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DNA barcoding of Austrian eulipotyphlans

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Abstract

DNA barcoding based on the cytochrome c oxidase subunit I (COI) gene is widely applied and a reliable method for species identification, but reference libraries are essential for this task. In this study, I present DNA barcodes of 100 individuals, spread over 28 mammalian species and an emphasis on eulipotyphlans. Each eulipotyphlan species formed a monophyletic clade with high bootstrap support and the analysis of intra- and interspecific genetic distances is in congruence with this result: the lowest interspecific genetic distance is still higher than the highest intraspecific and a barcoding gap is clearly distinguishable. Two different approaches were used to estimate the number of molecular taxonomic units (MOTUs): Automatic Barcode Gap Discovery and the Poisson tree processes model. Both came to the conclusion that each species forms its own MOTU.

In addition to fresh samples, mostly muscle tissue, historical samples were amplified in short fragments with partial success.

Kurzfassung

DNA-Barcoding basierend auf dem Gen Cytochrom-c-Oxidase ist eine weit verbreitete und verlässliche Methode zur Bestimmung von Arten, Referenzdatenbanken sind hierfür aber dringend nötig. In dieser Studie präsentiere ich DNA Barcodes von 100 Individuen aus 28 Säugetierarten, mit einem Schwerpunkt auf der Ordnung Eulipotyphla. Jede Art innerhalb der Eulipotyphla formte eine monophyletische Klade mit hohem Bootstrap-Support und die Analyse der intra- und interspezifischen genetischen Distanzen stimmt mit diesem Ergebnis überein: die geringste interspezifische Distanz ist höher als die höchste intraspezifische und ein Barcoding Gap ist deutlich erkennbar. Zwei verschiedene Methoden wurden zur Bestimmung der Anzahl der *molecular taxonomic units* (MOTUs) verwendet: *Automatic Barcode Gap Discovery* und *Poisson tree processes model*. Beide kamen zu dem selben Ergebnis: jede Spezies bildet ein eigenes MOTU.
Zusätzlich zu den Frischproben, großteils aus Muskelgewebe bestehend, wurden auch historische Proben in kurzen Fragmenten amplifiziert, was aber nur teilweise erfolgreich war.

Introduction

Although Austria is a rather small country, it is inhabited by 97 mammalian species (Spitzenberger 2001) and given that there are 194 known species in Europe (Mitchell-Jones et al. 1999), the Austrian species inventory contains 50% of them.
Mammals rank amongst the most studied animal groups, with their taxonomy well documented in the literature (Wilson & Reeder 2005). However, small mammals like eulipotyphlans and bats are often neglected.

The order Eulipotyphla comprises five families: the hedgehogs and gymnures (Erinaceidae), the true shrews (Soricidae), the moles, shrew-like moles and desmans (Talpidae), the solenodons (Solenodontidae) and the extinct Nesophontidae (Douady et al., 2002).
The Eulipotyphla are the third largest Eutherian order after Rodentia and Chiroptera (Ye et al., 2006).

Hedgehogs (subfamily Erinaceinae) and gymnures (subfamily Galericinae) form the family Erinaceidae. There are two species of hedgehogs in Austria: The Northern White-breasted Hedgehog (*Erinaceus roumanicus*) and the West European Hedgehog (*E. europaeus*). *E. roumanicus* inhabits Eastern Europe and Ponto-Mediterranean regions. The range of *E. europaeus* covers Western Europe and spreads to Scandinavia and European Russia (Hutterer, 2005). Both species are nocturnal and inhabit a variety of urban and rural habitats.

The family Talpidae is split into three extant subfamilies: Desmaninae, Uropsilinae and Talpinae. The latter subfamily is divided into five tribes (McKenna and Bell, 1997), of which the Old World fossorial moles (Talpini) are the most species rich and contain the only Austrian member of this family; the European mole (*Talpa europea*). *T. europea* is widespread throughout Eurasia from Britain to the Ob and Irtysh Rivers in Russia (Hutterer, 2005). They are usually found in areas with deep, soft soil, enabling them to dig tunnels. Other ecological parameters, like altitude and temperature, are of lesser importance (Nowak, 1999).

Shrews (Soricidae) are the fourth largest mammalian family (376 species; Hutterer, 2005). Their wide distribution, including Europe, Africa, Asia, North America and the north of South America, and their adaptation to various habitats, from tropical rain forest to arctic tundra, and from semi-aquatic regions to deserts, make them one of the most successful clades of extant mammals (Nowak, 1999). They are divided in two (Soricinae and Crocidurinae; Dubey et al., 2007d) or three subfamilies (Soricinae, Crocidurinae and Myosoricinae; Hutterer, 2005), of which only two are present in Austria; the Soricinae and Crocidurinae.

The red-toothed shrews of the subfamily Soricinae have a Holarctic distribution with 142 species from 13 genera living in the northern Hemisphere. The Crocidurinae are restricted to the Old World and represent 209 species from 10 genera (Hutterer, 2005).

In Austria, there are six species of soricine shrews in two genera; *Sorex* and *Neomys*, and three species of crocidurine shrews in the genus *Crocidura*.

Bats (order Chiroptera) are one of the largest monophyletic clades in mammals and account for 20% of extant mammalian species. They are present throughout most of the world and are important pollinators and insect predators (Patterson et al., 2003).

Their 1100 species (Simmons, 2005) are arranged in 20 families with three of them occurring in Austria: Vespertilionidae (25 species in A), Molossidae (1 species in A) and Rhinolophidae (2 species in A).

Eulipotyphlans and bats are important reservoirs for several diseases, even in Europe. Hilbe et al. 2006 reported the first unequivocal detection of Borna disease virus (BDV), a virus which causes fatal neurological disorders in horses and sheep, in *Crocidura leucodon* in Switzerland. Different species of hantaviruses, which cause potentially fatal diseases in humans, were found in shrews and moles in central Poland (Gu et al. 2014). Protozoan parasites like *Cryptosporidium parvum*, which is known to infect livestock, were found in British European hedgehogs (Sangster et al. 2016) and *Leptospira* ssp., which causes a febrile disease in animals and humans, was found in shrews in Germany (Mayer-Scholl et al. 2014).

Two bat lyssaviruses referred to as European bat lyssaviruses (EBLVs) 1 and 2, which are closely related to the classical rabies virus, where detected in different bats all over

Europe (Schatz et al. 2013, Moldal et al. 2017). Identifying these potential carriers of viruses is an important task.

While some of these species are easily identified by morphological characters, many closely related species are often difficult to distinguish based on phenotypic characters. For example, the red-toothed shrews *Sorex araneus* and *Sorex coronatus* are similar looking species and occur sympatrically in Vorarlberg. The differentiation of these two species is tricky and is either done by discriminant analysis of skull morphometric characters (Turni & Müller 1996), which makes the collection of dead specimen necessary, or by biochemical analysis of the urinary pepsin (Neet & Hauser 1991). These days, molecular techniques, like DNA barcoding, are the most promising ways to distinguish these species.

DNA barcoding is a system designed to provide accurate, fast and automatable species identification by using short and standardized gene regions as internal species tag (Hebert & Gregory, 2005). Since Hebert et al. (2003) first employed the mitochondrial cytochrome c oxidase subunit I (COI) for species identification, it has been shown that this gene could serve as DNA barcode in different kinds of animals (Clare et al., 2007; Waug, 2007).

DNA barcoding is a powerful tool in species identification, and has several advantages over a strict morphological approach: DNA extraction of samples undergone digestion is still possible (Long et al., 2013), there is only a small piece of tissue needed (Yang et al., 2012) and the DNA barcodes are constant throughout development.

DNA barcoding also helps in the detection of cryptic species. The incorporation of DNA barcoding into bat surveys has led to the taxonomic reevaluation of species groups and discovered several cryptic taxa (Borisenko et al., 2008; Francis et al., 2010). Two surveys on crocidurine shrews in Vietnam and Guinea found cryptic genetic diversity with potential new species (Bannikova et al. 2011, Jacquet et al. 2012).

