A new fast real-time PCR method for the identification of three sibling Apodemus species (A. sylvaticus, A. flavicollis, and A. alpicola) in Italy

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Abstract
The identification of field mice Apodemus flavicollis, Apodemus sylvaticus, and Apodemus alpicola represents a challenge for field scientists due to their highly overlapping morphological traits and habitats. Here, we propose a new fast real-time PCR method to discriminate the three species by species-specific TaqMan assays. Primers and probes were designed based on the alignment of 54 cyt-b partial sequences from 25 different European countries retrieved from GenBank. TaqMan assays were then tested on 133 samples from three different areas of Italy. Real-time PCR analysis showed 92 samples classified as A. flavicollis, 13 as A. sylvaticus, and 28 as A. alpicola. We did not observe any double amplification and DNA sequencing confirmed species assignment obtained by the TaqMan assays. The method is implementable on different matrices (ear tissues, tail, and blood). It can be used on dead specimens or on alive animals with minimally invasive sampling, and given the high sensitivity, the assay may be also suitable for degraded or low-DNA samples. The method proved to work well to discriminate between the species analyzed. Furthermore, it gives clear results (amplified or not) and it does not require any postamplification handling of PCR product, reducing the time needed for the analyses and the risk of carryover contamination. It therefore represents a valuable tool for field ecologists, conservationists, and epidemiologists.

Keywords
alpine field mouse, cytochrome B, probe, TaqMan, wood mouse, yellow-necked mouse

1 INTRODUCTION
Apodemus is a genus of field mice distributed in the Palearctic Region including 21 species and four groups: Sylvaemus, Apodemus, Gurkha, and Argenteus (Musser & Carleton, 2005). Seven species occur in Europe with more or less overlapping ranges. Many of these species are targeted by ecological, conservation, and epidemiological studies that have to face the need of distinguishing between co-occurring species, especially when they are similar.

In most Europe, in particular, the identification of yellow-necked mouse (Apodemus flavicollis) and wood mouse (Apodemus sylvaticus), both belonging to Sylvaemus group, represents a challenge for field scientists due to their highly overlapping morphological traits, habitats, and geographic range.
Apodemus sylvaticus is distributed throughout Europe (excluding Finland and northern parts of Scandinavia, the Baltics and Russia) and in some regions of North Africa (Schlitter et al., 2016). Apodemus flavicollis has a very similar distribution that extends northwards in southern Finland, in the Baltics, western Russia and some regions of Anatoly (whereas it is absent in Africa and Iceland) (Amori et al., 2016).

The physical resemblance of these two species is particularly marked in the southern part of their range, where discrimination based on the sole external characters such as body size and pelage color has proven to be unfeasible (Filippucci, Cristaldi, Tizi, & Contoli, 1984; Montgomery, 1980; Niethammer, 1978). Even though the two species have different ecologies, with A. flavicollis being more strictly associated to forested habitats and A. sylvaticus being also found in forest-edges, ecotones and in association with agricultural and anthropized environments, their ecological preferences partially overlap and the two species often live in syntopy (Marsh & Harris, 2000; Mitchell-Jones et al., 1999). The discrimination between these two siblings, albeit distinct species, is therefore very important for ecological, epidemiological, evolutionary, and B-chromosome studies. The situation is even more complex in the Alpine region, where a third very similar species, the Alpine field mouse (Apodemus alpicola) occurs in sympathy and often in syntopy with the other two. The Alpine field mouse is distributed throughout the Alps (France, Switzerland, Germany, Italy, and Austria).

