University of Natural Resources
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Phylogeography of two European hedgehog species (*Erinaceus europaeus* and *E. roumanicus*) and evaluation of their potential hybrid zone

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University of Natural Resources
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Declaration in lieu of oath

I herewith declare in lieu of oath that this thesis has been composed by myself without any inadmissible help and without the use of sources other than those given due reference in the text and listed in the list of references. I further declare that all persons and institutions that have directly or indirectly helped me with the preparation of the thesis have been acknowledged and that this thesis has not been submitted, wholly or substantially, as an examination document at any other institution.

19 May 2020 _____

Date Signature

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Abstract

European hedgehogs (genus *Erinaceus*) belong to the vast number of species showing a zone of secondary contact and potential hybridisation within Europe. The West European hedgehog (*E. europaeus*) – distributed throughout Western and Central Europe – and the Northern White-breasted hedgehog (*E. roumanicus*) – with its range reaching from Central to Eastern Europe – exhibit two zones of sympatric occurrence within Central and North-eastern Europe, respectively. Their mid-European contact zone reaches from Poland and the Czech Republic downwards Austria to the border between Italy and Slovenia.

The aim of this master thesis was to search for potential signals of admixture between *E. europaeus* and *E. roumanicus* individuals within the area of Austria on the one hand side and to characterize their contact zone, on the other side. In addition, the phylogeographic distribution pattern of the two species was examined on an inter- as well as intraspecific level, throughout Europe, and discussed in relation to existing knowledge on post-glacial recolonization patterns. On the basis of a denser sampling and the usage of an increased number of markers, compared to previous studies, a short sequence repeats genotyping by sequence (SSR-GBS) approach was applied. Subsequent population genetic and phylogeographic analyses were conducted using STRUCTURE v.2.3.4, as well as GenAlEx v.6.5.

Out of 264 samples 169 and 81 were assigned to *E. europaeus* and *E. roumanicus*, respectively. Fourteen samples showed admixed assignment patterns to various degrees and are regarded potential hybrid individuals. Further evaluation of their hybrid status within, inter alia, haplotype analysis is intended. In comparison to current knowledge on distribution ranges within Austria a rather broad zone of overlap within the provinces of Upper Austria, Lower Austria and Burgenland is indicated for the two species. Nine potential hybrid individuals were detected within this area.

Zusammenfassung

Europäische Igel (Gattung *Erinaceus*) gehören zu der Vielzahl an Arten, welche eine sekundäre Kontakt-, sowie potentielle Hybridisierungszone innerhalb Europas aufweisen. Der Braunbrustigel (*E. europaeus*) – verbreitet von West- bis Zentraleuropa – und der Nördliche Weißbrustigel (*E. roumanicus*) – mit einem Verbreitungsgebiet von Zentral- bis über Osteuropa – besitzen zwei Zonen sympatrischer Verbreitung innerhalb Mittel- und Nordosteuropas. Ihre mitteleuropäische Kontaktzone reicht von Polen und der Tschechischen Republik, südlich über Österreich bis zur Grenze zwischen Italien und Slowenien.

Das Ziel dieser Masterarbeit lag einerseits in der Suche nach potentiellen *E. europaeus x E. roumanicus* Hybrid-Individuen innerhalb Österreichs, sowie andererseits in der Charakterisierung ihrer Kontaktzone. Zusätzlich wurde das phylogeografische Verbreitungsmuster dieser beiden Arten auf zwischen-, sowie innerartlichem Niveau innerhalb Europas untersucht und unter Berücksichtigung bereits bestehender Literatur zu nach-eiszeitlichen Wiederbesiedelungsmustern diskutiert. Auf Grundlage einer höheren Probendichte und dem Einsatz einer erhöhten Zahl von Mikrosatellitenmarkern, im Vergleich zu bisherigen Studien, wurde ein "short sequence repeats genotyping by sequence" (SSR-GBS) Ansatz gewählt. Nachfolgende populationsgenetische und phylogeografische Analysen wurden mit Hilfe der Programme STRUCTURE v.2.3.4 und GenAlEx v.6.5 durchgeführt.

Von den insgesamt 264 analysierten Proben wurden 169 und 81 den Arten *E. europaeus* und *E. roumanicus*, respektive zugeordnet. 14 Proben zeigten ein gemischtes Muster, in unterschiedlichen Anteilen, und wurden als potentielle Hybrid-Individuen gehandelt. Eine weitere Evaluierung ihres Hybridstatus, durch u.a. Haplotypen-Analyse, ist geplant. Im Vergleich zu bereits bestehenden Erkenntnissen der Verbreitungsgebiete beider Arten innerhalb Österreichs wurde eine relativ breite Überlappungszone innerhalb der Bundesländer Oberösterreich, Niederösterreich und dem Burgenland gefunden. Neun der potentiellen Hybrid-Individuen wurden innerhalb dieser Zone gefunden.

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1. Introduction

1.1 Hedgehog classification and distribution

1.1.1 Hedgehog Classification – Classification of the family of Erinaceidae

Hedgehogs are nocturnal insectivores (Reeve 1994), belonging to the family of Erinaceidae, which is restricted to the Old World, occurring in Europe, Africa and Asia. Four to five distinct genera (Macdonald 2001; Lowen 2018) are being recognised within the subfamily of Erinaceinae (depending on the considered approach). The genus *Hemiechinus* represents the long-eared hedgehogs, which consist of six different species, that can be found in desert regions from the Middle East over to Central Asia. Four species build up the genus *Atelerix*, which is restricted to sub-Saharan and northern regions of Africa, with one exception (*A. algirus*) also having been introduced to Spain and France. Two species comprise the steppe hedgehogs (genus *Mesechinus*), which live in Mongolia and partly in China. European hedgehogs are classified to the genus *Erinaceus*, which is constituted by the species *E. europaeus*, *E. roumanicus*, *E. concolor* and the Chinese species *E. amurensis* (Morris 2018; Lowen 2018).

1.1.2 Distribution ranges of European *Erinaceus* species

European hedgehogs´ distribution range reaches across Europe, from the Iberian peninsula to the Urals. While the northwards distribution limit is to be found in Scandinavia, the most southernly individuals can be seen from the Mediterranean region over to Turkey and the near East (Aulagnier et al. 2009).

Until recently two species of European hedgehogs have been recognised – *Erinaceus europaeus* and *E. concolor* with a western and eastern distribution, respectively. However, various studies based on current genetic and morphological data (Filippucci and Simson 1996; Santucci et al. 1998; Seddon et al. 2001, 2002; Krystufek 2002) showed a "considerable level of divergence between *E. concolor* from Turkey and Israel and the former *E. concolor* from the Balkans" (Santucci et al. 1998) and hence classified the latter (taxon *roumanicus*) to an independent species – *E. roumanicus* (which has formerly been ascribed as subspecies). So that currently three different species of the genus *Erinaceus* are being recognized within the western palearctic zone (Aulagnier et al. 2009):

The (West) European hedgehog or brown-breasted hedgehog, *E. europaeus* (Linne, 1758), which inhabits Western and Central Europe, with its range spreading to the United Kingdom, (south) Scandinavia, the Baltic republics as well as Northern and Central Russia (see Fig. 1). The Northern White-breasted hedgehog, *E. roumanicus* (Barrett-Hamilton, 1900), covering the central and eastern parts of Europe, as well as the Ponto-Mediterranean regions and its sister species the Southern White-breasted hedgehog, *E. concolor* (Martin, 1837), which is distributed through Asia Minor and the

Levant, but is geographically isolated from the distribution area of *E. roumanicus* by the Bosporus strait and the Caucasus Mountains, forming barriers (Seddon et al. 2002) (see Fig. 2) (Corbet 1988; Reeve 1994; Hutterer 2005; Aulagnier et al. 2009).