The accuracy DNA barcodes rely heavily on a complete reference data base (Ratnasingham & Hebert, 2007). The Austrian Barcode of Life (www.abol.ac.at) tries to provide this kind of reference library for all species of animals, plants and fungi recorded for Austria (Sattmann et al., 2014).

In this study, I created DNA barcodes for most of Austria's Eulipotyphla, some Chiroptera and other mammals.

Material & Methods

Sample collection

Most of the tissue material was collected from already deposited, frozen voucher specimens at the national history museum Vienna, the Biologiezentrum Linz and Joanneum Graz. Specimen, brought to us by private donors and other institutions, were deposited at the Joanneum Graz. Each specimen was photographed and identified to species level by morphological characters. The tissue samples taken were mostly muscle tissue or wing tissue in the case of bats. These tissue samples were stored in 96% Ethanol at -23°C. Samples of dry historical material were also taken but kept dry.

DNA extraction

DNA was extracted using a rapid Chelex protocol (Richlen & Barber 2005). In addition, the DNA of historical material was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following a protocol for ancient nuclear DNA from toe pads (Fulton et al. 2012) with some modifications; after the 24-hour rehydration, the samples were washed three times in HPLC water, followed by two times in ethanol and again two times in HPLC water. The extraction of historical material was conducted in a separate sterile room under a laboratory extractor hood, with working materials exposed to UV light prior to every step and constant positive air pressure.

Table 2. Primers used for the amplification of the whole barcoding region. PCR primers were tagged with M13 tails (Messing 1983) to facilitate efficient sequencing.

Primer	Sequence	Reference
St_f	CYNCWAMCCACAARGAYATNGGNAC	Meusnier et al. 2008
E_r1	GTRKGAGATRATTCCGAAKCC	Schäffer et al. 2017
E_r2	ATNCCTATGTANCCGAATGGRTCTTT	Patel et al. 2010
E_r3	TANACNTCNGGNTGNCCNAANAATCA	Schäffer et al. 2017
C_VF1LFt1		Ivanova et al. 2007
LepF1_t1	TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG	Hebert et al. 2004
VF1_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG	Ivanova et al. 2006
VF1d_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG	Ivanova et al. 2006
VF1i_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG	Ivanova et al. 2006
C_VR1LRt1		Ivanova et al. 2007
LepR1_t1	CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA	Hebert et al. 2004

VR1_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA	Ivanova et al. 2006
VR1d_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA	Ward et al. 2005
VR1i_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA	Ivanova et al. 2006

PCR and DNA sequencing

PCR amplification was performed in a 10 µL volume reaction containing 1 µL of DNA extract, 0.35 µL dNTP mix (0.25 µM), 0.25 µL of each primer (0.25 µM), 1 µL Buffer with $MgCl_2$ and 0.1 µL Taq DNA polymerase (1 U, Biotherm).

The PCR conditions were as follows: 3 min at 95 °C; 45 cycles with 30 sec at 95 °C for denaturation, 30 sec at 46-52 °C (depending on the primers) for annealing and 1 min at 72 °C for extension. The final extension phase at 72 °C lasted for 7 min.

As an alternative, Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used. The 10 µL volume reaction contained 0.2 µL dNTP mix (0.25 µM), 0.5 µL of each primer, 2 µL 5X Phusion HF Buffer and 0.1 µL Phusion DNA polymerase (0.2 U).

The PCR conditions followed the manufacturer's protocol; an initial denaturation at 98 °C for 30 sec was followed by 45 cycles of 98 °C for 10 sec, 46 °C for 30 sec and 72 °C for 1 min. The final extension phase lasted for 10 min at 72 °C.

2 µL of PCR products were mixed with 2 µL of Xylene cyanol as tracking dye and then visualized by electrophoresis on 2% agarose gel containing PeqGreen, a fluorescence dye for staining DNA.

PCR products were purified using ExoSAP-IT (Affymetrix).

The purified PCR products were sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the 8 µL sequencing reaction consisted of 3.95 µL distilled water, 1.6 µL 5X BigDye Sequencing Buffer, 0.3 µL BigDye Terminator Reaction Mix, 0.25 µL M13 sequencing primer (0.25 µM) and 2 µL purified PCR product.

The sequencing reaction consisted of an initial denaturation for 4 min at 94 °C, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 30 sec at 50 °C and extension for 3 min at 60 °C. The final extension phase lasted for 7 min at 60 °C.

Sequenced DNA was purified with Sephadex G-50 (Amersham Biosciences) and visualized on an ABI 3130xl capillary sequencer (Applied Biosystems).

Table 3. Primers used for the amplification of the six overlapping fragments of the barcoding region.

Primer	Sequence (5'-3')	Fragment	Reference
St_f	CYNCWAMCCACAARGAYATNGGNAC	1	Meusnier et al. 2008
st_r	GAARATYATNAYGAANGCRTGNGC	1	Meusnier et al. 2008
In1f	GGNGAYGAYCARATNTACAATGT	2	Schäffer et al. 2017
In1r	GGNGGNAGNAGTCARAARC	2	Schäffer et al. 2017
A2f2	GNGCNCCNGAYATRGCNTTYCC	3	Schäffer et al. 2017
A2r2	CNGCNAGRTGNAGNGARAARATNGC	3	Schäffer et al. 2017
In3f	GGNGTNGGNACNGGNTGAAC	4	Patel et al. 2010
In3r	GATCANACGAANAGNGGNGTYTG	4	Patel et al. 2010
M4f	GCNGGNGTNTCNTCNATTYTAGG	5	Schäffer et al. 2017
M4r	ARGTTGTRTTYARGTTNCGGTCYGT	5	Schäffer et al. 2017
M5f1	CNCARTAYCAAACNCCNCTNTTYGT	6	Schäffer et al. 2017
E_r3	TANACNTCNGGNTGNCCNAANAATCA	6	Schäffer et al. 2017

Data analysis

The sequences were edited in MEGA 6.06 (Tamura et al. 2013) and were subjected to a blast search in GenBank to verify species identification. Due to poor coverage of most mammal lineages, the following in-depth analysis was only done with the eulipotyphlan dataset. The Kimura-2-parameter (Kimura 1980) model was used to calculate intra- and interspecific genetic distances, as it is most widely used in barcoding studies (Hebert et al. 2003). A Neighbor-Joining tree based on K2P was constructed in MEGA 6.06 with 1000 bootstrap replications (Felsenstein 2005).

To estimate the number of molecular operational taxonomic units (MOTUs, Goldstein & DeSalle 2011), two different approaches were used: Automatic Barcode Gap Discovery (Puillandre et al. 2012) and Poisson-Tree-Processes (Zhang et al. 2013).

ABGP was conducted under the following conditions: Pmin = 0.001, Pmax = 0.1, Steps = 10, relative gap width = 1, Nb bins = 20 and K2P.

bPTP was carried out with a Maximum Likelihood tree (model: TN93+G+I, model selection was based on corrected Akaike Information Criterion [AICc: 6814.832] and Maximum likelihood value [lnL: -3272.786] and carried out in MEGA 6.06) created in MEGA 6.06, with the following conditions: 100000 MCMC generations, 100 Thinning, 0.1 Burn-in and the seed 123.

Results

Sequencing

A total of 100 individuals, spread over 28 species, were successfully PCR amplified (Table 1). 67 of those 100 individuals were eulipotyphlans, while the rest mostly consisted of bats, carnivorans and artiodactyls.

Two other documented Austrian eulipotyphlans, *Sorex coronatus* and *Crocidura russula*, were not included in the analysis as no specimens with adequate DNA quality were obtainable.