To date, different approaches have been developed and discussed to tackle the need of discriminating between Apodemus species, but the debate is still far from being solved. Cranial measurements have proven to be effective in many cases (e.g., Barčiová & Macholán, 2002; Orlov, Bulatova, Nadžafova, & Kozlovsky, 1996; Vogel, Madalena, Mabile, & Paquet, 1991) and DNA analysis (e.g., sequencing, PCR), can instead be applied on samples from alive animals (e.g., a small sample of ear or tail tissue). Michaux et al. (2001) described an assay based on a conventional PCR with species-specific primers targeting a fragment of cytochrome b mitochondrial gene (cyt-b). Given its simplicity and clarity of results (PCR product present or not), this method is one of the most commonly used in the literature, being preferred to other expensive methods such as DNA sequencing. However, in their recent work, Bugarski-Stanojević, Blagojević, Adnadević, Jovanović, and Vujošević (2013) showed that the method by Michaux et al. (2001) is subject to a certain degree of misidentification, probably due to the low specificity of the chosen primers or to the existence of nuclear copies of mitochondrial genes (pseudogenes) which determine false-positive results (Dubey, Michaux, Brunner, Hutterer, & Vogel, 2009). In their work, Bugarski-Stanojević et al. (2013) compared the method with two alternative molecular assays with higher specificity: the arbitrarily primed-PCR (AP-PCR) and the intersimple sequence repeat-PCR (ISSR-PCR). Both methods result in species-specific DNA profiles that can be visualized through gel electrophoresis. Such methods, however, referring to arbitrary sequence PCR, generally exhibit poor interlaboratory reproducibility that hinders their widespread use. Moreover, postamplification handling of PCR products is required, increasing the time needed for the analyses and introducing the risk of carryover contamination.

Here, we propose a new fast real-time PCR method to distinguish between the three species. Real-time PCR using TaqMan probes has been reported by different authors as a fast and sensitive method for the identification of species (Cammà, Di Domenico, & Monaco, 2012; Di Domenico, Di Giuseppe, Wicochea Rodríguez, & Cammà, 2017; Overdyk, Braid, Naaum, Crawford, & Hanner, 2016). It does not require any postamplification step and can be easily automated allowing the analysis of large numbers of samples. Moreover, the application of specific primers in combination with fluorogenic probes considerably increases reaction specificity.

### 2 MATERIALS AND METHODS

#### 2.1 Design of primers and probes

A total of 54 cyt-b partial sequences of A. flavicollis, A. sylvaticus, and A. alpicola from 25 different countries were retrieved from GenBank (Accession numbers in Table 1). Sequences were aligned with software MegAlign (DNASTAR Lasergene 10) and species-specific primers and TaqMan probes were designed based on differences between species (Table 2). Primer Express Software 3.0.1 (Applied Biosystems) was also used to exclude the presence of secondary structures between primers and probes that would reduce reaction efficiency.
TABLE 1 Geographic origin, references and GenBank accession numbers of *Apodemus cyt-b* sequences used to design primers and probes for real-time PCR assays

| Species        | Geographic origin | Accession number | Reference                                      |
|----------------|-------------------|------------------|------------------------------------------------|
| *A. flavicollis* | Belgium           | Gembes           | AJ298601                                       | Michaux et al. (2001) |
|                | Belgium           | Ardennes         | AJ298598                                       | Michaux et al. (2001) |
|                | France            | Allier           | AJ311151                                       | Michaux, Chevret, Filippucci, and Macholan (2002) |
|                | Germany           | Bielefeld        | AJ298603                                       | Michaux et al. (2001) |
|                | Greece            | Mt. Olympus      | AJ631968                                       | Michaux, Libois, and Filippucci (2005) |
|                | Greece            | Peloponnese      | AJ605625                                       | Michaux, Libois, Paradis, and Filippucci (2004) |
|                | Italy             | Abruzzes         | AJ311150                                       | Michaux et al. (2002) |
|                | Spain             | Navarra          | AJ631969                                       | Michaux et al. (2005) |
|                | Sweden            | Gotland          | AJ631970                                       | Michaux et al. (2005) |
|                | Switzerland       | Champéry         | AB032853                                       | Serizawa, Suzuki, and Tsuchiya (2000) |
|                | Ukraine           | Chernobyl        | AF127539                                       | Makova, Nekrutenko, and Baker (2000) |
|                | Bosnia and Herzegovina | /          | JF819970                                        | Krystufek et al. (2012) |
|                | Czech Republic    | Karsperske       | AJ605609                                       | Michaux et al. (2004) |
|                | Hungary           | Debrecen         | AJ605634                                       | Michaux et al. (2004) |
|                | Macedonia         | Bistra           | AJ605644                                       | Michaux et al. (2004) |
|                | Romania           | Cheile garlistei | AJ605647                                       | Michaux et al. (2004) |
|                | Russia            | Voronezh         | AJ605654                                       | Michaux et al. (2004) |
|                | Slovenia          | Asan cesma       | AJ605657                                       | Michaux et al. (2004) |
| *A. sylvaticus* | Belgium           | Ardenes          | AJ298605                                       | Michaux et al. (2001) |
|                | France            | Eastern Pyrenees | AJ298599                                       | Michaux et al. (2001) |
|                | Italy             | Aspromonte       | AJ511923                                       | Michaux, Magnanou, Paradis, Nieberding, and Libois (2003) |
|                | United Kingdom    | Frenchay         | AF127536                                       | Makova et al. (2000) |
|                | Poland            | Pomorze          | KX159689                                       | Herman et al. (2016) |
|                | Ireland           | Galway           | KX159658                                       | Herman et al. (2016) |
|                | Portugal          | Vila Real        | KX159669                                       | Herman et al. (2016) |
|                | Luxembourg        | /                | KX159672                                       | Herman et al. (2016) |
|                | Norway            | Rogaland         | KX159647                                       | Herman et al. (2016) |
|                | United Kingdom    | York             | KX159644                                       | Herman et al. (2016) |
|                | United Kingdom    | Warwick          | KX159637                                       | Herman et al. (2016) |
|                | Iceland           | Shetland         | KX159653                                       | Herman et al. (2016) |

