A novel approach to field identification of cryptic Apodemus wood mice: calls differ more than morphology

A novel approach to field identification of cryptic *Apodemus* wood mice: calls differ more than morphology

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Abstract
Field identification of European wood mice *Apodemus* spp. is challenging, due to their morphological resemblance and frequent sympatry. We developed discriminant functions based on body mass and acoustic variables of distress calls to identify three cryptic species of wood mice (*Apodemus alpicola*, *A. flavicollis* and *A. sylvaticus*) in Italy. We achieved an overall correct classification rate of 86.4-98.1%, the best results (100% correct classification) were obtained for *A. sylvaticus* calls. This minimally invasive, effective and low-cost method highlights the potential role of bioacoustics as a powerful tool for field discrimination of cryptic species of terrestrial mammals.

Keywords
Species discrimination, *Apodemus alpicola*, *Apodemus flavicollis*, *Apodemus sylvaticus*, distress call

Word count: 2359

Introduction
The identification of ryptic species, i.e. two or more distinct taxa that are often erroneously classified within the same species name because of their strong morphological similarity (Bickford *et al*. 2006) – typically requires the analysis of non-morphological variables. The examination of animal sound may offer rapid and cost-effective species discrimination, and has been used for several cryptic taxa, e.g. insects (Henry 1994), amphibians (Narins 1983), birds (Cicero 1996), and mammals (Barlow & Jones. 1997, Barlow et al. 1997; Russo & Jones 2002; Zsebők *et al*. 2015). No researcher has so far used bioacoustics to identify any of the many cryptic rodent species known to science (Wilson & Reeder 2005).
The wood mouse *Apodemus sylvaticus* and the yellow-necked wood mouse *A. flavicollis* are two rodents that are widespread and abundant in Europe, occurring in sympatry in much of their ranges (Michaux *et al*. 2005). Their overlapping ecological niches and close morphological resemblance make field discrimination of these species difficult (Michaux *et al*. 2001). Although in Central Europe most adult wood mice can be identified by visual inspection (Niethammer 1978), elsewhere their strong similarity makes reliable identification challenging (Debernardi *et al*. 2003, Bartolommei *et al*. 2015). Species distinction is even more complicated in the Alps, where another cryptic wood mouse, the Alpine wood mouse *A. alpicola*, previously regarded as a subspecies of *A. flavicollis* (Musser *et al*. 1996), also occurs (Reutter *et al*. 2003).
At present these species can be reliably recognised only in the laboratory (Bugarski-Stanojević *et al*. 2013) by skull morphology (Amori *et al*. 1994, Reutter *et al*. 1999, Debernardi *et al*. 2003, Barčiová & Macholán 2009), enzyme (Filippucci *et al*. 1996) or DNA analyses (Michaux *et al*. 2001, Jojić *et al*. 2014). Besides being time-consuming and expensive, these methods also require the examination of skulls (e.g. obtained from dead specimens or extracted from owl pellets) or invasive tissue sampling (e.g. ear punch, tail biopsy or toe clipping).

The vocal repertoires of Apodemus sylvaticus and Apodemus flavicollis have been partly described (Hoffmeyer & Sales 1977, Gyger & Schenk 1984), but so far no research has been focused on their distress calls. In this work, we present a novel, highly effective and less invasive approach to the identification of wood mice in the field, based on a combination of distress call variables and body size and mass variables.

Methods

Fieldwork

We trapped wood mice between May and September in 2014 and 2015, at 14 sites in three Italian regions (Valle d'Aosta and Piedmont, Northern Italy; Latium, Central Italy). Sites were all characterised by woody vegetation, which differed according to latitude and elevation, including Mediterranean scrubland, mesophilous and conifer woodlands, and alpine scrubland. The three species were sympatric in the Alpine sites, whereas in the other sites *Apodemus sylvaticus* co-occurred with *Apodemus flavicollis* only. Mice were caught with Sherman and Longworth live traps spaced out 10 meters along transects and baited with nut cream, sunflower seeds and pieces of apples. Nesting material (a small amount of hydrophobic cotton) was also placed within the traps to provide mice with thermal insulation at night. At each site, traps were left in place for three nights and checked twice a day (at dawn and at dusk). For each subject we determined sex, measured body mass (BM, in g) and foot length (FL, in mm) and ear-punched a 3-mm tissue sample.

