Research Article

Faecal DNA template as non-invasive tools in order to distinguish the endangered Pyrenean desman (*Galemys pyrenaicus*, Eulipotyphla, Talpidae) from Mediterranean water shrews (*Neomys anomalus*, Soricomorpha, Soricidae)

José Luis Fernández-García*, María del Prado Vivas-Cedillo

Genetic and Animal Breeding, Universidad de Extremadura, 10071 Cáceres, Spain

Abstract

The molecular genetic tools using non-invasive samples such as faeces have an important role for the monitoring of wildlife and identification of elusive and rare species of semi-aquatic vertebrates. However, under aquatic environmental conditions the faeces undergo physical changes that harm the direct identification by visual inspection and leads to the confusion of the deposits from different cohabitating species. Consequently, the need of a refinement of molecular procedures is suggested. In this study this task was focused on the study of the reliability of the PCR-RFLP method to easily distinguish the faeces of the desman (*Galemys pyrenaicus*) from ecologically related species, including *Neomys anomalus*, but using an *in silico*-RFLP strategy. In this work, the faecal samples were collected from 2013 to 2014 from the LIC "Sierra de Gredos y Valle del Jerte" in the Mountain Central System of Spain, where the southernmost European population of the Pyrenean desman still survives. One primer pair was designed to amplify a very short portion of the mitochondrial cytochrome b gene containing targets for the restriction enzymes *Hinf* I, *Mbo* I and *Hpy* CH4 V, because the combination provides species-specific identifications. Pyrenean desman or Mediterranean water shrews were undoubtedly identified in the collected faeces. Moreover, the degree of homoplasy was studied under different scenarios of the three enzymes combination. Finally, the *in silico*-RFLP profiles given by the three enzymes combination have contributed with valuable information for a successful discrimination of faeces of the Pyrenean desman from those derived of *N. fodiens*, *N. anomalus*, *Nectogale elegans* and *Cinclus cinclus*.

Introduction

The desmans are part of the tribu Desmanini — within the subfamily Desmaninae Mivart, 1871 family Talpidae, order Eulipotyphla — with two genera and only one extant species each: (1) *Galemys pyrenaicus* E. Geoffroy, 1811; representing the Iberian or Pyrenean desman, and (2) *Desmana moschata* Linnaeus, 1758; the Russian desman. Recent molecular data support monophyly for these two desmanines which situate both as the last representatives of a relict lineage of great evolutionary and ecological interest (Igea et al., 2013). The Pyrenean desman (*G. pyrenaicus*) is a small semi-aquatic mammal endemic of the Iberian Peninsula. The species distribution roughly ranges from the Central Mountain System (Spain) and Sierra Da Estrela (Portugal) to the northern Iberian including the French side of the Pyrenees. The Pyrenean desmans live in typical mountain habitats but where the streams flow with clean cold well-oxygenated water and rich on benthic invertebrates (Nores et al., 2007; Igea et al., 2013). This species is characterized by large webbed hind feet, a double layered fur, a long tail and a mobile prehensile snout, which make it a specialist in finding and feeding on larvae of benthonic macroinvertebrates (Palmeirim and Hoffman, 1983; Richard, 1986). They live in pairs with a lifespan of about 3.5 years. The body size is between 11 and 16 cm in length; the tail measures between 12 and 16 cm and weighs between 35-80 g. It is

considered by the IUCN (Fernandes et al., 2008) as a vulnerable relict species and, recently, experiencing elevated historical extinction rates (Igea et al., 2013). The genetic variability found at the cytochrome b (Cyb) gene have permitted to regroup the Pyrenean desman in four phylogenetic lineages, with strong parapatric distribution and only one population declared to be admixed at contact zone (Igea et al., 2013). This strong signature of isolation among lineages has proved to be useful for unambiguously ascribing them to geographic localities (Igea et al., 2013).

Conservation of this endangered species is critically dependent on the knowledge of their populations (Frankham, 2003). Moreover, anthropogenic activities modify their natural habitat resulting in fragmentation, habitat loss or varying the distribution range of the species, which plays a role in shaping the structure of wildlife populations too (Gillet et al., 2014). All this information is essential to try to find molecular methods dedicated to the detection of this elusive species and to develop management strategies (Némoz and Bertrand, 2009) based on collected samples in as many localities as possible, rather than discrete populations (Igea et al., 2013).