(Continues)
2.2 | Sensitivity, specificity and repeatability of the real-time PCR assays

Three replicates of five tenfold DNA serial dilutions from 20 ng/ml to 2 pg/ml of each species were prepared to create the standard curve. The efficiency (E) of the real-time PCR was calculated according to the formula $E = (10^{-1/slope} - 1) \times 100$ (Vaerman, Saussuy, & Ingargiola, 2004). The lowest dilution in the linear dynamic range producing all positive results was considered to assess the limit of detection (LOD). The repeatability of the methods was estimated calculating the coefficient of variation (CV) relative to the analysis of twenty-four replicates, in three different runs for the different LOD DNA concentration relative to the target species A. sylvaticus, A. flavicollis, and A. alpicola. The analytical specificity was evaluated using the three target species as input DNA and DNA from human, Mus musculus, Myodes glareolus, and Rattus norvegicus as negative controls. Specificity was also evaluated in silico by comparing the designed PCR primers and probes with the sequences, deposited in NCBI public database, of other similar Apodemus species (A. uralensis and A. witherbyi, of the Sylvaemus group, sharing part of the distributional range with the target species).

2.3 | Samples and DNA extraction

We collected a total of 133 samples from three different areas of Italy to ensure a high genetic variability between samples. Two areas, where A. flavicollis and A. sylvaticus occur in sympatry, are located in Central Italy and are separated by the Apennine chain (TE: Teramo and VT: Viterbo; Figure 1). The third area is located in Northern Italy (GP: Gran Paradiso National Park; Figure 1), were the third species, A. alpicola, is sympatric with the other two. Samples from VT (N = 11) were provided as DNA extracts stored at −20°C (previously obtained with the salting-out protocol described by Aljanabi & Martinez, 1997 from ear tissues). Samples from TE (N = 85) were blood samples added with 10 μl sodium citrate (5%) and stored at +4°C before DNA extraction, while samples from GP (N = 37) were

| TABLE 1 (Continued) |
|----------------------|
| Species | Geographic origin | Accession number | Reference |
| A. alpicola | Austria | Klosterle | AY179495 | Reutter et al. (2003) |
| | | Stuben | AB032854 | Serizawa et al. (2000) |
| | | Stuben | AY179496 | Reutter et al. (2003) |
| | | Vorarlberg | AJ311153 | Michaux et al. (2002) |
| | France | Mt. Cenis | AY179497 | Reutter et al. (2003) |
| | | Savoie | AJ311152 | Michaux et al. (2002) |
| | Switzerland | Sanetsch | AY179494 | Reutter et al. (2003) |
| | | / | AF159391 | Martin et al. (2000) |