We recorded distress calls (Hogsted 1983) broadcast by mice in the field during handling (see video file, Appendix S1): while one operator gently manipulated the subject to take body measurements and tissue samples, another recorded calls with an ultrasound recorder (D500X, Pettersson Elektronik AB, Uppsala, Sweden) kept in manual mode and held at 40 cm from the mouse. The recorder covers a 1-130 kHz frequency range. Sound was sampled at a 300 kHz rate and saved as Waveform Audio Files onto four-gigabyte flashcards. Once 1-3 distress calls were recorded, the mouse was released *in situ*. To minimize stress, when a mouse did not emit calls in 2 min, it was allowed to rest in the trap for another 15 mins and then released after a second attempt. Less than 5% of subjects failed to vocalise during the process.

Molecular identification

DNA was extracted from tissue samples following the protocol described by Aljanabi and Martinez (1997) and the species were identified by the species-specific PCR amplification of cytochrome *b* following Michaux *et al*. (2001).

Sound analysis

To avoid pseudo-replication, only one good quality call/subject was considered for analysis (Hurlbert 1984). Calls were analyzed with BatSound 4.11 (Pettersson Elektronik AB, Uppsala, Sweden) using a Hanning window with a 512-point FFT and a 98% overlap. Maximum (Fmax) and minimum (Fmin) call frequencies and the number of harmonics (Harm) were taken manually from spectrograms, the frequency containing most energy in the call (peak frequency, PF) was measured from power spectra and call duration (Dur) was obtained from oscillograms (Appendix S2). Frequency values were expressed in kHz, duration in ms.

Statistical analyses

We used ANOVA to test for interspecific differences in distress calls as well as body size indicators (foot length: FL; body mass: BM). Ryan-Joiner and Bartlett tests were used to test for residual normality and homoscedasticity.
We used quadratic Discriminant Function Analysis using cross-validation to generate models including different subsets of acoustic variables, body mass and foot length. Only the models achieving the highest classification performances are shown here.
The sympatry of the three species is limited to the Alps, whereas *Apodemus sylvaticus* and *Apodemus flavicollis* co-occur in a wide geographical area. Therefore, we generated two models, one including all species, another excluding *Apodemus alpicola*. Model significance and the variables' discriminating power were tested with Wilk's λ. All tests were performed with MINITAB release 17.2 Significance was set at $p < 0.05$.

Results

We identified by molecular analysis 87 animals from which we recorded distress calls, including *A. sylvaticus* (n = 27), *A. flavicollis* (n = 27) and *A. alpicola* (n = 33). Calls were audible (7-10 kHz) quasi-constant frequency tonal emissions (Fig. 1). Except the number of harmonics ($F_{2,84}= 3.10$, $p>0.05$), all variables differed among species (PF: $F_{2,84}=98.14$, $p<0.001$; MaxF: $F_{2,84}=123.59$, $p<0.001$; MinF: $F_{2,84}=91.37$, $p<0.001$; Dur: $F_{2,84}= 47.18$, $p<0.001$). Body mass, but not foot length, also differed among species (FL: $F_{2,84}=2.67$, $p>0.05$; BM: $F_{2,84}=4.40$, $p<0.05$; Table 1).
The best multivariate discriminant model (Fig. 2) included MaxF, Dur and BM and achieved an 86% overall classification rate (79% for *Apodemus flavicollis*. 85% for *Apodemus alpicola* and 100% for *Apodemus sylvaticus*). The best model including *Apodemus sylvaticus* and *Apodemus flavicollis* provided a 98% overall correct classification rate (*A. flavicollis*: 96%; *A. sylvaticus*: 100%). Both models were significant (all species: Wilk's $\lambda=0.178$, $F_{6,164}=37.357$, $p<0.001$; two species: Wilk's $\lambda= 0.219$, $F_{3,50}=59.581$, $p<0.001$). The variables' ranking of discrimination power was Fmax> DUR > body mass.

Discussion

We showed that three cryptic species of European wood mice can be recognised based on a combination of acoustic variables and body mass. Our method requires easy field recording operations and basic sound analysis skills to be carried out. Moreover, subjects often broadcast distress calls when handled, making data collection easy. Our method was better than those relying on morphology alone. For instance, Bartolommei *et al*. (2015) achieved nearly 80% of correct classification for both *Apodemus flavicollis* and *Apodemus sylvaticus* based on foot length and body mass, whereas we identified all *Apodemus sylvaticus* and most *Apodemus flavicollis*; misclassification of *Apodemus flavicollis* was negligible (<4%) in absence of *Apodemus alpicola* (see also Appendix S3)
Although distributional studies might still require the adoption of other approaches where the three species co-occur, e.g. genetics (Michaux *et al*. 2001), our method is especially suitable for ecological studies, such as e.g. assessment of habitat selection or demography, as the few misclassified cases are unlikely to have significant influence over quantitative, statistically analysed patterns (Zsebők *et al*. 2015).
Overall, ours represents a promising approach to field identification of wood mice, due to its easy application, reduced operational time and invasiveness, and limited costs. We only analysed Italian *Apodemus* populations, so we encourage other researchers to test whether our method works throughout the vast European range of

of these species, and on other cryptic rodents – e.g. *Calomys* spp. (Gonzàles Ittig et al. 2002), *Mastomys* spp. (Britton-Davidian et al. 1995) and *Microtus agrestis* lineages (Paupério et al. 2012). We conclude that bioacoustics has great potential as a field approach to species recognition in terrestrial mammals.