Previous researches (Nores et al., 1998; González-Esteban et al., 2003) suggested that Pyrenean desman can be detected by faecal sampling or live-trapping. However, this small semi-aquatic vertebrate species is highly vulnerable to human management during live-trapping method (Gillet et al., 2014), which is supposed to be a potential risk for its survival. The development of non-invasive tech-

*Corresponding author

Email address: pepelufe@unex.es (José Luis Fernández-García)
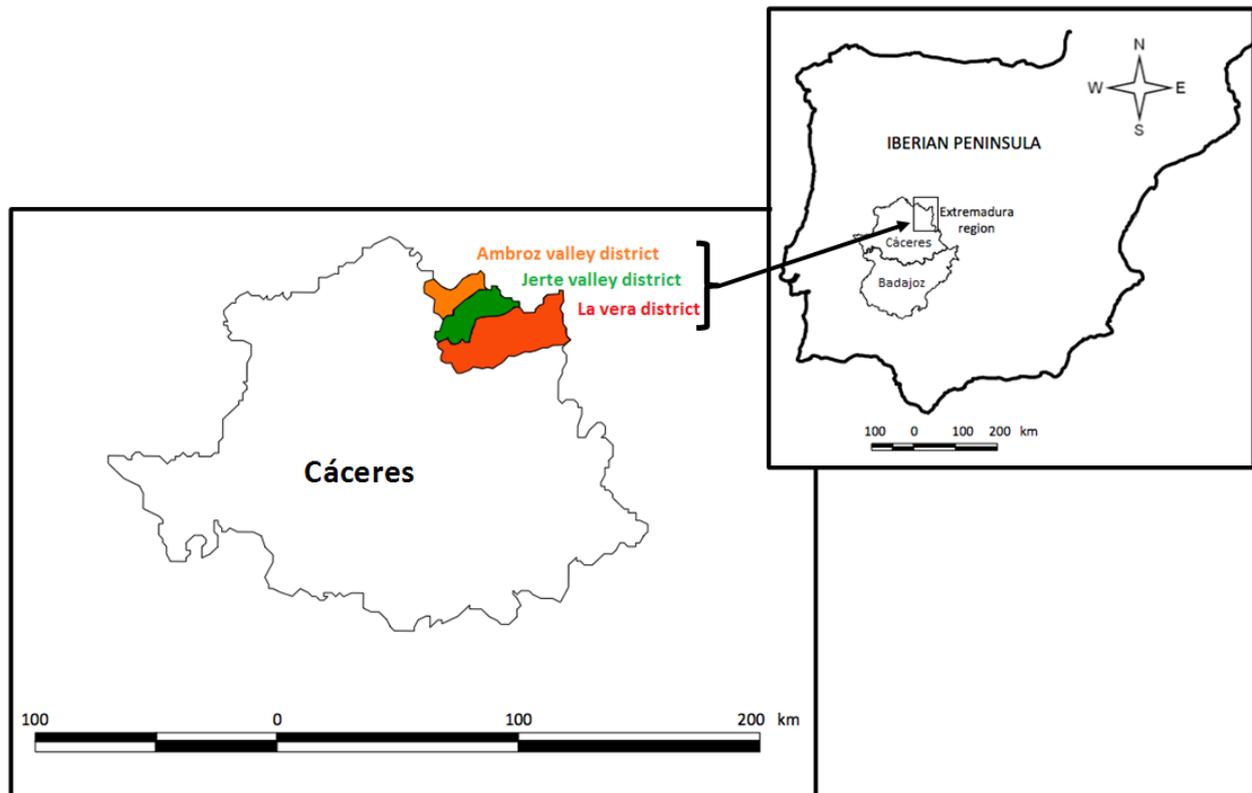
Figure 1 – Geographic position of the desman populations by districts within the LIC "Sierra de Gredos y Valle del Jerte": "Ambroz" Valley district, "Jerte" Valley district and "La Vera" district in orange, green and red, respectively. Site Location: Longitude -5.7211 Latitude 40.1928.