Concerning local distribution patterns of European hedgehog populations a connection and correlation with factors, such as predator (Doncaster 1992) and resource (Micol et al. 1994) density (food and shelter) has been observed. As it comes to ecological preferences of *E. europaeus* and *E. roumanicus* no statistically significant differences are to be found, according to Holz and Niethammer (1990a, b). They merely describe *E. roumanicus* to be more temperature sensitive to cold climate conditions (Holz and Niethammer 1990a, b). Additionally, Bolfíková and Hulva (2012) attribute *E. roumanicus* a linkage to lower altitudes and open landscape.

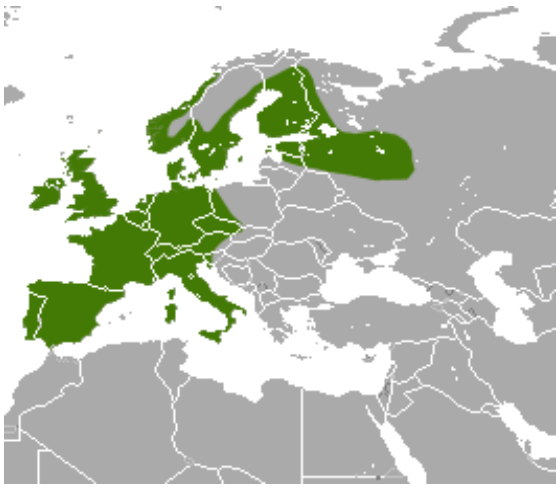

Fig. 1. Distribution range map of the West European hedgehog, *E. europaeus*. (Picture taken from https://upload.wikimedia.org/wikipedia/commons/4/48/European_Hedgehog_area2.png)

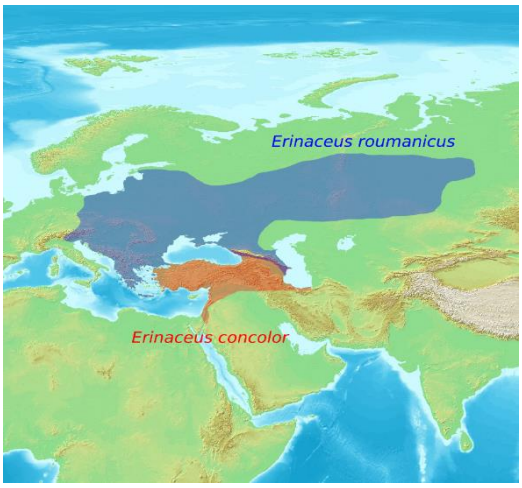

Fig. 2. Distribution range map of the Northern (*E. roumanicus*) and Southern White-breasted hedgehog (*E. concolor*). (Picture taken from https://de.wikipedia.org/wiki/Datei:Range_Erinaceus_concolor_and_Erinaceus_roumanicus.png)

1.2 Phylogeography

1.2.1 Phylogeography as a scientific discipline

According to Avise (2009) phylogeography can be described as the discipline or science of "explicit genealogical inquiries into the spatial and temporal dimensions of (micro-)evolution". It "deals with the spatial arrangements of genetic lineages within and among closely related species", while comparative phylogeographic assessments of various species serve the investigation of intraspecific evolution (Avise 2009). This way phylogeography can be considered an appraisal of the link(age) "between phylogenetic and geographic patterns of distribution among taxa" (Avise 2009 in Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography).

Phylogeographic studies are well established and play(ed) a major role in providing information on allopatric evolution and speciation, as well as observing distributional patterns of various species across time, in conjunction with glaciations (Santucci et al. 1998). As well as that they show extraordinary value in the identification of conservation units, consequently affecting management decisions on investigated species (Taberlet and Bouvet 1994). They work through the integration of (past) geographic information with the present genetic structure of a species, using methodology and sequence data (Avise et al. 1987). Some of the species that have been investigated within this context, in the concern of "environmentally induced range changes" (Santucci et al. 1998) are grasshoppers (Cooper et al. 1995), bees (Garnery et al. 1992), mice (Boursot et al. 1996; Din et al. 1996), rabbits (Hardy et al. 1995) and bears (Taberlet and Bouvet 1994; Kohn et al. 1995).

Also the field of molecular phylogeography made a huge contribution to extending our knowledge on speciation processes, as well as in the field of conservation biology. Due to it we know that most species can be defined in geographical populations (with the exception of highly mobile organisms, and such that show a continuous range distribution on a historical scale) and that their divergence might have happened on a time-scale anywhere between quite recently and long ago. A divergence like this leads to phylogenetical gaps, also between adjacent (regional) populations, when environmental barriers hinder gene flow over a longer time period (Avise 2001).

The initial `phylogeographic revolution`, that took place in the late 1970s and 1980s and the following formation of the discipline of phylogeography massively impacted and led to a build-up of bridges between research fields and major evolutionary disciplines which previously showed limited contact and communication - population genetics, phylogenetic biology and biogeography. The follow-up work was, among other things, motivated by taxonomic uncertainties and conservation concerns. An example of research focus can be found in comparative phylogeographic assessments of multi-species regional biotas. The objective herein being to reconstruct prominent historical incidents leading to trends, reflected in shared phylogeographic patterns, which characterize multiple species in regional faunas and floras (reflecting dispersal and speciation) (Avise 2009).

1.2.2 Phylogeographic structuring and genealogies (within geographically isolated populations)

Phylogeographic studies usually investigate multiple populations, which are distributed across a specific geographic area. Different populations of a species are often separated by physical or behavioural barriers, that pose limitations to their dispersal, now or in the past. Physical filters might arise from rivers or mountains, while limited movement capability can lead to isolation by distance alone (the former having the ability to promote the latter). Both, however, promote the genetical structuring of populations. While some movement impediments are semi-permeable ("permitting the occasional exchange of genetic lineages"), others constitute a permanent isolation, leading to a long-standing limitation of gene exchange. Thus, genealogical patterns largely correlate with and depend on

the period of time the dispersal impediment is lasting and are as well impacted by historical demographies of geographically separated populations (Avise 2009).

According to Avise (2009) (profound) phylogeographic structuring is represented through genealogical similarities, and such matches can be found on a 1) within-locus level, 2) multi-locus level, 3) multi-species level or 4) an agreement among those. Within his paper "Phylogeography: retrospect and prospect" (2009) he instanced a gene-tree split in geographical accordance with a permanent dispersal barrier as a deep phylogeographic split within the concerned species (Avise 2009).

The concomitant phylogeographic structuring is based on a long-term geographical isolation – like the restriction to isolated Pleistocene refugia - of affected populations (as described above). To acquire new mutations, required for such genetical sub-structuring and accumulation of genetic differentiation, hundreds of generations in local isolation are usually needed. Species succumbing to phylogeographic structuring generally show either limited dispersal behaviour, or profound dispersal barriers (like mountains, rivers, roads or other forms of human development) restricting their gene flow. Range expansions after elimination of dispersal impediments might, however, lead to secondary contacts. Also natural selection poses an impact source on phylogeographic structuring, as it might prohibit the exchange of haplotypes (now or in the past due to environmental gradients) and may as well have had its role in allopatric genetic divergence (Avise 2009).

Since phylogeographic structuring might lead to the formation of distinct gene pools and local adaptions in long-term isolated populations (due to reproductive isolation), it is also relevant for the identification of population units for conservation purposes – as subspecies are being recognised as separate conservation units. As well as that phylogeographical assessments are valuable for the identification of another type of conservation unit – biogeographic areas or provinces, displaying consistent phylogeographic patterns among multiple species and thereby possibly acting as a valuable source for nature reserves (Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography).

Not only species with limited dispersal capabilities can show genealogical structuring, also vagile organisms and species with high dispersal potential might display obvious population genetic structure. This form of genetic structuring is rather caused by philopatry (i.e. the "behavioural fidelity to specific locations"), than resulting from physical impediments (Avise 2009).

Not all species are, however, phylogeographically subdivided, but might show what is known as a `starburst` genealogical pattern. These portray a common and widespread haplotype (ancestral condition) linked to several rare haplotypes (obtained by different mutations), thereby representing a species, that just recently expanded from its geographic origin (Avise 2009).