| TABLE 2 | Primers and TaqMan probes designed for the identification of Apodemus species. Amplicon length and optimized concentrations of primers and probes are also reported |
|----------------------|
| Oligo | Sequence (5’→3’) | Concentration (nmol/L) | Amplicon length (bp) |
| Apodemus flavicollis | Forward | GCCGAGACGTAATATTGGATGAT | 150 |
| | Reverse | TCTTACGTGTAGAAATAAGCAATGAA | 150 |
| | Probe | FAM-AATTCGATATTTACACGAGCTC-TAMRA | 150 |
| Apodemus sylvaticus | Forward | ATCATGATGAAACTTCGGCTCAT | 200 |
| | Reverse | AGTCAGCCATAATTTAGCTCTGAC | 200 |
| | Probe | JOE-ATCCAAATCCTCACAGGCTTTTTCTAGCAATACA-TAMRA | 200 |
| | Apodemus alpicola | Forward | AATCAAAGACATTTCTAGGAGTACTCATATAATC | 600 |
| | Reverse | AGTATTATGTTGGTGGCAGGC | 600 |
| | Probe | FAM-TCTATTCCTTATATAACTAGTACTCTTTTCCCCGAGCCTTC-TAMRA | 250 |
FIGURE 1  Geographic origin of the 133 Italian Apodemus samples tested with real-time PCR for species discrimination. GP, Gran Paradiso National Park; VT, Viterbo area; TE, Teramo area

provided as ear/tail tissue samples stored in 70% ethanol. Samples from TE and GP were extracted with Maxwell 16 System instrument and Maxwell 16 Blood or Tissue DNA kits (Promega), according to producer’s protocols. DNA concentration of all samples was measured with NanoDrop 1000 V3.8.1 (Thermo Fisher Scientific) and then diluted up to 2 ng/μl.

2.4 | Real-time PCR

Real-time PCR reactions were carried out in 20 μl volumes including 10 μl of TaqMan Fast Universal PCR Master Mix 2× (Applied Biosystems) and 5 μl of 2 ng/μl DNA. The optimized concentration of primers and probes is reported in Table 2. A duplex assay, with the use of two different fluorescent markers, was optimized for A. flavicollis and A. sylvaticus (FAM and JOE, respectively). A simplex assay (FAM) was optimized for A. alpica. Reactions were performed in a 7900HT Fast real-time PCR System (Applied Biosystems) with the following thermal profile: 20 s at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 47°C and 30 s at 72°C plus 7 min at 72°C. PCR products were purified using the GeneAll ExpiTM PCR Kit (GeneAll) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the 3130XL Genetic Analyzer (Applied Biosystems) using the primers CB-AF and CB-AR2 in both directions. Sequences were assembled with SeqMan Pro (DNASTAR Lasergene 10). Based on sequence quality and overlapping of the two strands, we selected only the central part of the contig to be submitted on BLAST (Basic Local Alignment Search Tool) for species identification and to be deposited in GenBank. In the cases where we had poor-quality sequences or found possible clues for pseudogenes (i.e., double peaks), we discarded the uncertain sequence, thus only indisputable sequences were included in the analyses.

3 | RESULTS

3.1 | Sensitivity, specificity, and repeatability of the real-time PCR assays

The slope of the standard curve, the efficiency, and the correlation coefficient (R²) of all three assays are reported in Table 3. The LOD calculated for the different species ranges between 10 and 100 pg as reported (Table 3). Moreover, the methods revealed a very high level of repeatability as assessed by the low values of the coefficient of variations as shown in the same Table 3.

For each assay, the other two species were tested as nontarget DNA, using the same amount, and no cross-amplifications were observed; human, M. musculus, Myodes glareolus, and Rattus norvegicus DNA did not produce fluorescent amplification signal in any of the three new developed assays.

In addition, in silico analyses confirmed the specificity also against other Apodemus species. In particular, primers and probe designed for the three different assays showed sequence identity against A. whiterbyi and A. uralensis lower than 92% even for the best records. An exception was A. sylvaticus probe against A. uralensis showing a 94% sequence identity; however, both forward and reverse primers mismatched in two nucleotides at the 3’ end, limiting the aspecific amplification of this nontarget species.

3.2 | Real-time PCR and DNA sequencing

Real-time PCR reactions obtained a 100% success of amplification. Ninety-two samples were classified as A. flavicollis, 13 as A. sylvaticus and 28 as A. alpica (Table 3). We did not obtain any double amplification (Table 3).