niques like collecting faecal deposits (Igea et al., 2013), made sampling less harmful for monitoring rare or elusive wild species (Lerone et al., 2014). However, these techniques may pose different issues such as a mistakenly assigned Pyrenean desman sample belonging to other semi-aquatic insectivorous species which shares habitats and diet. In the case of the desman a differential assignment should be performed, respect to mainly, three extant *Neomys* species (Family Soricidae): *N. fodiens* (Pennant, 1771), *N. anomalus* (Cabrera, 1907) and *N. teres* (Miller, 1908) (Corbet, 1978; Spitzenberger, 1990; Hutterer, 1993), since they have overlapped geographic distribution. The Eurasian water shrew (*N. fodiens*) is a species of palaearctic distribution which can be found across Europe from the north of the Iberian Peninsula to lake Baikal (including the British Isles to Russia, Norwest Mongolia, China and Nord Korea) but it is absent in the middle and southern Spain. The Mediterranean water shrew (*N. anomalus*, Cabrera 1907) is a palaearctic species that occupies most of the woodlands from southern and central Europe and extends eastwards (Black Sea, Ukraine and Asia Minor) except for the absence in much of the south and west of France, which has caused the isolation of the Iberian and the French Pyrenees populations respect to the rest of the continental species. It is widely distributed in the northern half of the Iberian Peninsula and also in the eastern half of Andalusia and Extremadura (Spain). Based on somatic dimensions and genetic data, the two recognized subspecies differ significantly from the central European morphotype *N. a. milleri* minor than the nominal subspecies *N. a. anomalus* which is being considered the autochthonous morphotype of the Iberian peninsula (Ventura, 2002 and references therein). However, the geographic range of the transcaucasian water shrew (*N. teres*) is comparatively small, being found in Armenia, Azerbaijan, Georgia, Iran and Turkey. Other less related Soricomorpha species like *Nectogale elegans* (Milne-Edwards 1870) has been reported in China, North Sikkim (India) and Eastern Nepal (Molur et al., 2005) being only interesting as a molecular control. The White-throated dipper (*Cinclus cinclus*, Linnaeus 1758) is found throughout the Palaearctic region associated to fast flowing streams, so contributing to confound the desmans' faeces.

The PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) approach (where a PCR product is digested by restriction endonucleases digestions targeting short DNA motifs to generate a specific genetic profile) has been widely used to survey from faeces (Mukherjee et al., 2010) but also it has been proved to be useful to distinguish up to a maximum of 7 sympatric species (Cossíos and Anger, 2006; Bidlack et al., 2007). Hence, when a vast diversity of species occur in sympatry, a set of protocols like PCR-RFLP for assigning scats accurately to species is required (Mukherjee et al., 2010), mainly if PCR product sizes do not differ among species (Montes et al., in press). Recently, in order to discard the droppings of sympatric species, Gillet et al. (2014) have been implementing a PCR-RFLP technique when some confusion can occur with not-fresh, water-soaked faeces or other reasons. However, Gillet et al. (2014) did not include the *N. anomalus* among them. Accordingly, a most complete identification is therefore of great importance to detect the presence of the desman. This is the reason why the aim of this study was (1) to study the reliability of the PCR-RFLP method to avoid and control the wrong assignments of species in small semi-aquatic and ecologically related species, including specially *N. a. anomalus*, (2) to implement this procedure within the context of non-invasive methods able to easily distinguish desmans´ faeces and (3) to prevent mistake of assignations of species attributable to inter-specific homoplasy at some sites by using *in silico*-RFLP on disposable Gen-Bank sequences.

Material and methods

Sampling

All the samples from faeces and tissues were collected with the authorizations licensed by the Extremadura Government (see acknowledgement). The tissue samples of desman were collected from fortuitously dead specimens (personal communication of the senders). The faeces of supposedly desman (n=97) were collected by professionally qualified personnel from three geographical protected areas belonging to LIC ("Lugares de Importancia Comunitaria": officially declared as sites of community importance) of the Red Natura2000 "Sierra de

Gredos y Valle del Jerte": Ambroz Valley district, Jerte Valley district and La Vera district from north-western to north-eastern, respectively, of the Spanish Central Mountain System but inside the Extremadura autonomy (south-western Spain) (Fig. 1). Here the two semi-aquatic *N. anomalus* and *G. pryrenaicus* overlap. The field collection was done in the summer from 2013 to 2015 and the faeces preserved using a 1.8 ml sterile cryo-tube with screw cap (certified premium Line, LABBOX Germany) filled with 70% ethanol and stored at least to -20°C until the next step.

Tissue samples of the Pyrenean desman (n=6) and Mediterranean water shrew (n=1), were used here as PCR controls. Except water shrew sample, the rest of control samples were selected from populations genetically unrelated from those in the LIC sites (Igea et al., 2013) for best monitoring possible cross contaminations after sequencing positive scats. Tissues were preserved in ethanol 70% like described for faeces.

DNA extraction
Genomic DNA from faecal samples were extracted using the Real Pure Spin Food Stool (Real), following the manufacturer protocols (DUR-VIZT S.L, Spain). The genomic DNA from tissues were extracted using a salting out procedures (Fernández-García, 2012). All DNA extractions were made in a dedicated room keeping clean the working area and under a Bunsen burner during the entire procedure but a UV-sterilised cabinet where no tissue samples had been previously managed were used for faeces. DNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies from Thermo Fisher Scientific). In our faecal extraction the DNA concentration was 7–25 ng/µl or even less, but it was not possible to discard the presence of contaminant DNA as those from food of the desman or other sources. Then, the DNA from tissues should be diluted to around 10-20 ng/µl to accommodate to the most common DNA concentration from faeces.