Also historical biogeographic processes and forces seem to have shaped regional biotas and biotic communities. Genealogical structuring appears to be alike in species displaying similar distribution ranges or ecologies. Again, this might have happened during the Pleistocene era, when ancestral populations were restricted to refugia, which supported the formation of phylogeographic substructuring through the accumulation of mutations resulting in genetic differentiation (Avise 2009). Such consistent genealogical patterns within faunas and floras in Europe largely trace back to three European regions acting as refugia during ice ages. Those are namely the Iberian Peninsula, the Italian Peninsula, and the Balkan region (Hewitt 1996, 1999, 2000). Colonization routes to these Pleistocene refugia, as well as post-Pleistocene range expansions have been identified for numerous species, showing distinct genealogical substructuring across Europe, identified through their "phylogenetic footprints". In this context, also zones of secondary contact – sources for possible hybridisation – have been found (Avise 2009).

1.2.3 Examples of phylogeographic studies

The field of intraspecific phylogeography was initially pioneered by J. Avise and his colleagues, through their classic example of mitochondrial DNA phylogeography of the south-eastern pocket gopher, *Geomys pinetis* (Avise et al. 1979). This study indeed introduced phylogeography as an independent and autonomous research discipline. Within their genetical assessment of 87 pocket gophers, based on mtDNA haplotypes, they clearly demonstrated a deep genealogical split between animals from the eastern and western area of the examined species´ range. The 20 observed mtDNA haplotypes appeared to be distinct and spatially organized. While deep genetic separation divided animals that were geographically far apart, shallow splits were found between geographically close individuals ("routine isolation by distance") representing related haplotypes (Avise et al. 1979; Avise 2009).

This pattern of genealogical structuring can be found among a vast number of "low-vagility" animal species (small mammals, reptiles and amphibians), examined by phylogeographical assessments using mtDNA haplotypes. These species are naturally (more easily) subjected to dispersal impediments, as well as physical and/or behavioural barriers, resulting in greatly similar haplotypes localized in the same geographical regions. The deeper genealogical splits among regionally separated populations of a species might well date back to a differentiation in separate refugia, tracing back to known historical dispersal impediments (Avise 2000; Avise 2009).

1.2.4 The methodology of phylogeography

Phylogeographic assessments operate through gene/haplotype trees, which provide valuable tools, in this concern. They help with the exploration of underlying mechanisms and processes leading to the existing genealogical architecture within different populations of a species or several related subspecies (Avise 2009). Nested clade phylogeographic analysis (NCPA, Templeton 1998) acts as a widely used, but controversially considered, approach. While it is thought to "provide the correct inference about phylogeographic history", quantification of its correctness is a more critical concern (Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography). Papers like Beaumont et al. (2010) demonstrated model-based phylogeographic approaches to be of greater value and more efficient (Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography). Those, however, should be based on genetical information derived from multiple nuclear loci, as well as mitochondrial data, to constitute a "robust statistical phylogeography" (Knowles and Maddison 2002; Templeton 2004). The fusion of population genetic and phylogenetic methods, however, portray the fundament of those (Avise 2009).

1.3 Glacial refugia and postglacial recolonization

1.3.1 The Quaternary Period

Genetic variation that can be observed within and between different populations, subspecies as well as species and communities belonging to divers geographical regions has massively been shaped along time by climatic fluctuations, influencing their geographic distribution patterns (Hewitt 1996, 1999, 2000; Nichols and Hewitt 1994; Santucci et al. 1998). The Quaternary period has played a special and very important role within this concern (Hewitt 2000; Taberlet et al. 1998). It is divided into the Holocene, our current geological epoch, that began approx. 11.700 years BP (Before Present) (following the last glacial period), and the Pleistocene, which lasted from 2.58 Mya (Million years ago) to 11.700 years ago and constitutes the most recent period of repeated glaciations. The Pliocene is the epoch preceding the Quaternary, it lasted from 5.33 to 2.58 million years BP (Cohen et al. 2013). Speciation processes took place throughout the Pliocene and Pleistocene with various intensity (Bermingham et al. 1992; Zink and Slowinski 1995; Hewitt 1996; Taberlet et al. 1998) and various findings strongly suggest Pleistocene range contractions, i.e. expansions and restrictions to southern refugia, as a major impact on and shaper of genetic structure in today´s populations (Hewitt 1996, 1999, 2000; Santucci et al. 1998; Taberlet et al. 1998).

1.3.2 Pleistocene ice age refugia

Major Pleistocene ice ages and their accompanying climatic changes, that took place in Europe millions of years ago, led to a significant structuring of genomes in today´s species and are claimed to have profoundly modified distribution ranges of most living organisms, leaving distinctive marks on their genomes, which might persist for thousands of generations (Hewitt 1996, 1999, 2000; Taberlet et al. 1998). Consequently most European animal and plant taxa have been restricted to southern refugia, during those paleogeographic events. Resulting in recurrent periods of range contraction, i.e. isolation during glaciation, through advancing ice and tundra, and subsequent northwards expansions in times of climate amelioration, constantly searching for suitable habitat (Hewitt 1993b, 1996, 1999). Species that can be found within temperate and boreal regions today, found glacial refugia below the southern border of ice and permafrost. If temperate species did not retreat to survive in southern refugia or migrated to more hospitable areas, they went extinct (Hewitt 2000).

In this regard three regions have consistently been identified (through genomic distribution patterns pooled together with climate and pollen data), acting as such refugia for a wide variety of European species– the Iberian peninsula, Italy and the Balkans. (Hewitt 1993b, 1996, 1999; Seddon et al. 2001). The contribution of these refugia to Europe´s post-glacial colonization, varies on a broad scale (Hewitt 1999). While the Balkan and Iberic Peninsula gave rise to most of the northern species expansion (in postglacial times), producing diverse genomes, Italy hardly brought up lineages and species spreading into northern Europe (with the hedgehog as one exception – see Fig. 3 and section 1.3.3 Post-glacial colonization and its genetic consequences). The Alps might have acted as a substantial impediment and initial barrier in this regard, while the Pyrenees must have represented a rather remote obstacle (Hewitt 1999, 2000). Taberlet et al. (1998), who investigated 10 different taxa on their (dis)similarities in terms of 1) phylogeography and 2) post-glacial colonization (routes), in order to deduce general trends within Europe, found little concordance as it comes to 1), but some likeness within 2). These are in agreement with the before mentioned.

Mountain ranges, like those of the Alps, the Pyrenees and the Caucasus (as well as marine barriers) did pose substantial restrictions to the movement and colonization, as well as expansion behaviour of various European species, during interglacial warmings (Hewitt 1996, 1999; Seddon et al. 2002). Those mountain blocks, with their general east-west orientation (Taberlet et al. 1998), showed extensive glaciation, thereby creating land bridges (Hewitt 2000) and acting as dispersal barriers, isolating populations in the refugia of Iberia, Italy and Greece, as well as Turkey, which were thereby trapped due to the Mediterranean and Black Sea (respectively), prohibiting southern displacement (Hewitt 1999).

Such southern retreats, like the Balkan Peninsula (Hewitt 2000), and Italy (Taberlet et al. 1998) are considered important sources for genetic diversity and the formation of endemic species and lineages

(Krystufek and Reed 2004), since they acted as starting points for the expansion of many species after the Last Glacial Maximum (LGM) (Hewitt 2000).