Although there were many specimens of bats available, they showed poor results in PCR amplification and subsequently led to poor coverage of the Austrian species.

The DNA extraction of historical, dry samples was successful, but neither the whole barcode nor fragment 3 (A2f2 – A2r2) was possible to amplify and in further consequence, no complete barcode was achieved.

BLAST searches in GenBank confirmed the morphological identification of species and assured that even the historical samples were not contaminated with foreign DNA.

In most species, except for some bats, it was possible to obtain barcodes using either the primer pair St_f and E_r3 or the primer mix C_VF1LFt1 - C_VR1LRt1 (Table 2).

Table 1. Species, internal IDs and location for all successfully sequenced Specimen.

ID	Species	Collection localities
174	*Capreolus capreolus*	Styria
188	*Capreolus capreolus*	Styria
358	*Capreolus capreolus*	Styria
395	*Capreolus capreolus*	Burgenland
412	*Capreolus capreolus*	Tyrol
414	*Capreolus capreolus*	Tyrol
415	*Cervus elaphus*	Tyrol
142	*Crocidura leucodon*	Upper Austria
250	*Crocidura leucodon*	Styria
270	*Crocidura leucodon*	Styria
293	*Crocidura leucodon*	Styria
312	*Crocidura leucodon*	Styria
321	*Crocidura leucodon*	Styria
16	*Crocidura suaveolens*	Styria

166	*Crocidura suaveolens*	Upper Austria
249	*Crocidura suaveolens*	Styria
272	*Crocidura suaveolens*	?
275	*Crocidura suaveolens*	Styria
282	*Crocidura suaveolens*	?
313	*Crocidura suaveolens*	Styria
323	*Crocidura suaveolens*	Styria
325	*Crocidura suaveolens*	Styria
428	*Crocidura suaveolens*	Salzburg
429	*Crocidura suaveolens*	Vienna
139	*Erinaceus europaeus*	Upper Austria
159	*Erinaceus europaeus*	Upper Austria
160	*Erinaceus europaeus*	Lower Austria
189	*Erinaceus europaeus*	Vorarlberg
9	*Erinaceus roumanicus*	Styria
10	*Erinaceus roumanicus*	Styria
171	*Erinaceus roumanicus*	Upper Austria
248	*Erinaceus roumanicus*	Styria
310	*Erinaceus roumanicus*	Styria
403	*Martes martes*	Styria
350	*Mustela erminea*	?
31	*Mustela nivalis*	Styria
35	*Mustela nivalis*	Styria
185	*Mustela nivalis*	Styria
349	*Mustela nivalis*	?
404	*Mustela nivalis*	Styria
186	*Mustela putorius*	Styria
125	*Myotis bechsteini*	Upper Austria
165	*Myotis myotis*	Upper Austria
128	*Myotis mystacinus*	Upper Austria
285	*Myotis nattereri*	Styria
148	*Neomys anomalus*	Upper Austria
150	*Neomys anomalus*	Tyrol
151	*Neomys anomalus*	Upper Austria
155	*Neomys anomalus*	Upper Austria
180	*Neomys anomalus*	Styria
233	*Neomys anomalus*	Salzburg
245	*Neomys anomalus*	Styria

278	*Neomys anomalus*	Styria
144	*Neomys fodiens*	Upper Austria
145	*Neomys fodiens*	Lower Austria
146	*Neomys fodiens*	Upper Austria
120	*Nyctalus noctula*	Upper Austria
375	*Ovis gmelini musimon*	Tyrol
29	*Pipistrellus kuhlii*	Styria
218	*Pipistrellus pygmaeus*	Salzburg
132	*Rhinolophus hipposideros*	Lower Austria
265	*Rhinolophus hipposideros*	?
366	*Rupicapra rupicapra*	Styria
408	*Rupicapra rupicapra*	Tyrol
409	*Rupicapra rupicapra*	Tyrol
134	*Sorex alpinus*	Upper Austria
135	*Sorex alpinus*	Upper Austria
152	*Sorex alpinus*	Upper Austria
153	*Sorex alpinus*	Upper Austria
172	*Sorex alpinus*	Styria
276	*Sorex alpinus*	Styria
13	*Sorex araneus*	Styria
143	*Sorex araneus*	Upper Austria
147	*Sorex araneus*	Upper Austria
156	*Sorex araneus*	Upper Austria
157	*Sorex araneus*	Upper Austria
158	*Sorex araneus*	Upper Austria
269	*Sorex araneus*	Styria
274	*Sorex araneus*	Styria
277	*Sorex araneus*	Styria
305	*Sorex araneus*	Styria
257	*Sorex minutus*	Styria
273	*Sorex minutus*	Styria
279	*Sorex minutus*	Styria
280	*Sorex minutus*	Styria
292	*Sorex minutus*	?
294	*Sorex minutus*	Styria
370	*Sus scrofa*	Burgenland
25	*Talpa europaea*	Styria
161	*Talpa europaea*	Lower Austria

167	*Talpa europaea*	Upper Austria
168	*Talpa europaea*	Upper Austria
170	*Talpa europaea*	Upper Austria
178	*Talpa europaea*	Styria
234	*Talpa europaea*	Lower Austria
430	*Talpa europaea*	Lower Austria
137	*Vespertilio murinus*	Upper Austria
36	*Vespertilio murinus*	Styria
387	*Vulpes vulpes*	Tyrol
402	*Vulpes vulpes*	Styria

Alignment

The length of the sequences ranged from 375 to 747 bp, with a mean length of 591 bp.

The final eulipotyphlan alignment was trimmed and 4 sequences were excluded in consequence of their short length. The 446 bp long alignment had 191 polymorphic sites, of which 172 were parsimony informative. No insertions, deletions or Stop-Codons were found after translation.

Table 4. Primer combinations which led to the successful amplification of the barcoding region, sorted by species.

	Primers					
	fwd			rev		
Species	St_f	C_VF1LFt1	E_r1	E_r2	E_r3	C_VR1LRt1
Capreolus capreolus	✓	✓			✓	✓
Cervus elaphus	✓	✓			✓	✓
Crocidura leucodon	✓	✓			✓	✓
Crocidura suaveolens	✓	✓			✓	✓
Eptesicus nilsonii		✓			✓	
Erinaceus europaeus	✓				✓	
Erinaceus roumanicus	✓	✓			✓	
Martes martes	✓				✓	
Mustela erminea		✓				✓
Mustela nivalis	✓	✓			✓	✓
Mustela putorius	✓	✓	✓		✓	✓
Myotis bechsteini		✓			✓	✓

Myotis myotis		✓				✓
Myotis mystacinus		✓				✓
Neomys anomalus	✓				✓	
Neomys fodiens	✓				✓	
Nyctalus noctula	✓		✓			
Ovis orientalis musimon	✓				✓	
Pipistrellus kuhlii		✓			✓	
Pipistrellus nathusii		✓			✓	
Pipistrellus pygmaeus	✓				✓	
Rhinolophus hipposideros	✓			✓		
Rupicapra rupicapra	✓				✓	
Sorex alpinus	✓	✓			✓	✓
Sorex araneus	✓	✓			✓	✓
Sorex minutus		✓				✓
Sus scrofa	✓	✓	✓		✓	✓
Talpa europaea	✓	✓			✓	✓
Vespertilio murinus		✓			✓	
Vulpes vulpes	✓				✓	

Table 5. Successfully amplified fragments of the COI barcoding region.