Primer design
Due to the low quality of DNA extracted from faeces (Paetkau, 2003; Lerone et al., 2014) more efficient primer pairs should be designed under the following criteria: (1) to amplify a small size portion of the Cyb gene, since DNA extracted from faeces should be frequently degraded, (2) flanking species-specific Restriction Endonuclease (RE) targets for the species of interest, mainly those with overlapping geographic distribution as occurred for the desman and two shrews (*N. fodiens* and *N. anomalus*) in Spain, (3) that the number of RE cutting site inside the amplicon should have from zero to a maximum of three target, preferably around the middle point of the fragment so as to have a good resolution under agarose gel electrophoresis. The primer pair was designed for this study on the basis of the published Cyb sequences (Tab. S1) of *N. anomalus* (including two subspecies) because this species is the only one that has been described in the range of the Central Systems Mountains according to the distribution maps of the IUCN red list of threatened species. However, the template from several species in the annealing site of selected primer pair contains arbitrary distributed mispriming nucleotide events which do not guarantee amplification of the Cyb, but in the case of *G. pyrenaicus* and *N. anomalus* the PCR assays were successful after experimental adjustments. This event were also observed in species such as *D. moschata*, *N. fodiens*, *N. teres*, *N. elegans* and *C. cinclus* but for these ones only *in silico* analysis was performed. The primer mispriming by species can be seen in Tab. S1.

Species identification by Restriction Enzyme using *in silico*-RFLP
The BLAST tool (Zhang et al., 2000) at NCBI (www.ncbi.nlm.nih.gov) was used to download all the disposable sequences deposited in Gen-Bank of the mitochondrial Cyb (Tab. 1) from the following species: *G. pyrenaicus*, *D. moschata*, *N. anomalus*, *N. fodiens*, *N. teres*, *N. elegans* and *C. cinclus*. All Cyb sequences were trimmed to accommodate to the shorter portion flanked by the designed primer pair, that is, 202 bp long. The MEGA 4.0 software (Tamura et al., 2007) was used to align

the sequences of all these species (see Acc. number in Tab. 1). The RESTRICTIONMAPPER 3.0 software (http://www.restrictionmapper.org/) was used to predict suitable RE with species-specific target within the trimmed sequences. A panel of three RE (*Mbo* I, *Hinf* I and *Hpy* CH4 V) were selected on the basis of inter-species different cutting sites found and according to the criteria of primer selection (see primer design; Material & Methods). The checking of cleavage targets across all the downloaded sequences was conducted using MEGA 4.0 software (Tamura et al., 2007).