1.3.3 Post-glacial colonization and its genetic consequences

By now geographical range changes and consequent forced movements, caused by environmental conditions (such as climatic fluctuations), are recognised to result in the structuring of genomes (Nichols and Hewitt 1994; Hewitt 1996; Ibrahim et al. 1996). For instance, the loss of allelic diversity and corresponding homozygosity through fast range expansions leading to bottleneck situations, is expected to result in contrasting genetic diversity levels within refugial areas and posteriori colonized regions (Hewitt 1996, 1999, 2000; Santucci et al. 1998). Different levels of genetic diversity may be found at the following levels – "number of species, extent of subspecific division and allelic variation" (Hewitt 1999). This pattern, however, was caused by leading edge expansions (the spread of populations from the northern border of refugia into unpopulated areas suiting their needs, repeated multiple times along their route of expansion) of long-distance dispersers (Hewitt 1989, 1993a, 2000). They would dominate the genomes of colonized areas consequently, giving later migrants little chance of contribution (Hewitt 1996). Many of those expanded through what is known as `leptokurtic dispersal`, which is correlated with the formation of wide areas of homozygosity, that may not just survive, but also grow over generations (Ibrahim et al. 1996). Here one may as well refer to the term of "southern richness to northern purity" (Hewitt 1996). Southern glacial refugia contributed to the formation of several divergent genomes through their topography, an assortment of suitable habitats and repeated allopatry during several ice ages. Meanwhile fast range expansion into northern territories delimited allelic diversity (Hewitt 1996, 1999, 2000), additionally reinforced by repeated smaller climatic oscillations, that can create further bottlenecks (Hewitt 1999). A lower species diversity, while greater species´ range at higher latitudes may as well be explained by `Rapoport´s Rule` - the need for tolerance of multiple changing environmental conditions (Hewitt 1996).

In this concern it is also considered that "allopatrically diverged genomes may be distributed in geographical patchworks with hybrid zones where they meet" (Nichols and Hewitt 1994; Ibrahim et al. 1996; Santucci et al. 1998). Such hybrid zones arose where divergent genomes of a species met, due to the performed expansion out of diverse southern refugia (Hewitt 2000) – this includes the areas of the Alps, central Europe, the north Balkans and the Pyrenees (Hewitt 1993a, 1996, 1999; Taberlet 1998) (see Fig. 4), which all show records of subspecific contacts. Besides it is indicated that these zones stayed in a more or less consistent area during the whole Holocene (Hewitt 1999). Taberlet et al. (1998) found four main suture zones in this concern, when analysing various colonization routes from the main glacial refugia. And a classic example of post-glacial colonization is found with the house mouse (*Mus musculus* and *M. domesticus*), that shows a hybrid zone reaching through the centre of Europe (Boursot et al. 1993).

Moreover complete geographic (refugial) isolation and the subsequent limitation in gene flow resulting from Pleistocene climate oscillations are explanatory for various modes of speciation in European biota (Hewitt 2000; Coyne and Orr 2004). The interruption of gene exchange leads to genetical divergence among (sub)species and their geographically dispersed populations, resulting in various forms of speciation. Extinctions in northern regions occurred regularly, due to advancing ice ages, but southern refugia enabled the survival of populations retreating from mountains and easily finding new suitable habitat. Subsequently enhancing divergence, hybrid zone formation and speciation, in the follow-up of repeated allopatry (Hewitt 1996, 1999).

Genetic reorganization during colonization is associated with evolutionary adaption and the origin of divergence within genomes, as well as distinct genetic structures. Colonization rates within a species may differ considerably through its distribution range, due to physical impediments, and/or previous occupation (Hewitt 2000). Different modes of dispersal, range expansion and colonization, indeed show various genetical outcomes. For instance occasional long distance dispersers show little influence on already established populations under ´normal´ circumstances, while they contribute massively to gene flow during expansion events (Nichols and Hewitt 1994; Ibrahim et al. 1996). They pronounce the development of so called ´pocket populations´, leading to a long-term persistence of spatially clustered genotypes, which is even enhanced under leptokurtic dispersal. On the one hand side the formed patches are highly inbred, on the other side they show a high differentiation between them (Ibrahim et al. 1996). Long distance dispersal, in this regard, may as well result in (genetic) introgression, when an area is colonised by two genetically distinct and divers genomes (Nichols and Hewitt 1994). The founder effect of a long-distance migrant, however, may persist for many thousand generations (Boileau et al. 1992).

Three general patterns of species colonization can be seen as paradigms when it comes to the postglacial colonization history of Europe and the subsequent geographic distribution of genomes - ´the grasshopper´, ´the hedgehog´ and ´the bear´ (Hewitt 1999, 2000). The `grasshopper` (*Chorthippus parallelus*) represents an expansion out of the Balkan peninsula, with genomes from the other two refugia, being blocked at the mountain ranges of the Pyrenees and the Alps. The `bear` (*Ursus artos*) shows a spread from the Iberian peninsula and the eastern part of the Balkans, only (Hewitt 1999). The `hedgehog` post-glacial colonization pattern, however, will be outlined in detail, since it is of particular relevance for this work (also see section 1.3.4 Southern refugia and post-glacial expansion of European hedgehog species).

The two parapatric species *Erinaceus europaeus* and *Erinaceus roumanicus* seem to have initially separated at the beginning of the Quaternary. Three major splits along a west/east-axis can be identified for the hedgehog genome within Europe, corresponding to the three main glacial refugia of the species – the Iberian (western clade), the Italian (central clade) and the Balkan peninsula (eastern clade) (see Fig. 3). With Turkey and Israel acting as a fourth independent southern refugium (for *E.*

concolor). The mountain ranges of the Pyrenees and the Alps, however, did not pose a substantial physical restriction to their northward migration (Santucci et al. 1998; Hewitt 1999, 2000).

Nuclear as well as mitochondrial markers have been used to reconstruct glacial refugia and post-glacial colonization routes of various species worldwide, European hedgehogs among them (Sommer 2007). A variety of studies (e.g. Santucci et al. 1998; Seddon et al. 2001), investigating European hedgehogs mtDNA, do bear proof of glacially induced imprints within their genomes.

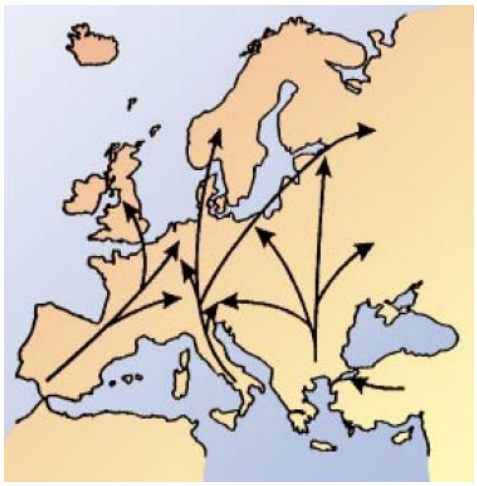

Fig. 3. Glacial refugia and post-glacial colonization routes of European hedgehog (*Erinaceus*) species (Picture taken from Hewitt 2000).

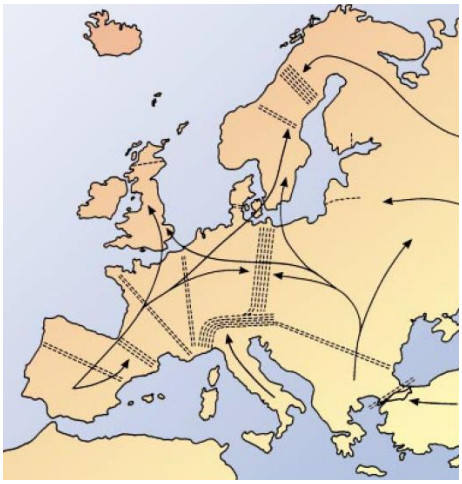

Fig. 4. Documented hybrid zones and areas of secondary contact within Europe (Picture taken from Hewitt 2000).

1.3.4 Southern refugia and post-glacial expansion of European hedgehog species

It is suggested that recurrent cycles of Pleistocene ice ages and interglacial warm periods forced hedgehogs to retract to and expand from refugia in approx. 100.000-year periods. Iberia and Italy, as well as the Balkans and Turkey (plus the near-East) presumably acted as such continual refugia for the parapatric species *E. europaeus* and *E. roumanicus*, as well as *E. concolor*, respectively (Hewitt 1999; Santucci et al. 1998; Seddon et al. 2001).