ID	Species	1	2	3	4	5	6
38	*Erinaceus roumanicus*	✓	✓			✓	✓
39	*Erinaceus roumanicus*	✓					✓
40	*Erinaceus roumanicus*	✓				✓	
48	*Sorex alpinus*	✓	✓				
75	*Neomys fodiens*	✓				✓	✓
78	*Crocidura suaveolens*	✓	✓		✓	✓	✓
80	*Crocidura suaveolens*	✓	✓		✓	✓	
81	*Crocidura leucodon*	✓	✓		✓	✓	
82	*Crocidura leucodon*	✓	✓		✓		

Neighbor-Joining tree

The NJ tree (Figure 1) yielded a topology representing the three families Talpidae, Erinaceidae and Soricidae. Each species formed its own clade with a bootstrap support of 100. The intraspecific distances in the tree are small with *Erinaceus roumanicus*, *Sorex minutus* and *Neomys fodiens* being the exceptions. In addition, a Maximum-Likelihood tree under the TN93+G+I model (Tamura & Nei 1993) was generated and depicted the same topology (Figure 3).

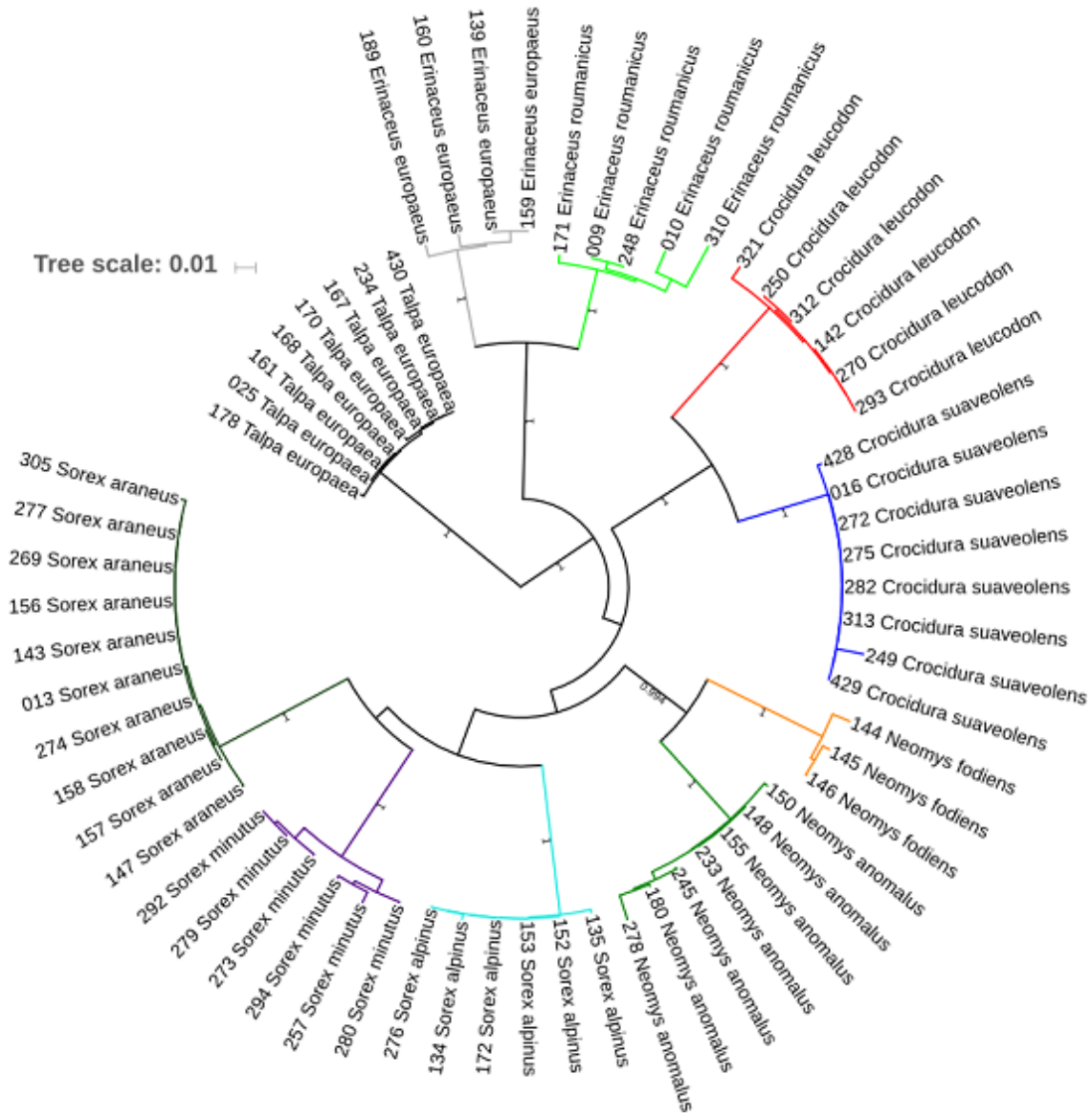

Figure 1. Neighbor-Joining tree of the eulipotyphlan dataset with bootstrap values over 99% shown.

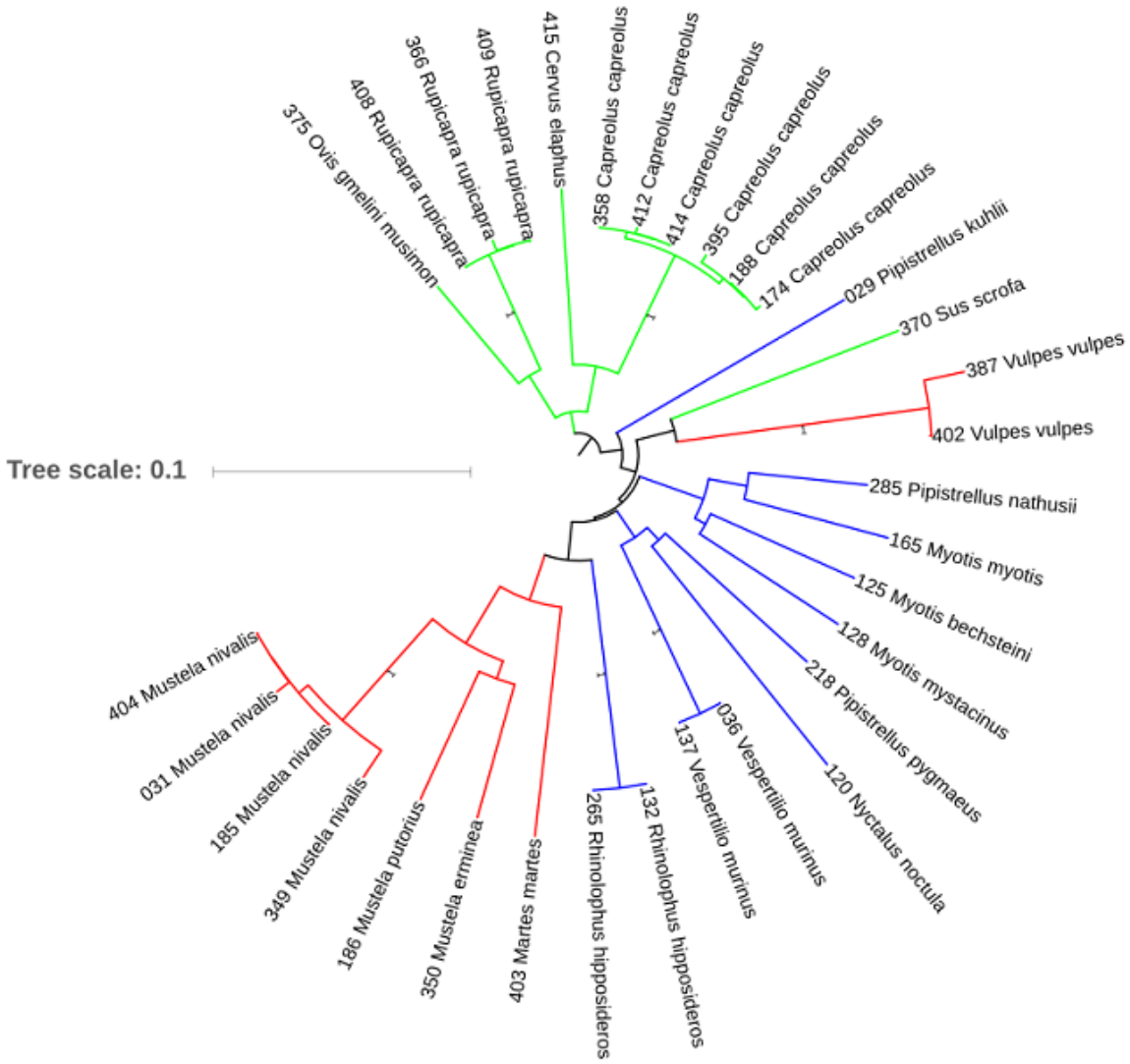

Figure 2. Neighbor-Joining tree of the other mammalian barcodes created in this study with bootstrap values over 99% shown. Green branches indicate artiodactyls, red branches carnivorans and blue ones chiropterans.

