lanes 1, 4, 7, 10, 13 and 16) and *A. alpicola* (ALPUP and ALPDN, lanes 3, 6, 9, 12, 15 and 18). Examples of six individuals demonstrate the specificity of this technique.

Fewer numbers of *A. alpicola* specimens were used because of their great rarity and difficulties in trapping them. However, as the genetic variability is low within this species (Vogel *et al*. 1991; Filippucci 1992; Filippucci, unpublished data) and as the tested animals were collected throughout the distribution area, this sampling is likely to be a good representation of the species.

These data indicate that any specimen of the three west European *Apodemus* species can be confidently identified

using the present method. Moreover, as our approach requires only small quantities of total DNA, it can be performed on a small piece of ear or tail fragment that can be taken from live animals, making it available for routine identification in field studies. Although the amplified fragment sizes are rather long (265–290 bp; it was impossible to define specific primers for smaller fragments), the test has also been successfully performed on eight museum specimens preserved in ethanol for 25 (Filippucci collection), 64 and 72 (Heim de Balsac collector, Nancy Museum collection) years. Thus, this test is feasible as long as the samples were not previously treated with formol.

Finally, although the aim of our study was based on west European *Apodemus*, control tests were performed with each of the three pairs of specific primers on five specimens of *A. uralensis* ( JRM-257; JRM-267; JRM-283; JRM-284; JRM-289), a fourth similar woodmouse species living in central Europe and Russia. Importantly, the PCR results were always negative, further suggesting the specificity of this approach.

In conclusion, the feasibility of this method for discriminating between *Apodemus* appear very promising.

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## References

Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. *Cell*, **26**, 167–180.

Catzeflis F (1991) Animal tissue collections for molecular genetics and systematics. *Trends Ecology and Evolution*, **6**, 168.

Filippucci MG (1992) Allozyme variation and divergence among European, Middle Eastern and North African species of the genus *Apodemus* (Rodentia, Muridae). *Israël Journal of Zoology*, **38**, 193–218.

Filippucci MG, Cristaldi M, Tizi L, Contoli L (1984) Dati morfologici e morfometrici in popolazioni di *Apodemus* (*Sylvaemus*) dell'Italia centro-meridionale determinati elettroforeticamente. In: *Recenti Acquisizioni Sul Genere Apodemus in Italia* (eds Contoli L, Cristaldi M, Filippucci MG, Tizi L, Vigna-Taglianti A), pp. 85–126. Supplemento alle Ricerche di Biologia della Selvaggina. University of Roma, Roma, Italy.

Gemmeke H (1981) Albumin differences in the long-tailed field mice and yellow-necked field mice (*Apodemus sylvaticus* and *Apodemus flavicollis*) electrophoretically determined in dry muscles and skin. *Zeitschrift Fur Säugetierkunde*, **46** (2), 124–125.

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, **86**, 6196–6200.

Montgomery WI (1999) Apodemus sylvaticus *and* Apodemus flavicollis. In: *The Atlas of European Mammals* (eds Mitchell-Jones AJ *et al.*). Academic Press, London.

Musser GG, Brothers EM, Carleton MD, Hutterer R (1996) Taxonomy and distribution records of Oriental and European *Apodemus*, with a review of the *Apodemus Sylvaemus* problem. *Bonner Zoologische Beiträge*, **46**, 143–190.

Niethammer J (1978) *Apodemus flavicollis* (Melchior, 1834) — Gelbhalsmaus; *Apodemus sylvaticus* (Linnaeus, 1758) — Waldmaus. In: *'Handbuch der Säugetiere Europas.'* (eds Niethammer J, Krapp F), pp. 325–358. Akademische-Verlagsgesellschaft, Wiesbaden.

Philippe H (1993) MUST: a computer package of Management Utilities for Sequences and Trees. *Nucleic Acids Research*, **21**, 5264–5272.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Storch G & Lütt O (1989) Artstatus der Alpenwaldmaus, *Apodemus alpicola* Heinrich, 1952. *Zeitschrift für Säugetierkunde*, **54**, 337–346.

Suzuki H, Tsuchiya K, Takezaki N (2000) A molecular phylogenetic framework for the Ryuku endemic rodents *Tokudaia osimensis* and *Diplothrix legata*. *Molecular Phylogenetics and Evolution*, **15**, 15–24.

Vogel P, Maddalena T, Mabille A, Paquet G (1991) Confirmation biochimique du statut spécifique du mulot alpestre *Apodemus alpicola* Heinrich, 1952 (Mammalia, Rodentia). *Bulletin Société Vaudoise Sciences Naturelles*, **8**, 471–481.