Based on the similar expansion patterns of hedgehogs and oak (Ferris et al. 1998; Hewitt 2000), as well as silver fir (Hewitt 2000), along the three glacial refugia, Seddon et al. (2001) claim that hedgehogs followed the establishment of deciduous woodland, as a limiting factor regarding climate and food sources. Which is also set in concordance with the observed dependency of *Erinaceus* hedgehogs on deciduous woodland (Rautio et al. 2014) by Bolfíková et al. (2017).

During the Quaternary period hedgehogs must have been massively influenced by climate-dependent changes, since they belong to the insectivores and thereby greatly rely on insect availability (Bolfíková and Hulva 2012). These provoked range shifts clearly reflect in their genetic structure, as has been shown by various studies (Filippucci and Simson 1996; Santucci et al. 1998; Seddon et al. 2001, 2002; Berggren et al. 2005).

There are specific patterns, that seem to be associated with the expansion of hedgehogs from their glacial refugia (Seddon et al. 2001). Among them is the loss of genetic diversity through bottleneck situations induced by long distance colonization (Nichols and Hewitt 1994; Hewitt 1996). This was already proven by Seddon et al. (2001), who found less genetic diversity within the northern regions of expansion, i.e. UK and Ireland for *E. europaeus* and Estonia for *E. roumanicus*.

While hedgehogs did show cyclic restrictions to glacial refugia, alternating with periods of expansion during climate ameliorations, only populations located within refugia are suggested to actually have survived ice ages. The last of those ended approx. 16.000 years ago. This is considerably recent, suggesting that hedgehog´s mitotypes must have survived within the afore mentioned refugia. This circumstance prompts Seddon et al. (2001) to conclude that such refugial populations persist as a number of smaller isolated populations, containing the whole genetic diversity across populations. Past-glacial dispersion from those populations must have contributed to the genetic set-up of today's populations through colonization (Hewitt 1996, Seddon et al. 2001).

Sommer (2007) speaks of a "well-known example in the zoogeography of the Holartic" or a "unique and well-known example in the zoogeography of the Paleartic", when it comes to the separate refugia of *E. europaeus* and *E. roumanicus* during the Pleistocene and subsequent expansions. The West European hedgehog seems to have been strongly restricted to the areas of Iberian and Italian Peninsula – acting as glacial refugia – until the end of the LGM (between 23.000 – 16.000 years BP), which is supported by fossil records. This was followed by (a gradual dispersion) and expansion into the southern parts of France (early Late Glacial, 16.000 – 12.500 years BP. While the first occurrence of *E. europaeus* within Central Europe is estimated to the Boreal (8.600 – 7.100 cal. BC), Great Britain and Ireland might well have been populated already by the end of the Pre-Boreal (9.600-8.600 cal. BC) (Sommer 2007). As it comes to *E. roumanicus* little data on Pleistocene distribution is available, but it seems like the species survived within the Balkan peninsula (Seddon et al. 2001; Sommer 2007). It also seems that both species´ colonization modes differed largely, validated by genetic and fossil data (Berggren et al. 2005). West European and Northern White-Breasted hedgehog presumably met in the area of Central Europe during the Early Holocene, with their distribution area largely remaining the same over time (Sommer 2007). Sub-fossil data confirming an occurrence of *E. europaeus* and *E. roumanicus* in the LGM and the Holocene are indeed in accordance with their current distribution (see section 1.1.2 Distribution ranges of European *Erinaceus* species) (Seddon et al. 2001; Sommer 2007). While both species reached each other north of the Alps during the Boreal, their meeting at the most

southern point of the current hybridisation zone took place far earlier, due to its proximity to the glacial refugia of the Italian Peninsula (Sommer 2007). Furthermore it is indicated that *E. roumanicus* colonized central Europe later than *E. europaeus*, whereby Bolfíková and Hulva (2012) combine this colonization process with the "large-scale deforestation of the Neolithic period".

1.4 Studies on genetic differentiation between *E. europaeus* and *E. roumanicus* and within the respective species in a phylogeographical context

As morphological classifications largely failed to distinguish discrete subspecies of European hedgehogs, there was an urge to clear up hedgehog taxonomy through genetical and molecular - based analysis (Corbet 1988). Early examples of such investigations are offered by Becher and Griffiths (1997) as a more methodological approach, or Filippucci and Simson (1996) and Santucci et al. (1998) in a phylogeographic context, for example.

Various studies support the view of a high level of genetic divergence between and within each of the two species – *E. europaeus* and *E. roumanicus* (e.g. Filippucci and Simson 1996; Santucci et al. 1998; Seddon et al. 2001).

1.4.1 Phylogeography and genetic differentiation of *E. concolor* and *E. roumanicus*

As already mentioned under "1.1 Hedgehog classification and distribution" Filippucci and Simson (1996) claimed to divide the "original" *E. concolor* into *E. concolor*, from Asia Minor and the Levant, and *E. roumanicus* from the Balkans – leading to a new classification of *Erinaceus* species within Europe, based on an allozyme study. This was confirmed by an analysis on mitochondrial DNA phylogeography of European hedgehogs from Santucci et al. (1998). The authors here showed haplotypes from Balkan populations to be distinctive from Anatolian and Israeli samples. The species is suggested to have split into a Balkan lineage (Greece, Balkan and northeast samples) on the one hand and an Anatolian and Israeli (Turkey, as well as Israel) lineage, on the other hand side. Whereby the Bosporus is indicated as a long-time naturally occurring border by Santucci et al. (1998). And also the Caucasus Mountains must have acted as physical impediment to the northward expansion of *E. concolor*, as indicated by Seddon et al. (2002). Seddon et al. (2001, 2002) as well subdivided the original "*E. concolor"* into two clades. While mitotypes from one clade were "found in eastern Europe from Turkish Thrace and Greece, northwards through Austria and Hungary to Estonia", the second one was constituted by individuals from Turkey and Israel. This way also indicating two separate colonization routes and refugia (Seddon et al. 2002). Their divergence time, however, is calculated to date back to approx. 0.4-1.4Mya (Bannikova et al. 2014).

1.4.2 Genetic differentiation of *E. europaeus* and *E. roumanicus*

In 1998 Santucci et al. conducted investigations on the phylogeography of the two European hedgehog species *E. europaeus* and *E. roumanicus* on the basis of mitochondrial DNA analysis and were able to detect a major divergence between those two species and a contact zone separating this divergence between eastern and western Europe. As well as that, they classified a "western and eastern clade" for both, *E. europaeus* and *E. roumanicus* (*E. concolor*), and claimed further contact zones between the lesser diverged clades (Santucci et al. 1998). This deep divergence between the two *Erinaceus* species was repeatedly confirmed in a 2001 study from Seddon et al.

According to their genetic distance it has been suggested that the original divergence of *E. europaeus* and *E. roumanicus* took place in the late Miocene to early Pliocene (around 5.8Mya), when the original population with their common ancestors ("ancestral European hedgehog genepool") was restricted to east and west refugia (Santucci et al. 1998). A pre-Pleistocene/Pliocene differentiation (3.2-4.5Mya) of refugial populations is further presumed by Seddon et al. (2001). Suchentrunk et al. (1998), however, speak of a Pleistocene divergence, 435.000 – 495.000 years BP (based on calculations of interspecific Nei genetic distance).

1.4.3 Phylogeography and intraspecific genetic divergence of *E. europaeus* and *E. roumanicus* (*concolor*)

Based on findings of mitochondrial DNA, i.e. haplotype analysis, the next divergence within each of the separate species had given rise to three (two) mitochondrial clades within *E. europaeus* and two within *E. concolor* (Santucci et al. 1998; Seddon et al. 2001). While *E. europaeus* clades apparently split up around 2.7Mya, the divergence of *E. roumanicus* and *E. concolor* is estimated back to around 3Mya according to Santucci et al. (1998). Nuclear DNA analysis could not reflect the three detected clades for *europaeus*, as seen in the mitochondrial data, but discriminated *E. roumanicus* and *E. concolor* (Seddon et al. 2001).