The NJ tree of the sequenced mammals excluding eulipotyphlans yielded a topology with poorer representation of the actual evolutionary relationships between these species. The wild boar (*Sus scrofa*) formed a clade together with the red fox (*Vulpes Vulpes*), while the other artiodactyls and carnivorans formed their own clades, respectively. The chiropterans do not form a monophyletic clade but seem to form basal sister taxons of the carnivorans, with *Pipistrellus kuhlii* being the sister taxon to all sequenced specimen except the clade of artiodactyls. These higher order relationships show low bootstrap support as opposed to the clades consisting of multiple specimens of the same species.

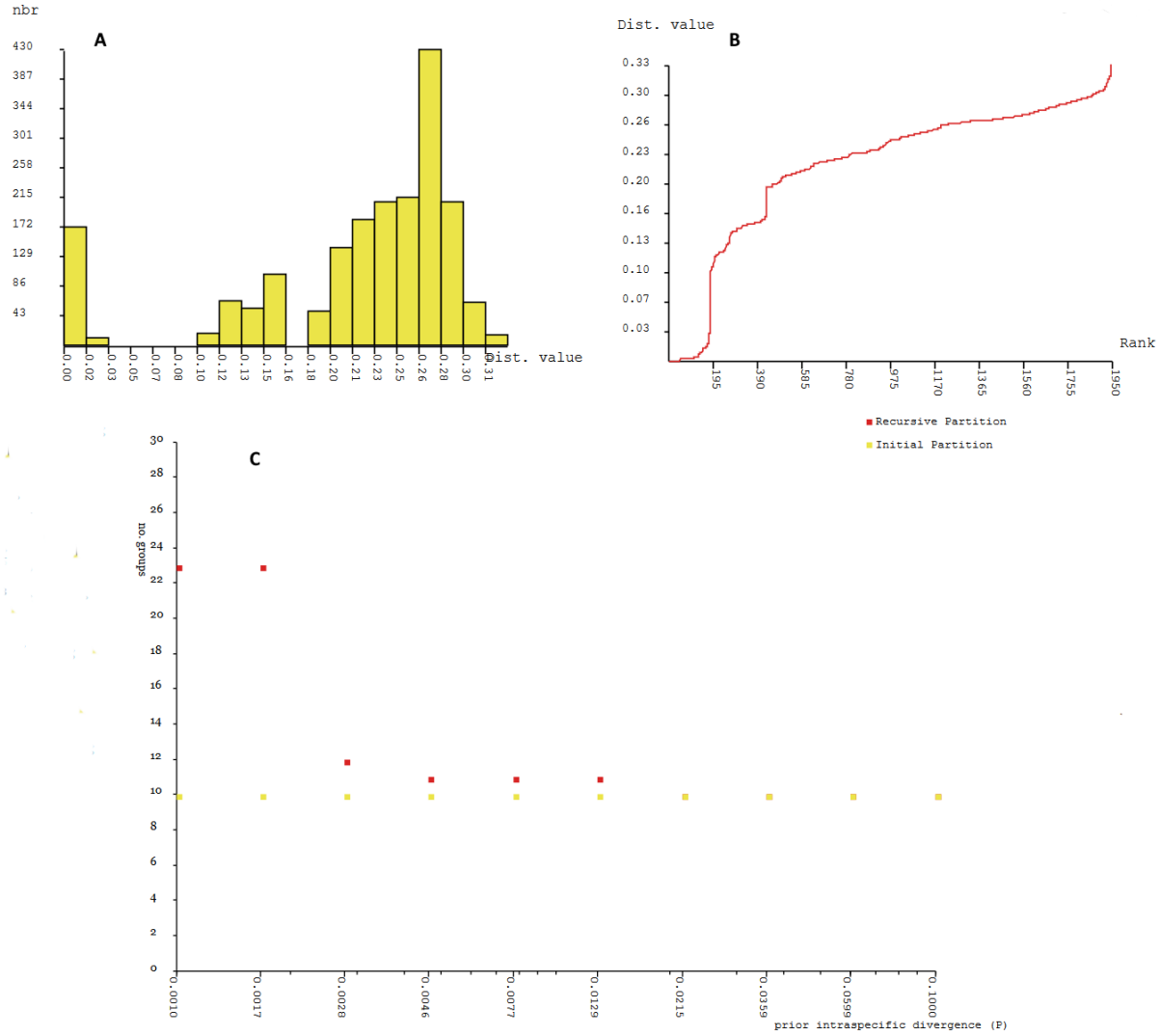

Figure 2. Distribution of pairwise distances (A), ranked distances (B) and the number of MOTUs generated by Automatic Barcode Gap Discovery under K2P according to prior intraspecific divergence(C).