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Tables

Table 1. Descriptive statistics (mean±SD; min-max range is indicated below, in parentheses) of acoustic parameters of distress calls and body size indicators in three cryptic species of wood mice (n=sample size). PF: peak frequency; MaxF: maximum frequency; MinF: minimum frequency; Dur: duration; Harm: number of harmonics.

	A. alpicola n=33	*A. flavicollis* n=27	*A. sylvaticus* n=27
PF (kHz)	6.73±1.24 (4.3-9.6)	7.39±0.86 (6-9.1)	10.13±0.61 (9.1-11.5)
MaxF (kHz)	7.74±1.27 (5.5-9.8)	8.39±1.88 (7.1-11.3)	11.61±0.68 (10-13)
MinF (kHz)	5.60±1.29 (3.3-8.7)	5.47±0.98 (4.5-8.8)	8.86±0.76 (7.5-10)
Dur (ms)	221.31±81.02 (62-350)	342.41±100.46 (240-660)	433.89±66.99 (270-580)
Harm	7.09±1.68 (4-11)	6.11±1.37 (4-10)	6.15±1.74 (4-11)
Foot length	24.36±1.72 (20.1-28.4)	23.29±0.8 (21.9-24)	22.13±0.9 (20.0-23.6)
Body mass	27.30±6.61 (19-40)	30.78±2.55 (25-34)	27.63±4.0 (23-35)

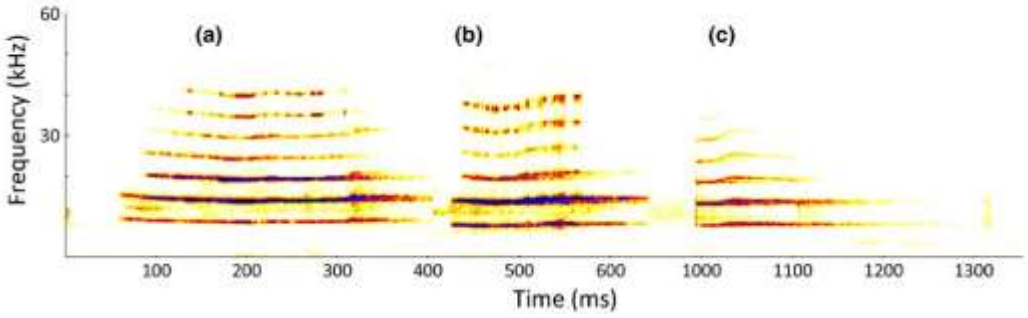

Figure 1. Spectrogram of a distress calls emitted by *Apodemus alpicola*.

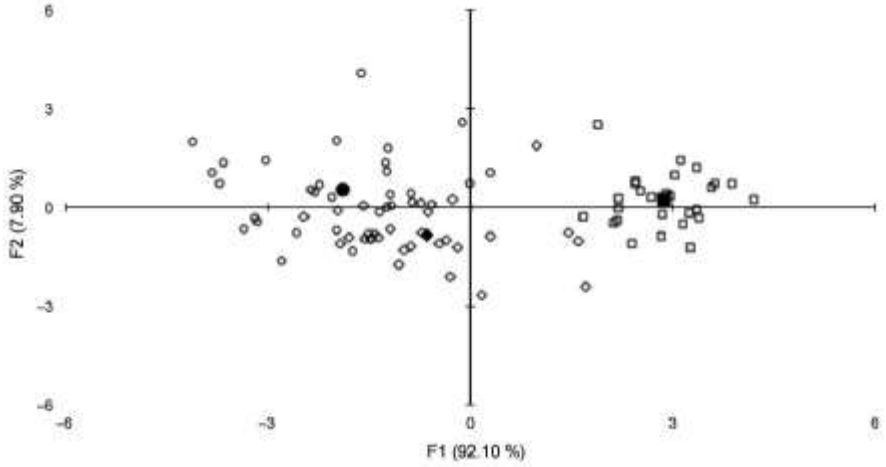

Figure 2. Signal space of distress calls by three species of wood mice of the genus *Apodemus* by the first two discriminant functions. Filled triangles: group centroids; empty symbols indicate single distress calls: diamonds=*A. alpicola*; circles=*A. flavicollis*; squares=*A. sylvaticus*.