Berggren et al. (2005) focussed on yet another possibility for determination of post-glacial expansion patterns within *Erinaceus* hedgehogs. They recognized that the results of these analyses differ for mitochondrial and nuclear DNA and decided to use two loci of the major histocompatibility complex (MHC) within their study. Their investigations resulted in a division into subgroups only between the samples of *E. concolor* and *E. roumanicus*, which is in concordance with analyses implemented by other nuclear markers (Berggren et al. 2005). Also Berggren et al. (2005) recognized the two distinct lineages of *E. roumanicus* and *E. concolor* separated by the Bosphorus, giving rise to the Northern and Southern White-breasted hedgehog. The conclusion can be drawn, that derivation of refugial clades leads to differing results when using either mtDNA or nuclear DNA data. The authors therefore presume an "additional older separation in refugia in *E. concolor*" or "a more rapid expansion from

refugia for *E. europaeus*" and "slower expansion patterns for *E. concolor* in the past, maintaining MHC variation" and thereby indicating recent bottlenecks to be less severe (Berggren et al. 2005).

The distribution of *E. europaeus* and *E. roumanicus* species appears to be in accordance with the northward migration from the three postulated southern refugia, the Iberian, Italian and Balkan peninsula (Santucci et al. 1998; Seddon et al. 2001). And while the north-south genomic division is demonstrating postglacial colonization from these refugia, the original refugial separation appears to have happened earlier, when considering Seddon et al. (2001). They calculated the division between *E. europaeus* and *E. roumanicus* to 3.2-4.5Mya and the division within each separate species to 1.7-2.2Mya.

As it comes to *E. europaeus* subdivision Santucci et al. (1998) inferred one clade from Sicily, Spain, France and the UK, while the other one is based on samples from mainland Italy and Germany. Herein, the level of observed divergence based on haplotype analysis is higher for the firstly and lower for the subsequently mentioned clade. Based on these findings they suggest an "east-west geographical partitioning down central Europe between France and Germany" (Santucci et al. 1998). These findings prompted Hewitt (1999) to speaks of a division "into three major genome strips" (see section 1.3.3 Post-glacial colonization and its genetic consequences, Fig. 3) and a distinction of Iberian, as well as Italian and Balkan haplotypes, that colonized Northern Europe from their ice age refugia. Also a more recent genetic substructuring (0.5Mya) on smaller geographical scales has been observed, within the eastern clade of *E. europaeus* (further subdividing German and Italian populations from each other) (Santucci et al. 1998).

Suchentrunk et al. (1998) as well speak of a "substructuring" within each of the species *E. europaeus* and *E. roumanicus* due to assumptions based on allele frequencies, still they only found "low genetic variability … compared to mammalian standards".

Seddon et al. 2001 distinguish three deeply divided clades within *E. europaeus*, based on mitotype analysis (indicating past fragmentation). Mitotypes from the first clade "are found from Italy northwards, through Austria, Switzerland, Germany, the Netherlands, Scandinavia and Estonia"; while the second clade is to be found only within Western Europe (populations from Spain, France, the Netherlands and the UK, as well as Ireland). A third clade is restricted to Sicily. Based on these results the researches proposed a trichotomy. Long distance colonization has been identified between southern Italy and Switzerland, as well as between northern Scandinavia and the Netherlands, while more recent range expansions seem to have happened within north-central Europe (Seddon et al. 2001).

Within *E. europaeus* Filippucci and Simson (1996) furthermore showed a clear differentiation of hedgehog populations originating from the Iberian peninsula (based on their allozyme study) and thereby asserting *E. hispanicus* (taxon *hispanicus*) to be a distinct species.

Concerning *E. roumanicus´* expansion out of the southern refugium of the Balkans Bolfíková et al. (2017) hypothesize the existence of a rather complex division into "subpopulations located mainly in the Pannonian Basin and the Adriatic and Pontic coast". Populations in their study, originating from the areas of Crete, Cyclades and Peloponnese, Euboea and southern, as well as central Greece, show strongly diverged genomes. Insular populations, in this concern, have been detected with rather low values of genetic diversity, indicating strong founder effect and genetic drift. They furthermore found genetic differentiation between individuals from the eastern and western (coastal) edges of the Balkan Peninsula. They deduce this from isolation-by-distance and the Carpathian Arc as a physical impediment to expansion. Also individuals from the Pannonian Basin appear as a separate division, leading the authors to attribute this circumstance to it being a distinct biogeographic region and a suspected "continental interglacial refugium", possibly leading to "geographical isolation and ecological niche differentiation" in the Northern White-breasted hedgehog (Stewart et al. 2010; Bolfíková et al. 2017). Geographical isolation, resulting in limited gene flow, is also found within the Slovenian population, which is (possibly) separated from the Balkans by the Alps and Dinarids. The results of their study also led them to the conclusion of genetic differentiation among hedgehog populations during interglacial range expansions. They recognize microevolutionary forces tackling at peri- and parapatric range edges as a powerful shaper in this concern (Bolfíková et al. 2017).

Estonia and Russia, now being inhabited by *E. roumanicus* seem originally to have been hosting *E. europaeus*, colonizing the area from central Europe through the Balkans. The contact zone present in this region is assumed to be of later origin than the central European one (Seddon et al. 2001). Due to this fact, and based on results from their study in 2012, Bolfíková and Hulva concluded that within the mid-European contact zone complete reproductive isolation (and "a recent range expansion of *E. roumanicus*") led to the development of a zone of sympatry, which did not yet happen in the Russian contact zone (Bolfíková and Hulva 2012).

1.5 Current knowledge on hybridisation occurrence between *E. europaeus* and *E. roumanicus*

Adaptive evolution, which represents the response of individuals, species and populations to changes within their environment, portraying selection effects, is one way of driving diversification within allopatric speciation (Coyne and Orr 2004). Secondary contact zones forming among allopatrically evolved populations lead to species interaction and further adaptive processes (Hewitt 2004). Where postglacial expansions from glacial refugia meet such zones of secondary contact and consequent hybridisation can be formed. As "a crossroad of postglacial colonisation routes" Central Europe possesses many such suture zones, where distinct phylogroups can get into secondary contact (Hewitt 2004; Avise 2009). Specific areas, like the Alps, the Pyrenees, central Europe and Scandinavia, represent crucial places for possible hybridisation events, triggered by post-Pleistocene range expansions out of common ice-age refugia and guided by physical dispersal barriers (Hewitt 1996;

Avise 2009). Taberlet et al. (1998) describe four main suture- zones, which are in accordance with the before mentioned regions.

Numerous species within central Europe – like the house mouse (*Mus domesticus* and *M. musculus*; Ferris et al. 1983; Boursot et al. 1996), the yellow-bellied and fire-bellied toad (*Bombina variegata* and *B. bombina*; Szymura 1993), the hooded and carrion crow (*Corvus cornix* and *C. corone*; Mayr 1963), grass snakes (*Natrix natrix*; Thorpe 1984) and shrews (*Sorex araneus* group; Taberlet et al. 1994) – show contact zones with hybrid occurrence along a north-south axis, where "divergent eastern and western genomes" meet (Santucci et al. 1998).

Such hybrid zones, however, defined according to Barton and Hewitt (1985) are "narrow regions in which genetically distinct populations meet, mate and produce hybrids". Remington (1968), however, speaks of suture zones, where hybrids arise through "interaction between recently joined biotas" (Hewitt 1996).

E. europaeus and *E. roumanicus* show a parapatric distribution range on a macrogeographical scale and a mid-European zone of sympatry with contact (and overlapping) zones situated in 1) Central Europe, reaching from Poland and the Czech Republic downwards Austria to the border between Italy and Slovenia on a north/south axis; as well as in 2) North-eastern Europe, situated in Latvia, Estonia and Russia to the Ural mountains. In both contact zones, the occurrence of hybridisation is termed possible (Bolfíková and Hulva 2012; also see Figure 5).