Partial cytochrome b amplifications using conventional PCR and PCR-RFLP
All faeces samples were subjected to conventional PCR amplification with *CybNeA-marker* (this study) for after positive *G. pyrenaicus* extract being studied with *Cyb1-marker*, *Cyb2-marker*, *Cyb3-marker* according to Igea et al. (2013) (data not showed). The primer of the *CybNeA-marker* described here amplified a fragment of 202bp that expanded from the nucleotide 368 to 569 of the Cyb gene. This 202 bp fragment overlapped the last and the first portion amplified with *Cyb1-marker* and *Cyb2-marker*, respectively (Igea et al., 2013). Positive reactions to *CybNeA-marker* were subjected to PCR-RFLP analysis as described below. Positive samples to desman were further amplified with *Cyb1* to *Cyb3–marker* and positive PCR were sequenced to identify desman haplotypes (unpublished data). PCR reactions were prepared in a final volume of 25 µl containing 4-5 µl DNA, 1 µM of each primer (forward primer NeAF and reverse primer NeAR; CybNeA-marker), 0.25 µM dNTPs, 1.5 mM MgCl2, 1X buffer reaction and 0.5 U of Biotaq DNA polymerase (Ecogen®). Amplifications were performed with a 2720 thermal cycler (Life Technologies®) under the next protocol: one denature step at 94°C for 5 min followed by 40 cycles (denaturation at 94°C for 30 seconds, annealing at 54°C for 60 seconds, extension at 72°C for 30 seconds) and final extension step at 72°C for 7 minutes. The protocol used when the DNA template consisted of DNA from faecal samples was as follow: the first and penultimate tubes of the racks were reserved as negative controls but the final one was loaded with the positive control using *N. anomalus* template. PCR products were electrophoresed on 1.6% low melting agarose gels (ethidium bromide stained, 0.5 µg/ml) for 1 h at 100 V, but using the first slot for the molecular weight marker (100bp Ladder; NZItech®).
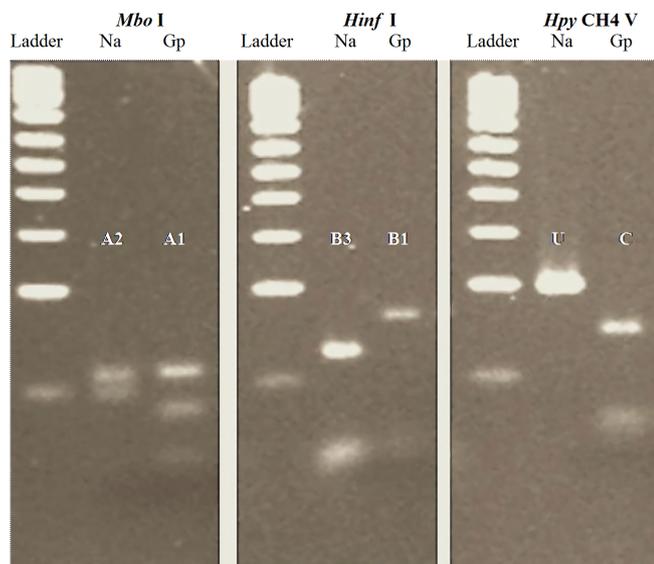
Finally, the surplus PCR reaction from positive tissue and faecal samples (including positive controls) were divided into aliquots of 5 µl to be digested with each one of the selected restriction enzymes. The positive enzymatic digestions were performed in 10 µl volume (5 µl of the PCR product) according to manufacturer recommendations. Digestions were performed for 4 hours at 37°C in a thermal cycler. 10 µl of the digestion were electrophoresed on 2% (Low Melting) agarose gel (ethidium bromide stained, 0.5 µg/ml) for 1 hour at 100 V and, again, the first slot loading with molecular weight marker (100 bp Ladder; NZItech®).

Results and discussion
The prospected area corresponded to the southernmost and the most endangered European population of Pyrenean desman (Fig. 1) where the species only overlapped with the Mediterranean water shrew but specifically with the nominal subspecies *N. a. anomalus*. In the present study and under the described PCR protocols, positive amplifications were obtained with template DNA extracted from tissues samples of both species and faeces. However, in this stage of the protocol the conventional PCR cannot distinguish what species dropped the faeces: the Mediterranean water shrew or the Pyrenean desman. A 60.8% of faeces yielded positive amplification. The percentage of positives amplifications by district was as follows: 72%, 68% and 49% within Ambroz valley, La Vera and Jerte valley district, respectively. Moreover, in the subsequent RFLP assay it was obtained more species-specific information. In accordance with *in silico*-RFLP (Tab. 1), from the positive PCR (n=59) it was determined that 83.01% and 17.00% were identified as belonging to *G. pyrenaicus* and *N. a. anomalus*, respectively. RFLP profiles of any other species were not obtained. The geographic distri-

Table 1 – Species, subspecies, size as sequence numbers and geographic locations of the GenBank sequences used for the *in silico*-RFLP study. Below this first information, the *in silico*-RFLP haplotypes and its corresponding fragment size (FS) for each restriction enzyme. Acc. N. = Accession Numbers. FS = Fragment size. * Asterisk for *Cinclus cinclus* because analyzed sequences was treated as a whole which ranged from Northern Africa to Eastern Europe.