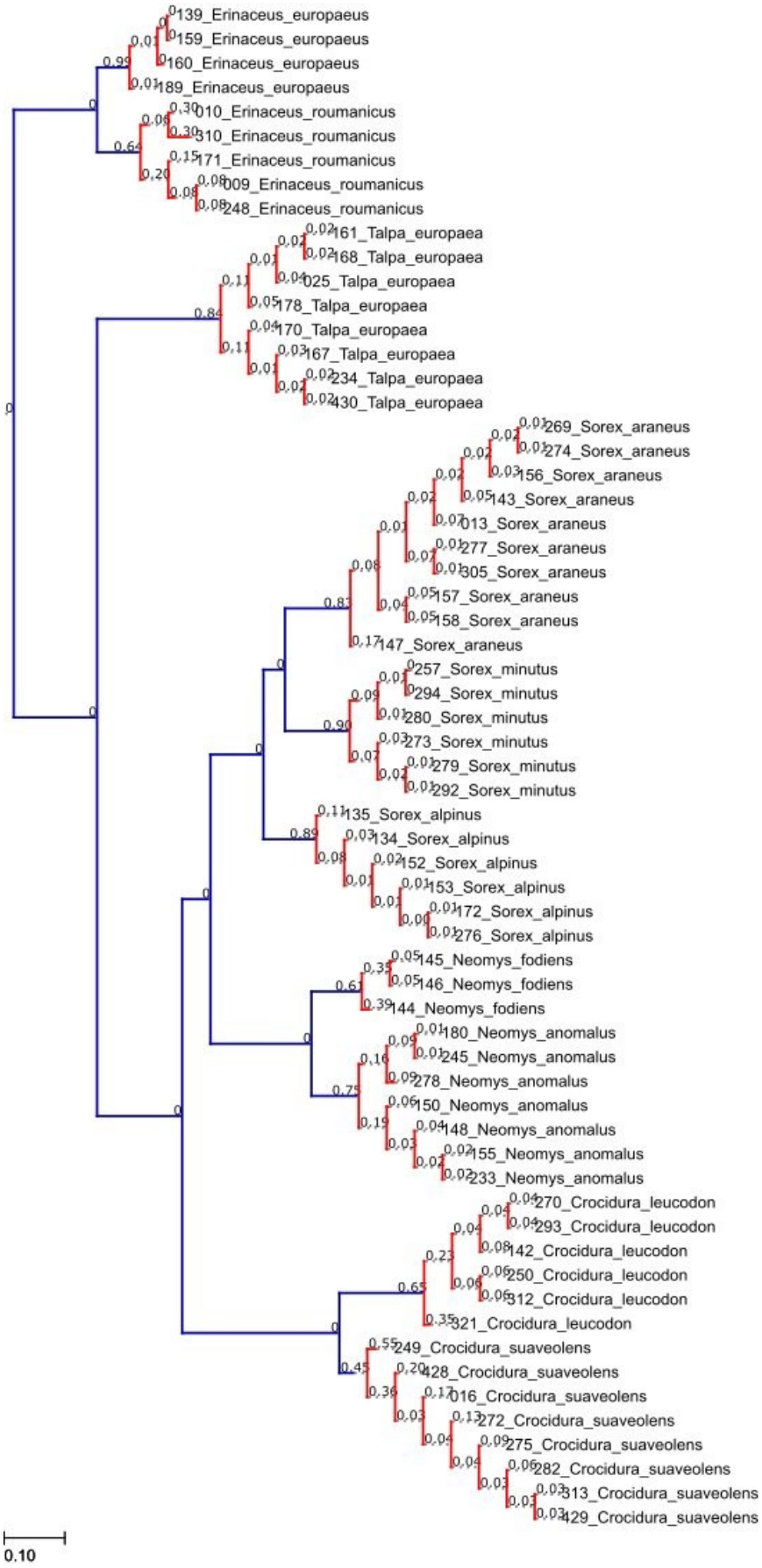

Figure 3. Maximum Likelihood tree of the eulipotyphlan dataset with bptp results. Blue branches indicate MOTUs.

MOTUs and genetic distance

The mean intraspecific K2P genetic distances ranged from 0.2% (*Sorex araneus* and *S. alpinus*) to 2.2% (*Erinaceus roumanicus*), with the maximum intraspecific distance in *Crocidura suaveolens* (7.6% between specimen 249 and 323, Table 7) and *Erinaceus roumanicus* (4.9% between specimen 171 and 310).

The mean interspecific distance ranged from 10.87% (*Erinaceus europaeus* and *Erinaceus roumanicus*) to 29.1% (*Neomys anomalus* and *Talpa europaea*), with the minimal interspecific distance in *Erinaceus europaeus* and *Erinaceus roumanicus* (9.7%, specimen 159 and 171) and in *Neomys anomals* und *Neomys fodiens* (11.4%, specimen 155 and 146).

The two methods to estimate the number of MOTUs came to similar results and were in congruence with the Neighbor-Joining tree.

ABGD generated 10 to 23 MOTUs. Initial partition produced 10 MOTUs at all prior intraspecific divergence levels (P = 0.001 – 0.1). The barcoding gap is clearly distinguishable on the frequency histogram of pairwise distances (Figure 2A).

bPTP estimated 10 MOTUs with support values ranging from 0.44 (*Crocidura suaveolens* MOTU) to 0.988 (*Erinaceus europaeus* MOTU) as seen in Figure 3.

Table 6. Estimates of mean intra- and interspecific K2P distances.

		1	2	3	4	5	6	7	8	9	10
1	*Crocidura leucodon*	0.004									
2	*Crocidura suaveolens*	0.12	0.004								
3	*Erinaceus europaeus*	0.27	0.27	0.01							
4	*Erinaceus roumanicus*	0.27	0.28	0.11	0.022						
5	*Neomys anomalus*	0.24	0.21	0.28	0.25	0.007					
6	*Neomys fodiens*	0.24	0.21	0.25	0.28	0.13	0.011				
7	*Sorex alpinus*	0.25	0.21	0.23	0.26	0.21	0.22	0.002			
8	*Sorex araneus*	0.26	0.22	0.26	0.24	0.2	0.22	0.16	0.002		
9	*Sorex minutus*	0.26	0.24	0.28	0.27	0.21	0.23	0.15	0.15	0.012	
10	*Talpa europaea*	0.26	0.26	0.27	0.27	0.29	0.26	0.22	0.28	0.24	0.003

Table 7. Maximum intraspecific and minimum interspecific K2P distances within and among Austrian eulipotyphla species.

Specimen 1	Specimen 2	distance
135 *Sorex alpinus*	152 *Sorex alpinus*	0,007
178 *Talpa europaea*	167 *Talpa europaea*	0,007
147 *Sorex araneus*	269 *Sorex araneus*	0,014
160 *Erinaceus europaeus*	189 *Erinaceus europaeus*	0,014
144 *Neomys fodiens*	145 *Neomys fodiens*	0,016
150 *Neomys anomalus*	278 *Neomys anomalus*	0,023
150 *Neomys anomalus*	278 *Neomys anomalus*	0,023
321 *Crocidura leucodon*	293 *Crocidura leucodon*	0,028
292 *Sorex minutus*	280 *Sorex minutus*	0,032
171 *Erinaceus roumanicus*	310 *Erinaceus roumanicus*	0,049
323 *Crocidura suaveolens*	249 *Crocidura suaveolens*	0,076
159 *Erinaceus europaeus*	171 *Erinaceus roumanicus*	0,097
155 *Neomys anomalus*	146 *Neomys fodiens*	0,114
312 *Crocidura leucodon*	313 *Crocidura suaveolens*	0,118
157 *Sorex araneus*	273 *Sorex minutus*	0,143
152 *Sorex alpinus*	157 *Sorex araneus*	0,150
153 *Sorex alpinus*	430 *Talpa europaea*	0,216

Discussion

Specimen acquisition

Many species were not obtainable as I had to rely mostly on already deposited species in museums, where some taxa were overrepresented while others were completely missing. *Crocidura russula*, which occurs in Austria only in Vorarlberg and *Sorex coronatus*, which occurs only in Vorarlberg too (Spitzenberger 2001), were only available as stuffed or tanned hide. Historical material like these samples have not been preserved with DNA analysis in mind and thus are highly degraded by both natural processes (e.g. depurination, deamination) and preservation techniques, resulting in highly fragmented DNA (Hofreiter et al. 2001).

The ability to generate barcodes from historical samples is dependent on the size of the amplicon, the smaller the amplicon size, the higher the rate of success. Therefore, complete DNA barcodes can be obtained by targeting overlapping smaller fragments and assemble them (Millar et al. 2008). Approaches like this are nowadays frequently used in barcoding (Patel et al. 2010, Mitchell et al. 2015, Schäffer et al. 2017).

Although mini-barcodes with a length of 100-200bp show success in species identification in some insect genera and rodents, this does not have to be universally true for other taxons (Hajibabaei et al. 2006, Meusnier et al. 2008, Galan et al. 2012).

The inability to produce fragment 3 out of the six overlapping fragments led to the exclusion of all dry samples in this study.

Overall, some parts of Austria were represented good in the museum collections, while others, especially the western parts, were highly neglected.

It would have been interesting to analyze the obtained data in a broader geographic context by adding eulipotyphlan sequences of other parts of Europe but there were either no sequences available (*Sorex alpinus*, *Neomys anomalus*, *Crocidura leucodon*, *Crocidura suaveolens*, *Talpa euopaea*, *Erinaceus roumanicus*) or only with disjunct geographic coverage (e.g. *Erinaceus europaeus*, where only 3 specimen from Norway were available and *Sorex araneus*, where specimen were only available from Norway or Russia).

Sequencing success

The sequencing of full DNA barcodes was successful in most specimen regardless of taxon, apart from bats. There are two potential factors which could be responsible for the low amplification success in bats: high variability in the COI gene and, therefore, bad primer binding (Schäffer et al. 2017) or low DNA concentration in the extract. Bats were the only species where wing tissue was used instead of muscle tissue which could be a reason for lower DNA concentration in these samples.

