ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 64 (3), 2012: 283-288

Molecular Discrimination and Morphological Description of *Apodemus sylvaticus* and *A. uralensis* from Cefa Nature Reserve (Romania)

***Philippe Gil de Mendonça*[1], *Ana-Maria Benedek*[2]**

[1] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Yuri Gagarin Str., BG-1113 Sofia, Bulgaria; E-mail: philippe.mendonca@iber.bas.bg
[2] Department of Ecology and Environment Protection, Lucian Blaga University, RO-550337 Sibiu, Romania

Abstract: Using sequences available on GenBank, a PCR protocol was optimized and applied to discriminate between *Apodemus sylvaticus* and *A. uralensis*. Molecular discrimination is more efficient than methods based on external morphology. The combination of live-trapping and molecular identification revealed that *A. uralensis* is relatively abundant in the wettest parts of Cefa Nature Reserve.

Key words: *Apodemus (Sylvaemus) sylvaticus, Apodemus (Sylvaemus) uralensis*, PCR, species identification

Introduction

Cefa Nature Reserve is situated in Crişul Repede River Basin, in the Western Plain of Romania. It is made of a variety of contrasting habitats, e.g. salt marshes, fish ponds, canals, cultivated fields, pastures and forest.

Four *Apodemus* species are present in the Cefa area, one within the subgenus *Apodemus* (*A. agrarius*), and three within the subgenus *Sylvaemus* (*A. flavicollis, A. sylvaticus* and *A. uralensis*, formerly known as *A. microps*). However, there were no published records of presence of the latter species within Cefa Nature Reserve until now. *Apodemus uralensis* is a little studied and poorly known species. It closely resembles its congeneric *A. sylvaticus*, and is often misidentified as *A. sylvaticus*. Until recently, it even remained undetected in some parts of its range (Juškaitis 1999, 2003). Field studies based on CMR (capture, marking, recapture) cannot rely on cranial morphology for accurate species identification. However, DNA may be sampled by means of little to moderately invasive methods (e.g. blood sampling or ear punching). Genetic markers discriminating between the two species are therefore a most appropriate modern tool for unambiguously identifying these two species. The present study aimed to use such markers to identify specimens from Cefa Nature Reserve.

Materials and Methods

Small mammals were captured in various types of live-traps set in the woodland, hedgerows and along ditches and fish ponds near Cefa Visitor Centre (Cefa Nature Reserve, Western Romania). Traps were baited with a mixture of seeds and pieces of apple. Hey was provided as bedding material. Traps were set at dusk and checked at dawn. Upon capture, mice were identified to the species level, sexed, weighed to the nearest 0.5 g, measured with a pair of callipers, and released at the place of capture. Standard measurements were taken on manually restrained mice. A small tissue sample was collected from each individual by ear notching. This permanent marking flags recaptures, thus preventing pseudo-replication due to repetitive sampling of the same individuals. The skin sample collected as

part of the marking procedure was immediately preserved in 80% ethanol for subsequent genetic analyses. Sampling started in 2005 and is still ongoing.

DNA was extracted by alkaline lysis followed by Chelex stabilization. Tissue samples were individually placed in 500 µL of 1.25% ammonia and incubated at 99 °C in tightly closed tubes for 30 min. Tubes were then centrifuged to remove drops from the lid, opened and incubated further at 99 °C to evaporate the ammonia and reduce the volume of liquid to 250 µL. The supernatant was then pipetted into a new tube containing 50 µL of a 20% suspension of Chelex 100 (molecular biology grade, Bio-Rad, Hercules CA) in 1xTE and thoroughly mixed. Extracted and stabilized DNA was then stored at 4 °C.

Reference samples of five *Apodemus* species from 13 countries from Europe, Asia and North Africa were used to test the primers (Table 1). Tissue samples included skin, muscle, kidney, liver and blood. In order to remove PCR inhibitors (e.g. haemoglobin), DNA from reference samples was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Hilden Germany) following the manufacturer's instructions. DNA integrity was checked by PCR using molecular marker pairs p0033/p0049 and p0066/p0067 targeting the vertebrate 18S rRNA gene (modified after Pichon *et al*. 2003).

For species identification, target DNA was amplified using a touchdown PCR program. Primer sequences targeting the mitochondrial cytochrome b gene were obtained from GenBank and are provided in Table 2 along with their GenBank accession numbers. Master-mix composition and thermocycling conditions are given in Tables 3 and 4 respectively. Amplicons were separated by agarose gel electrophoresis and visualized by UV transillumination at 300 nm using GelRed (Biotium, Hayward CA) as a fluorescent marker. Amplicon size was estimated by means of an *ad hoc* analysis software (Gene Tools 4.01, Syngene, Cambridge, UK).