Species	*G. pyrenaicus*	*D. moschata*	*N. anomalus*	*N. a. milleri*	*N. a. anomalus*	*N. fodiens*	*N. teres*	*N. elegans*	*Cinclus cinclus**
Subspecies				*N. a. milleri*	*N. a. anomalus*				
Size	(n=135)	(n=2)	(n=13)	(n=5)	(n=8)	(n=21)	(n=6)	(n=6)	(n=79)
Acc. N. by location									
Unspecified			AJ000466			AJ000465			AM502077 to AM502084, AM502084, AM502091, AM502094 to AM502096, AM502099 to AM502101, AM502104, AM502110 to AM502113, AM502116, AM502118 to AM502123, AM502127, AM502129, AM502130, AM502132, AM502135, AM502137, AM502140 to AM502142, AM502144, AM502145, AM502146, AM502148, AM502149, AM502150, AM502154 to AM502170, AM502172 to AM502176, AM502178 to AM502182, AY397731 to AY397733, AY397746, AY397748, AY397750, AY228054, FJ177319
Caucasus		AB076836 JX290581					HQ621858 to HQ621860	HQ621861	
Central Asia						AB175071, KM092492		AB175095, GU981291 to GU981294, KC503902	
Eastern Asia									
Central Europe			AB175099, AB175100, DQ630407, DQ630409			AB175098, DQ630410, DQ065611, DQ991063, GU981295, JF318987, DQ630421			
Eastern Europe						AB175096, AB175096 7	LK936675 LK936676		
Northern Europe									
Southern Europe	AY833419, JX290582 to JX290601, JX290603 to JX290631, JX290633 to JX290715, JX290632, JX290602		DQ630408, DQ991049 to DQ991055	LK936667 to LK936671	DQ991055, DQ630408, LK936659 to LK936666	DQ991056 to DQ991060, DQ991062, LK936672 to LK936674			
Combined									
RFLP-*Haplotype*	A1B1C, A1B2C, A2B3C	UBdCd	UB3U	UB3U	A2B3U	UB3U, UB4U	UB1U, UUU	UB4U	UB5U
Mbo I (FS)	A1:107,14,81	U:uncut	U:uncut	Uncut	A2:107,95	U:uncut	U:uncut	U:uncut	U:uncut
Hinf I (FS)	B1:163,39 / B2:38,125,39 / B3:121,42,39	Bd:121,81	B3:121,42,39	B3:121,42,39	B3:121,42,39	B3:121,42,39 / B4:121,39,30,12	B1:163,39	B4:121,30,12,39	B5:133,39,30
Hpy CH4 V (FS)	C:142,60	Cd:190,12	U:uncut	U:uncut	U:uncut	U:uncut	U:uncut	U:uncut	U:uncut

Figure 2 – Agarose gel electrophoresis of RFLP-haplotypes for *Neomys anomalus* (*Na*) and *Galemys pyrenaicus* (*Gp*) after *Mbo* I, *Hinf* I and *Hpy* CH4 V digestion. The first run showing 100 bp ladder.

bution by species and by district within the LIC can be seen in Fig. S2 as deduced from our result. With the support of *in silico*-RFLP analysis (Tab. 1), all field samples carried the most common RFLP-haplotype from desman (A1B1C; Fig. 2). Once the positive faeces were assigned as desman, a next stage was performed. These samples were used to be amplified by PCR using the three Cyb primers pairs described in Igea et al. (2013) and those successful amplifications subjected to sequencing methods so as to be assessed again (Fernández-García et al., 2015). The 17% of faeces assigned as shrews showed exclusively the A2B3U RFLP-haplotype (Tab. 1) which may be highly frequent in the *N. a. anomalus* (Fig. 2) from the Iberian Peninsula as suggested by the *in silico*-RFLP assay. Consequently, these samples were rejected for further PCR assays.

The *in silico*-RFLP analysis of the available GenBank sequences belonging to the desman and the nominal subspecies of the water shrew were always consistently concordant with the tissue controls. According to all these results, the digestion with three restriction enzymes leads to an unambiguous identification of both species, desman vs Mediterranean water shrew. In spite of this fact, it was demonstrated that genetic variability of the Cyb across different species can cause some confusing species assignments (Hansen and Jacobsen, 1999; Gomez-Moliner et al., 2004; Gillet et al., 2014). For example, this happened in the cases of RFLP-haplotypes UB3U and UB3U deduced for *N. anomalus* and *N. fodiens* or *N. elegans* and *C. cinclus*, respectively (Tab. 1). Although conflicting profiles for the identification of species can occur mainly with limited number of digestion (e.g. when it was used *Hinf* I and *Mbo* I for desman and shrews in Tab. 1), it may sometimes be infrequent in one of the species. However, it can occur that some enzymes may be determining for identification purposes as *Hpy* CH4 V, which showed higher discrimination power between desman and the rest of species tested, both *in silico*-RFLP analysis and empirically. As stated by Galtier et al. (2006), the mtDNA is greatly characterized by a high level of homoplasy, i.e., phylogenetic/genealogic conflict between sites, which should be solved using exhaustive data set whatever the origin of the homoplasy: the existence of mutation hot spots or ancestral polymorphism. However, the combination of the three restriction patterns was found a valid diagnostic method as deduced from all known sequences of the Pyrenean desman, that is, in the 45 haplotypes described by Igea et al. (2013) and Cabria et al. (2006). Moreover, *in silico*-RFLP should be performed in other species as *N. fodiens* vs. *N. anomalus*, *N. elegans* vs *C. cinclus* (Tab. 1) in order to study and to compare others conflicting genetic profiles. But the last

species should be considered important because it is also a frequent habitant of Iberian rivers.