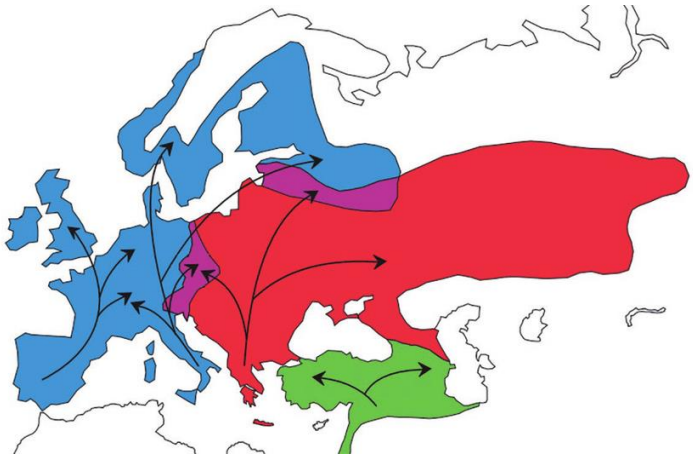

Fig. 5. Sympatric and potential hybridization areas of *E. europaeus* and *E. roumanicus*. Purple colour indicates areas of overlap (Picture taken from Bolfíková and Hulva (2012)).

West European and Northern White-breasted hedgehog both possess compatible karyotypes with a chromosome number of 2n = 48, enabling them to potentially interbreed (Gropp et al. 1969; Sokolov et al. 1991 in Bolfíková and Hulva 2012).

Within the 1970s and 1980s hybrids between *E. europaeus* and *E. roumanicus* had only been described, based on morphological observance within the mid-European contact zone (Kratochvil 1975 in Bogdanov 2009). The occurrence of hybrids in the wild, based on such analyses, was considered rare (Ruprecht 1972). A limited interspecific mating in nature, was also indicated by Corbet (1988), who referred to multivariate analyses of captive-bred hybrids (Holz 1978).

Both species were artificially crossed within laboratories, obtaining F1 litter in captivity and backcrosses with *E. roumanicus*. Interspecies hybrids represented profound variability among their intermediate morphological characteristics (Poduschka and Poduschka 1983). At this time evidence of hybridization (interspecific mating) through genetic analysis was still absent, resulting in no direct proof of hybrid individuals in nature and an unknown status in concern of their frequency of gene exchange (Bogdanov et al. 2009; Bolfíková and Hulva 2012).

So, by the 1990s hybridisation of *E. europaeus* and *E. roumanicus* in the wild was of limited evidence and due to the assumption of limited overlap, generally not hypothesised (Corbet 1988; Reeve 1994).

One of the first authors to investigate *E. europaeus* and *E. roumanicus* contact zone within Austria and adjacent regions on a genetical basis were Suchentrunk et al. (1998) using allozymes for their investigation of genetic variability and divergence between hedgehogs within central Europe. No proof of introgressive hybridisation was found by them. However, one should consider the limited geographical coverage of their study area (Bolfíková and Hulva 2012). In concordance with these results are, the findings of Filippucci and Lapini (1988) who investigated the zone of overlap in north-eastern Italy (Suchentrunk et al. 1998).

Seddon et al. (2001), who made profound investigations on genetic differentiation between *E. europaeus* and *E. roumanicus*, did not detect hybridization among their examined samples. But they found *europaeus* as well as *roumanicus* mitotypes within the boundaries of Italy and Lower Austria ("confirming the known north-south boundary between the species") (Seddon et al. 2001). Also Santucci et al. (1998) assumed a possible hybridization between the two species.

The first to find genetic proof of a naturally occurring hybrid within the region of Moscow, in Russia – where both species occur (Eastern European contact zone) – were Bogdanov et al. (2009). They detected an individual that possessed the nucleotide sequence of the observed 1 TTR intron from *E. roumanicus* on the one, and the mitochondrial DNA of *E. europaeus* on the other hand (Bogdanov et al. 2009).

As it comes to the Central European contact zone, investigations on the possible hybridisation of *E. europaeus* and *E. roumanicus* were undertaken by Bolfíková and Hulva, in 2012. They, however, were not able to find proof of recent hybridisation or introgression on a genetical basis in 2012. Which led them to the assumption of absence of hybridisation or interspecific mating on a very low level in consequence of prezygotic reproductive isolation or selection against hybrids in the wild. What needs

to be taken into consideration, in this concern, is a possibly varying abundance and density of both species in different regions, as well as the influence of artificial habitat fragmentation due to human settlements etc. (Bolfíková and Hulva 2012).

The same authors, however, were able to detect a hybrid individual of *E. europaeus x E. roumanicus* "for the first time in Central Europe" (Slovakia), by 2017 (Bolfíková et al. 2017). Bolfíková et al. (2017) thereby presume that when the West European hedgehog and the Northern White-breasted hedgehog came into contact for the first time interspecific mating and consequent hybridisation regularly happened, due to a not fully completed reproductive isolation. They hypothesise as well that interspecific hybridisation diminished after a subsequent reinforcement phase, due to the prevention of hybrid formation by adaptive evolution. The formation of the mentioned contact zone and consequent development of parapatry among the two related species can thereby be seen as a driving force limiting range expansion. Thus hybridisation and introgression along the suture zones of *E. europaeus* and *E. roumanicus* are considered to have farther developed and formed allopatrically evolved lineages (Bolfíková et al. 2017).

The postglacial recolonization of Europe out of major glacial refugia led to the formation of many different geographical distribution schemes and underlying genetical patterns (modes of speciation) for numerous species. The case of European hedgehogs constitutes just one out of many different examples, ranging from sole zones of hybridisation (e.g. the mice *Mus musculus* and *M. domesticus*; Selander et al. 1969) to wide-ranging and extensive zones of sympatric evolution (e.g. the bats *Pipistrellus pipistrellus* and *P. pygmaeus*; Hulva et al. 2010) (Bolfíková and Hulva 2012).

1.6 Research questions and hypothesis

Even though *E. europaeus* and *E. roumanicus* are regarded `least concern` by the IUCN Red List of Threatened Species (Amori 2016 and Amori et al. 2016), the species are of conservation interest on a more local geographical scale. The special importance of investigations on hedgehog (genus *Erinaceus*) phylogeography and assessment of genetically admixed individuals within their Central European contact zone can be traced back to several reasons. Hedgehogs are mainly to be found in close proximity to human settlements, rural as well as urban, occupying diverse man-made habitats (Amori 2016; Amori et al. 2016). This may lead to impacts on their population structure through human infrastructure and anthropogenic barriers, like roads, fragmenting the landscape. Thereby posing potential risks of limited migration possibilities and consequently disturbed gene flow (Huijser and Bergers 2000; Orlowski and Nowak 2004). This fact of a disturbed isolation-by-distance pattern on small geographical scales has already been proven in the UK (Becher and Griffiths 1998). Moreover, as likeable and well-known garden species, hedgehogs are in the centre of attention, when it comes to human-mediated rescue actions. They are frequently translocated and among one of the

most abundant species, to be found in wildlife or animal shelters (Molony et al. 2006). Subsequent releases, after successful rehabilitation, may result in (unintentional) translocation, if the individual´s origin is unknown or a release at this site not possible. Such uncoordinated translocations may have severe consequences for the wild population at the release site. Especially in *E. europaeus* and *E. roumanicus´* sympatric zone the impact on the genetic level might include promoted hybridization and outbreeding depression ("the reduction in fitness of hybrids compared with parental types" (Allendorf et al. 2013)) (Edmands 2007), as well as the loss of local adaptions and a general decrease in fitness (Allendorf et al. 2013).