Tree analysis

The mitochondrial COI gene is a good tool to identify species, but has its limitations when it comes to creating phylogenetic trees and showing deeper phylogenetic signals. Mitochondrial genes show a four times higher genetic drift than nuclear genes (Birky et al. 1989) and trees based on only one gene do not necessarily depict the real species tree (Pamilo & Nei 1988). This seems to be true for the tree containing mammals of different orders which does not depict evolutionary relationships but forms monospecific clades for all species. The polyphyly of *Pipistrellus* ssp. was unexpected and could be either caused by nuclear mitochondrial pseudogenes or mtDNA introgression, as incomplete lineage sorting would need closely related sister species to apply. Nuclear mitochondrial pseudogenes are paralogous copies of mitochondrial genes translocated to the nuclear genome and can be accidently amplified (Bensasson et al. 2001), but BLAST searches confirmed that the acquired sequences are indeed mitochondrial. Introgression between different genera seems to be rather unlikely and was only observed in sister species of the same genus (Berthier et al. 2006). Nesi et al. 2011 found similar patterns of polyphyly in two morpholologically distinct species of African fruit bats and hypothesized that either multiple mtDNA introgression events or incomplete lineage sorting of mtDNA haplotypes explains these patterns as they were not present in the dataset containing a nuclear gene. The Neighbor-Joining tree of the eulipotyphlans arranged every species in a monophyletic unit, indicating that the generated barcodes are suitable to discriminate Austrian eulipotyphlans. While some clades contained individuals, which significantly differed from the rest, most of them were rather uniform and it was not possible to link this differences to geographic patterns.

The *Sorex araneus* group, represented in Austria by *Sorex araneus* and *Sorex coronatus*, is one of the most interesting taxa in Europe due to its complex evolutionary history: they were restricted to three refugia in the south during Quarternary cold periods and one of the suture zones, were the post-glacial colonization routes meet, lies in the Alpine regions of Austria (Taberlet et al. 2002). Even within its type species, *Sorex araneus*, there are at least 60 chromosomal species distinguishable in Europe and Siberia and make this species one of the most chromosomally polymorphic among all mammals (Wójcik et al. 2002). The relatively low distances for *Sorex araneus* in the Neighbor-Joining tree are due to the fact that samples of this species were only taken in a rather small geographic region, only covering Upper Austria and Styria, while specimen of the suture zone on the western border of Austria were not available.

MOTUs and genetic distance

I used two MOTU picking methods because they implement different strategies and either over- or underestimate the number of MOTUs.

Automatic Barcode Gap Discovery sorts the sequences into MOTUs based on the barcode gap (Ward & Holmes 2007), which occurs when interspecific pairwise genetic distances are significantly larger than intraspecific distances.

The Poisson tree processes model is a coalescent-based species delimitation method which assumes that intra- and interspecific substitutions follow two Poisson processes. It needs a gene tree as input at adds Bayesian support to this particular topology and seems to outperform other coalescent-based methods like the General Mixed Yule Coalescent (GMYC) model (Zhang et al. 2013).

In the eulipotyphlan dataset both methods came to the same conclusion which is no surprise given that the lowest mean interspecific genetic distance is still five times higher than the highest mean intraspecific and the minimum interspecific distance still higher than the maximum intraspecific. Specimen 249 (*Crocidura suaveolens*) seems to be one of the few exceptions with a rather high distance and nearly an own MOTU in the bptp analysis (which lowers the support for the whole *C. suaveolens* MOTU). This specimen was in rather poor quality and the obtained sequence was partially not readable.

Bannikova et al. 2011 explored the mitochondrial diversity of the genus *Crocidura* in Vietnam and found six divergent lineages, corresponding to morphological and cryptic

species, with mean interspecific p-distances of >10% for the COI gene, which is in line with the data of the European white-toothed shrews (mean interspecific p-distance for *C. suaveolens* and *C. leucodon*: 12%).

Another study about barcoding in white-toothed shrews explored the diversity on Mount Nimba in Guinea (Jacquet et al. 2012) and detected two cryptic species. They tested different markers with COI displaying the highest discrimination power and mean interspecific distances 24.9-fold higher than the intraspecific ones.

Conclusion

The results of this study provide further confirmation that DNA barcoding is a powerful tool for species identification and reference libraries are essential for this task. The barcodes created in this study are linked to voucher specimens deposited in museums and therefore remain valid even after taxonomic changes. The here presented barcodes will play a vital role in the conservation and management of biological resources, in pest control and in the detection of potential carriers of diseases.

Most of the examined species showed low intraspecific genetic distances, however, geographic coverage was poor. Additional individuals from underrepresented parts of Austria are needed and the mammalian species inventory of Austria is far from being covered.

References

Bannikova AA, Abramov AV, Borisenko AV, Lebedev VS, Rozhnov VV (2011) Mitochondrial diversity of the whitetoothed shrews (Mammalia, Eulipotyphla, Crocidura) in Vietnam. Zootaxa 2812, 1–20.

Bensasson D, Zhang DX, Hartl DL, Hewitt GM (2001) Mitochondrial pseudogenes: evolution's misplaced witnesses, Trends in Ecology and Evolution 16, 314–321.

Berthier P, Excoffier L, Ruedi M (2006) Recurrent replacement of mtDNA and cryptic hybridization between two sibling bat species Myotis myotis and Myotis blythii, Proceedings of the Royal Society B: Biological Sciences 273, 3101–3109.

Birky CWJ, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, and drift: Equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes, Genetics 121, 613–627.

Borisenko AV, Lim BK, Ivanova NV, Hanner RH (2008). DNA barcoding in surveys of small mammal communities: a field study in Suriname. Molecular Ecology Resources 8, 471–479.

Clare EL, Lim BK, Engstrom MD, Eger JL, Hebert PDN (2007) DNA barcoding of neotropical bats: species identification and discovery within Guyana. Molecular Ecololgy Notes, 7, 184–190.

Douady CJ, Chatelier PI, Madsen O, de Jong WW, Catzeflis F, Springer MS, Stanhope MJ (2002) Molecular phylogenetic evidence confirming the Eulipotyphla concept and in support of hedgehogs as the sister group to shrews. Molecular Phylogenetics and Evolution 25, 200–209.

Dubey S, Salamin S, Ohdachi N, Barrière SD, Vogel P (2007d) Molecular phylogenetics of Soricidae (Mammalia, Eulipotyphla) reveals timing of transcontinental colonizations. Molecular Phylogenetics and Evolution 44, 126–137.

Felsenstein J (2005) Accuracy of coalescent likelihood estimates: Do we need more sites, more sequences, or more loci? Molecular Biology and Evolution 23, 691–700.

Francis CM, Borisenko AV, Ivanova NV, Eger JL (2010). The role of DNA barcodes in understanding and conservation of mammal diversity in southeast Asia. PLoS One 5: e12575.

Fulton TL, Wagner SM, Shapiro B (2012). Case Study: Recovery of Ancient Nuclear DNA from Toe Pads of the Extinct Passenger Pigeon. Methods in molecular biology 840, 29–35.

Galan M, Pagès M, Cosson JF (2012) Next–generation sequencing for rodent barcoding: species identification from fresh, degraded and environmental samples. PLoS One 7, e48374.

Goldstein PZ, DeSalle R (2011) Integrating DNA barcode data and taxonomic practice: determination, discovery, and description, Bioessays 33, 135–147.

Gu SH, Hejduk J, Markowski J, et al. (2014) Co–circulation of Soricid– and Talpid–borne Hantaviruses in Poland. Infection, genetics and evolution 28, 296–303.

Hajibabaei M, Smith AM, Janzen DH, et al. (2006) A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes 6, 959–964.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270, 313–321.