Melting point of each amplicon was determined by dissociation curve analysis using SYBR Green I (QuantiFast SYBR Green, QIAGEN, Hilden, Germany) following the manufacturer's instructions.

Results

All DNA samples from reference specimens of *A. sylvaticus* were successfully amplified using the marker pair for that species. No cross-reactions were observed with the markers for *A. uralensis*. Conversely, all DNA samples from reference specimens of *A. uralensis* were successfully amplified using the marker pair for that species. No cross-reactions were observed with the markers for *A. sylvaticus*. Amplicon size ranged between 306 bp and 318 bp for *A. sylvaticus* and between 292 bp and 307 bp for *A. uralensis* (Fig. 1). Melting ranges are 81.3 - 81.5 °C for *A. sylvaticus* and 80.1 - 81.8 °C for *A. uralensis* (Fig. 2). No cross-reactions with samples from *A. flavicollis* and *A. agrarius* were observed with any of the marker pairs. However, the markers for *A. uralensis* very efficiently amplified all reference samples of *A. alpicola*, with similar amplicon size (292-303 bp) and overlapping melting range (79.6-80.3 °C).

A total of 52 specimens captured in Cefa Nature Reserve matching the external description of either *A. sylvaticus* or *A. uralensis* were investigated by PCR. Six of them were identified molecularly as *A. sylvaticus*, and the remaining 46 mice were identified molecularly as *A. uralensis*. Based on morphological criteria only, 3 out of 6 *A. sylvaticus* and 23 out of 46 *A. uralensis* were correctly identified. Details of the external morphology of all *Apodemus* (*Sylvaemus*) mice from Cefa are given in Tables 5 and 6.

Discussion

The touchdown PCR program, thanks to higher annealing temperatures during the early steps of DNA amplification, reduces non-specific priming while generating a large number of copies of the intended target DNA. All samples from *A. sylvaticus* and *A. uralensis*, whichever their geographical origin, produced a strong signal without cross-reactions between these two target species.

Data from GenBank indicate very high levels of homology between *A. uralensis* and *A. alpicola*. Therefore, and as expected, a strong cross-reaction was observed between these two species. Since the geographical ranges of *A. uralensis* and *A. alpicola* do not overlap (Panteleev 1998), the strong cross-reaction observed between these two species is actually advantageous. Indeed, a single pair of primers is required to amplify DNA from both species and unambiguously distinguish them from *A. sylvaticus*.

The observed overlap in all external measurements from *A. sylvaticus* and *A. uralensis* makes it difficult for the inexperienced biologist to accurately discriminate *A. sylvaticus* and *A. uralensis* in the field.

Table 1. Geographical origin of the reference samples used to test the species-specific primers.

Species	Geographical origin of reference samples
A. sylvaticus	Algeria, Wales, England, Switzerland, Germany, Slovakia, Byelorussia, Romania, Bulgaria, Russia
A. uralensis	Slovakia, Romania, Russia, Kazakhstan
A. alpicola	Switzerland
A. flavicollis	Wales, England, Switzerland, Germany, Poland, Slovakia, Romania, Bulgaria, Russia
A. agrarius	Latvia, Slovakia, Romania, Bulgaria, Russia, Kazakhstan

Table 2. Sequence of primers used for the PCR amplification of DNA from *Apodemus sylvaticus* and *A. uralensis*.

Target species	Primer sequence	GenBank Accession number, position on sequence, Authors of GenBank entry
A. sylvaticus	F 5'-GAGGAGGATTCTCAGTAGAC-3'	AB033695.1, nt 494-513, Suzuki and Serizawa (2000)
A. sylvaticus	R 5'-TTAATATGGGGTGGGGTGTTA-3'	AB033695.1, nt 806-786, Suzuki and Serizawa (2000)
A. uralensis	F 5'-TAACAGCATTCTCTTCAGTCACA-3'	AB032854.1, nt 179-201, Serizawa and Suzuki (2000)
A. uralensis	R 5'-TATGGGATAGCTGATAGTAAG-3'	AB032854.1, nt 464-444, Serizawa and Suzuki (2000)

Table 3. Reaction mix for the PCR amplification of DNA from *Apodemus sylvaticus* and *A. uralensis*. Total volume is 25µL, including 5µL template DNA.