After sequencing, based on sequence quality and overlapping of the two strands, we selected a good central fragment of 677 bp (of 866 bp). For some samples, sequencing ended with poor-quality sequences that could not be used for the analysis or deposited. Other samples showed some clues of pseudogenes (i.e., double peaks) and also these sequences were excluded. We selected 33 good
sequences (about 25%) to be deposited in GenBank. All the 33 sequences were submitted on BLAST and confirmed the classifications obtained by TaqMan assays (Table 4). Accession numbers for deposited sequences are provided in the Data accessibility section.

4 | DISCUSSION

In this work, we provided a new fast real-time PCR method for the discrimination of three Apodemus species. It can be used on dead specimens or on alive animals with minimally invasive sampling, a characteristic often required by ecology or conservation studies. The three assays proved to be a useful tool on different matrices (tissues from ear, tail, and blood). Real-time PCR is characterized by a much higher sensitivity compared to conventional PCR, being able to detect even very low copies of DNA (Angelone-Alasaad et al., 2015). For this reason, this modern method is now commonly used to amplify small or degraded samples (Holt et al., 2016; Lee, McCord, & Buel, 2014) or for diagnostic purposes (Caraguael, Stryhn, Gagne’, 2013). Alternatively, real-time PCR is characterized by a higher sensitivity compared to conventional PCR, being able to detect even very low copies of DNA (Angelone-Alasaad et al., 2015). For this reason, this modern method is now commonly used to amplify small or degraded samples (Holt et al., 2016; Lee, McCord, & Buel, 2014) or for diagnostic purposes (Caraguael, Stryhn, Gagne’, 2013).

### Table 3

| Species                  | Slope  | Efficiency (E), % | R²     | LOD (pg) | CV  |
|--------------------------|--------|------------------|--------|----------|-----|
| Apodemus flavicollis⁴     | −3.50  | 90               | .99    | 100      | 1.7 |
| Apodemus sylvaticus⁴     | −3.32  | 100              | .99    | 10       | 0.8 |
| Apodemus alpicola        | −3.33  | 99               | .99    | 10       | 0.5 |

Efficiency (E) was calculated with the formula \( E = \frac{1}{\text{slope}} \times 100 \). Slope and coefficient of determination (R²) were calculated by the standard curve. Limit of detection (LOD) was calculated on the latest DNA dilution giving all positives results. CV was calculated by the ratio between standard deviation and mean of Ct values at LOD DNA concentration.

⁴Data referred to the duplex real-time PCR assay.

### Table 4

| Area   | Sample type | N   | TaqMan assays | DNA Sequencing |
|--------|-------------|-----|---------------|----------------|
|        |             |     | FLA | SYL | ALP | Confirmed/sequenced |
| TE     | Blood       | 79  | +   | −   | −   | 6/6 |
|        | Blood       | 6   | −   | +   | −   | |
| VT     | Ear tissue  | 7   | +   | −   | −   | 5/5 |
|        | Ear tissue  | 4   | −   | +   | −   | 2/2 |
| GP     | Ear/tail tissue | 6 | +   | −   | −   | 6/6 |
|        | Ear/tail tissue | 3 | −   | +   | −   | 3/3 |
|        | Ear/tail tissue | 28 | −   | −   | +   | 11/11 |
| Total  |             | 133 | 92  | 13  | 28  | 33/33 |

FLA = A. flavicollis, SYL = A. sylvaticus, ALP = A. alpicola.
species, we did not directly test them and we cannot exclude that a certain level of cross-amplification could occur. In all these hypothetical cases, the comparison of $C_t$ values (not possible with traditional methods relying on visual interpretation of electrophoresis band patterns) may be a useful approach to identify correct species assignment even with doubtful amplifications.

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CONFLICT OF INTEREST

None declared.

DATA ACCESSIBILITY

DNA sequences: Genbank accession numbers KU975553-KU975564.

AUTHOR CONTRIBUTIONS

GS, CC, MDD, and IP conceived and designed the study; GS collected samples; GS, VC, and MDD performed analyses; GS and MDD wrote the manuscript; all the authors contributed to substantial manuscript improvement.

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