Hebert PDN, Gregory TR (2005) The promise of DNA barcoding for taxonomy, Systematic Biology 54, 852–859.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes

fulgerator. Proceedings of the National Academy of Sciences of the United States of America 101(41), 14812–14817.
Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London, Series B: Biological Sciences 270, 96–99.

Hilbe M, Herrsche R, Kolodziejek J, Nowotny N, Ehrensperger F (2006) Shrews as reservoir hosts of Borna disease virus. Emerging Infectious Diseases 12, 675–7.

Hofreiter M, Serre D, Poinar HN, Kuch M, Paabo S (2001) Ancient DNA. Nature Reviews Genetics 2, 353–359.

Hutterer R (2005) Order Soricomorpha. In: Wilson, D.E., Reeder, D.A. (Eds.), Mammal Species of the World: A Taxonomic and Geographic Reference, 220–311.

Ivanova NV, Dewaard JR, Hebert PDN (2006) An inexpensive, automation–friendly protocol for recovering high–quality DNA. Molecular Ecology Notes 6(4), 998–1002.

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes 7(4), 544–548.

Jacquet F, Nicolas V, Bonillo C, Cruaud C, Denys C (2012) Barcoding, molecular taxonomy, and exploration of the diversity of shrews (Soricomorpha: Soricidae) on Mount Nimba (Guinea). Zoological Journal of the Linnean Society 166, 672–687.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2), 111–120.

Long BL, Kurta A, Clemans DL (2013) Analysis of DNA from feces to identify prey of big brown bats (Eptesicus fuscus) caught in Apple Orchards. The American Midland Naturalist 170, 287–297.

Mayer–Scholl A, Hammerl JA, Schmidt S, Ulrich RG, Pfeffer M, Woll D, et al. (2014) Leptospira spp. in Rodents and Shrews in Germany. International Journal of Environmental Research and Public Health 11, 7562–7574.

McKenna MC, Bell SK (1997) Classification of Mammals above the Species Level. Columbia University Press, New York.

Messing J (1983) New M13 vectors for cloning. Methods in Enzymology 10, 20–78.

Meusnier I, Singer GA, Landry JF, Hickey DA, Hebert PD, et al. (2008) A universal DNA mini–barcode for biodiversity analysis. BMC Genomics 9, 214.

Meusnier I, Singer GAC, Landry JF, Hickey DA, Hebert PDN, Hajibabaei M (2008) A universal DNA mini–bar–code for biodiversity analysis. BMC Genomics 9, 214.

Millar CD, Huynen L, Subramanian S, Mohandesan E, Lambert DM (2008) New developments in ancient genomics. Trends in Ecology & Evolution 23, 386–393.

Mitchell A (2015) Collecting in collections: a PCR strategy and primer set for DNA barcoding of decades–old dried museum specimens. Molecular Ecology Resources 15, 1102–1111.

Mitchell-Jones AJ, Amori G, Bogdanowicz W, Kryštufek B, Reijnders PJH, Spitzenberger F, Stubbe M, Thissen JBM, Vohralík V, Zima J (1999) The Atlas of European Mammals. Academic Press, London.

Moldal T, Vikøren T, Cliquet F, et al. (2017) First detection of European bat lyssavirustype 2 (EBLV–2) in Norway. BMC Veterinary Research 13, 216.

Neet CR, Hausser J (1991) Biochemical analysis and determination of living individuals of the alpine karyotypic races and species of the Sorex araneus group. Mémoire de la Société Vaudoise Sciences Naturelles 19, 97–106.

Nesi N, Nakouné E, Cruaud C, Hassanin A (2011) DNA barcoding of African fruit bats (Mammalia, Pteropodidae). The mitochondrial genome does not provide a reliable discrimination between Epomophorus gambianus and Micropteropus pusillus. Comptes Rendus Biologies 334, 544–554.

Nowak RM (1999) Walker's Mammals of the World. Johns Hopkins University Press.

Pamilo P, Nei M (1988) Relationships between gene trees and species trees, Molecular Biology and Evolution 5, 568–581.

Patel S, Waugh J, Millar CD, Lambert DM (2010) Conserved primers for DNA barcoding historical and modern samples from New Zealand and Antarctic birds. Molecular Ecology Resources 10, 431–438.

Patel S, Waugh J, Millar CD, Lambert DM (2010) Conserved primers for DNA barcoding historical and modern samples from New Zealand and Antarctic birds. Molecular Ecology Resources 10, 431–438.

Patterson BD, Willig MR, Stevens RD (2003) in Bat Ecology, Kunz TH, Fenton MB, Eds. (Univ. of Chicago Press, Chicago) 536–579.

Puillandre N, Lambert A, Brouillet S, Achaz G (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology 21, 1864–1877.

Ratnasingham S, Hebert PDN (2007) BOLD: the barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes 7, 355–364.

Richlen ML, Barber PH (2005) A technique for the rapid extraction of microalgal DNA from single live and preserved cells. Mol Ecol Notes 5, 688–691.

Sangster L, Blake DP, Robinson G, Hopkins TC, Sa RC, Cunningham AA, et al. (2016) Detection and molecular characterisation of Cryptosporidium parvum in British European hedgehogs (Erinaceus europaeus). Veterinary Parasitology 217, 39–44.

Sattmann H, Haring E, Zachos F, Kruckenhauser L, Zimmermann D, Gamauf A (2014). Land der Vielfalt: ABOL – Austrian Barcode of Life. Das Naturhistorische 6, 10–11.

Schatz J, Fooks AR, McElhinney L, Horton D, Echevarria J, Vazquez–Moron S, et al. (2013) Bat rabies surveillance in Europe. Zoonoses Public Health 60, 22–34.

Schäffer S, Zachos FE, Koblmüller S (2017) Opening the treasure chest: A DNA–barcoding primer set for most higher taxa of Central European birds and mammals from museum collections. PLoS ONE 12(3), e0174449.

Simmons NB (2005) in Mammalian Species of the World: A Taxonomic and Geographic Reference, Wilson DE, Reeder DM, Eds. (Johns Hopkins Univ. Press, Baltimore).

Spitzenberger F (2001) Die Säugetierfauna Österreichs. Grüne Reihe des Bundesministeriums für Land– und Forstwirtschaft, Umwelt und Wasserwirtschaft, Band 13.

Taberlet P, Fumagalli L, & Wust–Saucy AG, Cosson JF (2002) Comparative Phylogeography and Postglacial Colonization Routes in Europe. Molecular Ecology 7, 453–464.

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512–526.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30, 2725–2729.

Turni H, Müller EF (1996) Unterscheidung der Spitzmausarten Sorex araneus L., 1758 und Sorex coronatus, Millet, 1828 mit Hilfe einer neuen Diskriminanzfunktion. Zeitschrift für Säugetierkunde 61, 73–92.

Ward RD, Holmes BH (2007) An analysis of nucleotide and amino acid variability in the barcode region of cytochrome c oxidase I (cox1) in fishes. Molecular Ecology Notes, 7, 899–907.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1847–1857.

Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. BioEssays 29, 188–197.

Wilson DE, Reeder DM (2005) Mammal species of the world: a taxonomic and geographic reference. Baltimore, Maryland: Johns Hopkins University Press. 2142 p.

Wójcik JM, Ratkiewicz M, Searle JB (2002) Evolution of the common shrew Sorex araneus: chromosomal and molecular aspects. Acta Theriologica 47, 139–167.

Yang YC, Huang YW, Hsieh CH, Huang YR, Chen CH (2012) A unique specification method for processed unicorn filefish products using a DNA barcode marker. Food Control 25, 292–302.

Ye JP, Biltueva L, Huang L, Nie W, Wang J, Jing M, Su W, Vorobieva NV, Jiang X, Graphodatsky AS, Yang F (2006) Cross–species chromosome painting unveils cytogenetic signatures for the Eulipotyphla and evidence for the polyphyly of Insectivora. Chromosome Research 14, 151–159.

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29, 2869–2876.

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