Finally, in the present study our conclusion is that the accuracy of the PCR-RFLP methods with the aim to identify ecologically cohabiting species depends critically on the abundance and quality of the molecular data sets tested. Hence, apart from genetic information (Fagundes, 2005), well identified faeces provide a wealth of information to conduct ecological studies (Costa et al., 2016) focusing on habitat use (Rivero et al., 2005), diet (Stewart et al., 2003; Nájera-Hillman and Mandujano, 2013), density (Periago and Leynaud, 2009) or endocrinological research (Pereira et al., 2006). ✍

References

Bidlack A.L., Reed S.E., Palsbøll P.J., Getz W.M., 2007. Characterization of a western North American carnivore community using PCR-RFLP of cytochrome b obtained from fecal samples. Conserv. Genet., 8: 1511–1513.

Cabria M.T., Rubines J., Gomez-Moliner B., Zardoya R., 2006. On the phylogenetic position of a rare Iberian endemic mammal, the Pyrenean desman (*Galemys pyrenaicus*). Gene 375: 1–13.

Corbet G.B., 1978. The mammals of the Palaearctic region: a taxonomic review. British Museum (Natural History), London.

Cossíos D., Angers B., 2006. Identification of Andean felid species using PCR-RFLP. Mastozool. Neotrop. 13: 239–244.

Costa E.B.V., Oliveia M.L., Peres P.H.F., Grotta-Neto F., Vogliotti A., Piovezan U., Duarte J.M.B., 2016. Low accuracy of identifying Neotropical deer species by scat morphology. Studies on Neotropical Fauna and Environment, 52(1): 37–42. doi:10.1080/01650521.2016. 1263418

Fagundes V., 2005. Conservation genetics of the muriqui: past, present and future. Neotrop. Primates 13: 85–91.

Fernandes M., Herrero J., Aulagnier S., Amori G., 2008. *Galemys pyrenaicus*. The IUCN Red List of Threatened Species. Version 2014.3. Available at www.iucnredlist.org

Fernández-García J.L., Díaz Caballero J.A., Palacios-González M.J., 2015. Linajes mitocondriales del desmán (*Galemys pyrenaicus*) presentes en el LIC "Sierra de Gredos y Valle del Jerte". XII Congreso de la SECEM, 4 al 7 diciembre, Burgos, España.

Fernández-García J.L., 2012. The endangered *Dama dama mesopotamica*: genetic variability, allelic loss and hybridization signals. Contrib. Zool. 81(4): 223–233.

Frankham R., 2003. Genetics and conservation biology. C. R. Biol. 326: S22–S29.

Galtier N., Enard D., Radondy Y., Bazin E., Belkhir K., 2006. Mutation hot spots in mammalian mitochondrial DNA. Genome Res. 16(2): 215–222.

Gillet F., Cabria M.T., Némoz M., Blanc F., Fournier-Chambrillon C.,Sourp E., Vial-Novella C., Aulagnier S., Michaux J.R., 2014. PCR-RFLP identification of the endangered Pyrenean desman, *Galemys pyrenaicus* (Soricomorpha, Talpidae), based on faecal DNA. Mammalia 79(4): 473–477. doi:10.1515/mammalia-2014-0093

Gomez-Moliner B.J., Cabria M.T., Rubines J., Garin I., Madeira M.J., Elejalde A., Aihartza J., Fournier P., Palazón S., 2004. PCR-RFLP identification of mustelid species: European mink (*Mustela lutreola*), American mink (*Mustela vison*) and polecat (*Mustela putorius*) by analysis of excremental DNA. J. Zool. 262: 311–316.

González-Esteban J., Villate I., Castién E., 2003. A comparison of methodologies used in the detection of the Pyrenean desman *Galemys pyrenaicus* (E. Geoffroy, 1811). Mamm. Biol. 68:387–390.

Hansen M.M., Jacobsen L., 1999. Identification of mustelid species: otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples. J. Zool. 247: 177–181.

Hutterer R., 1993. Order Insectivora. In: Wilson D.E., Reeder D.M. (Eds.) Mammal species of the world: a taxonomic and geographic reference. 2nd ed. Smithsonian Institution Press, Washington, DC, pp. 69–130.

Igea J., Aymerich P., Fernández-González A., González-Esteban J., Gómez A., Alonso R., Gosálbez J., Castresana J., 2013. Phylogeography and postglacial expansion of the endangered semi- aquatic mammal *Galemys pyrenaicus*. BMC Evol. Biol. 13: 115.