10xQIAGEN CoralLoad PCR buffer (at 15 mM $MgCl_2$)	2.500µL
QIAGEN $MgCl_2$ (at 25 mM)	1.000µL
QIAGEN dNTPs (10 mM)	1.000µL
Forward primer (10 µM)	1.000µL
Reverse primer (10 µM)	1.000µL
H_2O	13.375µL
QIAGEN Hot Start Taq Plus (5U/µL)	0.125µL

The use of molecular markers makes is possible to accurately identify specimens to the species level after the latter ones have been released in the field. This is a major advantage for field studies involving CMR.

A limitation of this method, however, lies in the inability to combine the two marker pairs in a duplex PCR, as amplicon sizes are similar and melting ranges overlap.

Based on trapping and molecular data, it seems that *A. uralensis* and *A. agrarius* are the numerically dominant small rodent species in the wettest parts of Cefa Nature Reserve. This is an important piece of information which reinforces the view that *A. uralensis* tends to be relatively abundant in wet areas. Furthermore, this adds a new element to the widespread view that this species is predominantly associated with steppe or agricultural fields. Indeed, most of the *A. uralensis* specimens were captured along a narrow bank separating one of the fishponds and the canal bordering the forest. In summer, this approximately 3 m wide bank is covered with vegetation up to 2 m tall consisting mainly of *Urtica dioica*. The common point between this ecotone, agricultural fields and steppe is the presence of a rather dense herbaceous stratum. Based on these elements, it looks like *A. uralensis* may be linked to areas with a well developed herbaceous layer. Finally, these results suggest that the niche overlap between *A. uralensis* and *A. agrarius* may be wider than believed thus far. Further studies ought to improve our knowledge and understanding of the ecology of this neglected species.

Individual contributions:

Field work in Cefa Nature Reserve: AMB.
DNA extraction: PGM, AMB.
Molecular identification (PCR and electrophoresis): PGM.
Data analysis: PGM.

Table 4. Touchdown PCR program for the amplification of DNA from *Apodemus sylvaticus* and *A. uralensis.*

Activation of Hot Start Taq	95 °C	5 min	
Denaturation	94 °C	20 sec	**1°C/cycle**
Annealing (touchdown)	from 60 °C to 50 °C	30 sec	
Extension	72 °C	1 min	
Denaturation	94 °C	20 sec	**25 cycles**
Annealing	50 °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	10 min	

Table 5. External morphology (quantitative data: extrema, mean ± s.d.) of *Apodemus* (*Sylvaemus*) specimens captured in Cefa Nature Reserve. For comparative purposes, morphological data from *A. flavicollis* are also included here. Juveniles were excluded from this dataset.

	Apodemus uralensis	***Apodemus sylvaticus***	***Apodemus flavicollis***
Body mass (g)	12.0 - 22.5; 16.19 ± 2.24; n = 44	14.5 - 23.0; 18.83 ± 4.25; n = 3	19.0 - 47.5; 29.00 ± 7.28; n = 15
Hind foot length (cm)	1.72 - 2.03; 1.89 ± 0.07; n = 38	1.56 - 2.23; 1.99 ± 0.37; n = 3	2.12 - 2.47; 2.35 ± 0.11; n = 13
Tail length (cm)	6.90 - 9.65; 7.94 ± 0.58; n = 33	7.28 - 9.25; 8.50 ± 1.06; n = 3	8.87 - 11.36; 10.16 ± 0.88; n = 10
Head and body length (cm)	6.90 - 9.23; 8.23 ± 0.48; n = 37	8.00 - 8.94; 8.61 ± 0.53; n = 3	9.14 - 11.12; 9.90 ± 0.66; n = 12
Ear length (cm)	1.10 - 1.40; 1.28 ± 0.11; n = 15	No data	No data
Ratio tail: head+body	0.83 - 1.09; 0.98 ± 0.06; n = 33	0.91 - 1.04; 0.98 ± 0.07; n = 3	0.94 - 1.20; 1.04 ± 0.07; n = 10
Ratio hind foot: head+body	0.21 - 0.26; 0.23 ± 0.01; n = 37	0.20 - 0.25; 0.23 ± 0.03; n = 3	0.21 - 0.26; 0.24 ± 0.02; n = 12

Acknowledgements. We gratefully acknowledge the facilities made available to us by the authorities of Cefa Nature Reserve during our field investigations.

For providing additional reference samples for cross-reaction testing, thanks are due to (in non-preferential alphabetical order): M. Jurčovičova, N. Khammes, I. Mitre, G. Orazbaeva, V. Stakheev and A. Varshavskiy

This study was partially supported by a EU grant to the WETLANET project (EU FP7 CSA - SUPPORT ACTION, GA 229802). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

Table 6. External morphology (qualitative data) of *Apodemus* (*Sylvaemus*) specimens captured in Cefa Nature Reserve. For comparative purposes, morphological data from *A. flavicollis* are also included here. Juveniles were excluded from this dataset. Numerical values express the proportions of the observations.

	A. uralensis	*A. sylvaticus*	*A. flavicollis*
Neck type	n = 46	n = 6	n = 15
Complete collar	0.00	0.00	0.40
Incomplete collar (no tie)	0.00	0.17	0.40
Laterally enlarged tie (pseudo-collar)	0.00	0.00	0.00
Thin tie	0.00	0.50	0.00
Small but well marked spot	0.02	0.00	0.20
Faint yellowish smear	0.20	0.17	0.00
No mark at all	0.78	0.17	0.00
Flank line	n = 44	n = 6	n = 15
Sharp	0.70	0.67	0.93
Blurry	0.30	0.33	0.07
Belly color	n = 46	n = 6	n = 15
Pure white	0.02	0.17	0.60
Greyish white	0.57	0.50	0.33
Grey	0.35	0.17	0.07
Yellowish	0.07	0.17	0.00

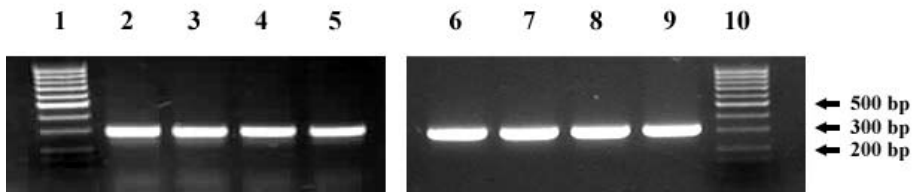

Fig. 1. 1.5% Agarose gels containing 1xGelRed. The first gel contains samples of *A. uralensis* (lanes 2 to 5). The second gel contains samples of *A. sylvaticus* (lanes 6 to 9). Lanes 1 and 10 contain a reference DNA ladder (size marker).

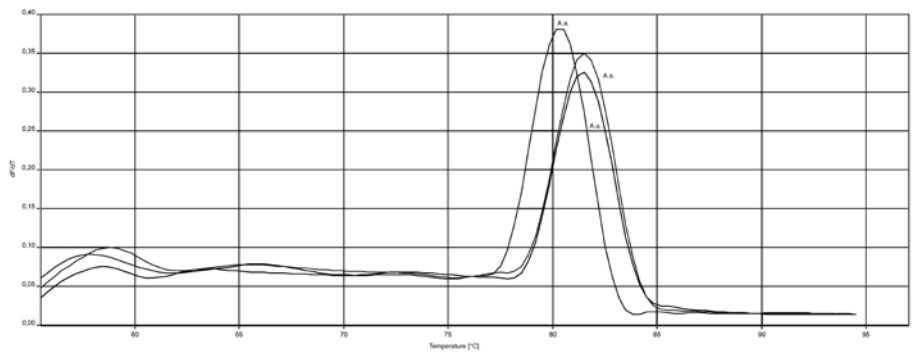

Fig. 2. Melting curves for *A. uralensis* (*A. u.*), *A. sylvaticus* (*A. s.*) and *A. alpicola* (*A. a.*).

References

Juškaitis R. 1999. Pygmy field mouse (*Apodemus uralensis* Pallas): A new mammal species in Lithuania. – *Acta Zoologica Lituanica, Biodiversity*, **9** (3): 118-119.

Juškaitis R. 2003. New data on the geographical distribution of the wood mouse, *Apodemus sylvaticus*, and the pygmy field mouse, *Apodemus uralensis*, in Lithuania. – *Folia Zoologica*, **52** (2): 222–224.

Panteleev P. A. 1998. The rodents of the Palaearctic. Moscow (Russian Academy of Sciences), 117 p.

Pichon B., D. Egan, M. Rogers, J. Gray 2003. Detection and identification of pathogens and host DNA in unfed host-seeking *Ixodes ricinus* L. (Acari: Ixodidae). – *Journal of Medical Entomology*, **40**: 723-731.

Received: 23.01.2012
Accepted: 13.04.2012