Lerone L., Mengoni C., Carpaneto G.M., Randi E., Loy A., 2014. Procedures to genotype problematic non-invasive otter (*Lutra lutra*) samples. Acta Theriol. 59(4): 511–520. doi:10.1007/s13364-014-0195-8

Molur S., Srinivasulu C., Srinivasulu B., Walker S., Nameer P.O., Ravikumar L., 2005. Status of South Asian non-volant small mammals: Conservation Assessment and Management Plan (CAMP), Workshop Report. Coimbatore, India: Zoo Outreach Organization/CBSG-South Asia.

Montes M.G., Fernández-García J.L., Habela M.A., in press. Genetic Variation of the β-tubulin gene of *Babesia caballi* strains. J. Arthropod Borne Dis.

Mukherjee S., Ashalakshmi C.N., Home C., Ramakrishnan U., 2010. An evaluation of the PCR-RFLP technique to aid molecular-based monitoring of felids and canids in India. BMC Res. Notes, 3(1): 159.

Nájera-Hillman E., Mandujano S., 2013. Faecal stable isotope analysis in white-tailed deer (*Odocoileus virginianus*), an alternative method for alimentary ecology studies. Wildl. Biol. Pract. 9(2): 63–75.

Némoz M., Bertrand A., 2009. Plan National d'Actions en faveur du Desman des Pyrénées (*Galemys pyrenaicus*), 2010–2015. Société Française pour l'Etude et la Protection des Mammifères / Ministère de l'Ecologie, de l'Energie, du Développement Durable et de l'Aménagement du Territoire, Toulouse. pp. 159.

Nores C., Ojeda F., Ruano A., Villate I., Gonzalez J., Cano J.M., 1998. Estimating the population density of *Galemys pyrenaicus* in four Spanish rivers. J. Zool. 246: 454–457.

Nores C., Queiroz A.I., Gisbert J., 2007. *Galemys pyrenaicus* (E. Geoffroy Saint-Hilaire, 1811).In: Palomo L., Gisbert J., Blanco J. (Eds.) Atlas y libro rojo de los Mamíferos terrestres de España. Dirección General para la Biodiversidad – SECEM – SECEMU, Madrid, Spain. pp. 92–95.

Paetkau D., 2003. An empirical exploration of data quality in DNA-based population inventories. Mol. Ecol. 12: 1375–1387.

Palmeirim J.M., Hoffmann R.S., 1983. *Galemys pyrenaicus*. Mamm. Species 207: 1–5.

Pereira R.J.G., Duarte J.M.B., Negrão J.A., 2006. Effects of environmental conditions, human activity, reproduction, antler cycle and grouping on fecal glucocorticoids of free-ranging Pampas deer stags (*Ozotoceros bezoarticus bezoarticus*). Horm. Behav. 49: 114–122.

Periago M.E., Leynaud G.C., 2009. Density estimates of *Mazama gouazoubira* (Cervidae) using the pellet count technique in the arid Chaco (Argentina). Ecol. Austral. 19(1): 73–77.

Richard P.B., 1986. Le Desman des Pyrénées. Un mammifere inconnu a découvrir. Editions Le Rocher, Monaco.

Rivero K., Rumiz D.I., Taber A.B., 2005. Differential habitat use by two sympatric brocket deer species (*Mazama gouazoubira* and *Mazama americana*) in a seasonal Chiquitano forest of Bolívia. Mammalia 69(2): 169–183.

Spitzenberger F., 1990. Gattung *Neomys* Kaup, 1829. In: Niethammer J., Krapp F. (Eds.) Handbuch der Säugetiere Europas: Insektenfresser, Herrentiere. AULA-Verlag, Wiesbaden, Germany, pp. 313–316.

Stewart K.M., Bowyer R.T., Kie J.G., Dick B.L., Ben-David M., 2003. Niche partitioning among mule deer, elk, and cattle: do stable isotopes reflect dietary niche? Ecoscience. 10(3): 297–302.

Tamura K., Dudley J., Nei M., Kumar S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599

Ventura J, 2002. Musgaño de Cabrera (*Neomys anomalus* Cabrera, 1907). In: Palomo L.J., Gisbert J. (Eds.) Atlas de los Mamíferos Terrestres de España. Dirección General de Conservación de la Naturaleza-SECEM-SECEMU, Madrid, pp. 94–97.

Zhang Z., Schwartz S., Wagner L., Miller W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7(1–2): 203–214.

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Primers used to amplify the cytochrome b fragment, and mismatch distribution of primer bias in different sequences of each species or subspecies within the homologous selected primer sites.

Figure S2 Number of PCR positive samples distributed by species.