Under this background the following issues – and corresponding research questions – will be processed within the present master thesis:

- Investigation of potential hybrid zone dynamics within Austria on the basis of dense sampling and the usage of multiple markers
    - Testing for signals of admixture between *E. europaeus* and *E. roumanicus*
      H0: No hybrid occurrence expected
    - Characterization of *E. europaeus* and *E. roumanicus´* contact zone within Austria
      H0: A narrow zone of overlap between eastern Upper and western Lower Austria is expected, based on previous paleontological and morphological assessments

- Investigations on the phylogeography of *E. europaeus* and *E. roumanicus* within Europe (from the Iberian peninsula to the Balkan peninsula) on an inter- and intraspecific level
    - Evaluation of factors influencing local colonization processes in consideration of post-glacial colonization patterns
    - Testing for potential isolation-by-distance within the respective species
      H0: No isolation-by-distance is expected

2. Material and Methods

2.1 Sample Collection and Preparation

In the course of a more comprehensive study on hedgehog genetic diversity, conducted at the Institute for Integrative Nature Conservation Research (INF, Department of Integrative Biology and Biodiversity Research, University for Natural Resources and Life Sciences Vienna) a total of 716 hedgehog samples have been collected and analysed since today. Sampling was largely conducted by employees and previous master students of the INF, as well as institutions that collaborate with the INF. Most samples stem from road fatalities and hedgehogs that have been sampled within animal shelters. A small portion of the original amount of samples has been prepared (seven samples DNA isolation, 45 samples multiplex PCR) and analysed (264 samples within population genetic analysis) by the author of the present master thesis. All mentioned samples underwent a similar treatment that will be outlined in the following sections. If treatment differed from the one performed by the author of the thesis, it followed the methodology described in Curto et al. (2019).

Individuals were either sampled through mouth swabs (animals that were still alive, corresponding to shelter animas) or muscle tissue (from road fatalities or samples that have been collected by scientific institutions). All samples were stored in Ethanol until further preparation and analysis (Curto et al. 2019).

2.2 Sample origin and geographical background

The majority of samples (224) has been collected within Austria, either as road fatalities or through sampling within animal shelters. 126 of the Austrian samples have been collected within the borders of Upper Austria, 5 in Lower Austria, 7 in Burgenland, and 1 in Salzburg, all of them wild animals (partly sampled by Plass Jürgen from the Biologiezentrum Linz, Upper Austrian State Museum; 1 sample contributed by the Natural History Museum Vienna). Samples from animal shelters were taken in Bludenz (31), Innsbruck (24) and Carinthia (30) (all of them contributed by Anna Seiter), whereby all shelter animals were found in a radius of max. 100 km around the shelter and within the same province (Curto et al. 2019). Non-Austrian samples were collected in Portugal (4 samples collected by members of the Research Centre in Biodiversity and Genetic Resources, CIBIO), Spain (4 samples collected by the CIBIO), Berlin (5 samples collected by Leon Barthel, Leibniz Institute for Zoo and Wildlife Research, IWZ), in proximity to the animal shelter “Igelburg Mossautal e.V” (10 samples contributed by Lea Ficker), in southeast Bavaria close to the Austrian border (2), in the Czech Republic (5), Poland (1), Slovakia (1), Hungary close to the Austrian border (1), Slovenia (2), Croatia (1), North Macedonia (1) and Greece (3). Institutions that have been providing the analysed samples are to be found in Table S2. Fig. 6 indicates the locations of all 264 samples used for population

genetic and phylogeographic analyses (Google LLC, Google Earth Pro Version 7.3.2.5776), exact coordinates of the location where each sample was taken, can be found in the Appendix (Tab. S2).

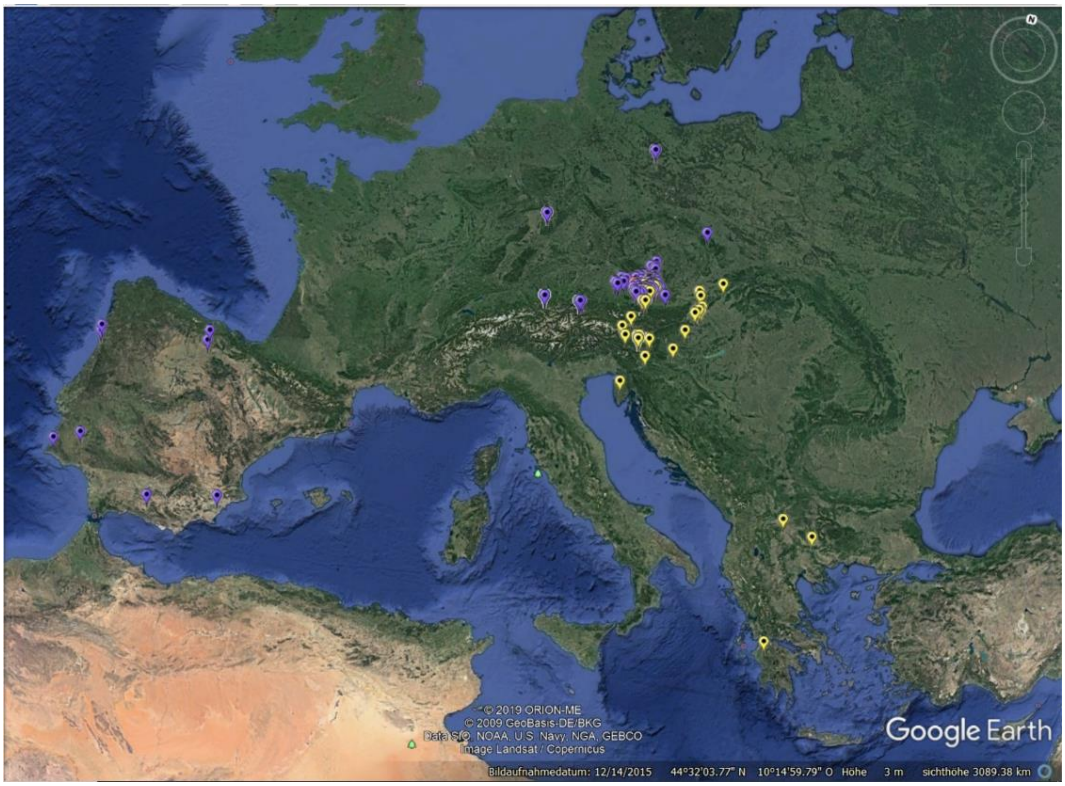

Fig. 6. Sample locations of all 264 hedgehog samples that were available for population genetic and phylogeographic analyses after exclusion of samples with too much missing data and usage restrictions. Coordinates and exact locations have been available for all samples from free-living or wild hedgehogs. Shelter individuals stem from no further than 100 km around the corresponding shelters. The map was created using Google Earth Pro Version 7.3.2.5776. The colour assignment is a reference to the detected species [blue = *E. europaeus*; yellow = *E. roumanicus*; red = potential hybrid]

Sample locations, including coordinates, that have been used for the creation of Fig. 6 were available for most of the samples that had actually been collected from free-living individuals. For some samples no coordinates were available (indicated as NA in Tab. S2) and they were assigned to a central random point of the city they were collected in, for graphical demonstration (see Fig. 6). Samples from shelter individuals were either given the coordinates of the shelter location, if no actual coordinates were available for them, or a random central point in the city they had been collected in, if such information was present. Furthermore it is known and has been corresponded that no shelter individual was collected further than 100 km around the shelter location or in a different province (Curto et al. 2019). Exact locations for each individual (if available) can be found in Table S2.

Tab. 1 gives an overview on the artificially created “populations” hedgehog individuals have been assigned to for further population genetics and phylogeographical analyses in STRCUTURE v.2.3.4 and GenAlEx v. 6.5. Samples have largely been grouped together, according to their geographical origin based on political barriers and (federal) state barriers.

Tab. 1. Artificially designed "populations" for further population genetic and phylogeographical analyses of hedgehog samples in STRUCTURE v. 2.3.4 and GenAlEx v. 6.5. Depending on their geographical origin and sample locations animals were assigned to 20 artificially created populations. The table shows the population name + subsequently used abbreviation, the number of samples to be found in each population, the geographical basis for the assigned population, i.e. the range spanned up by the corresponding samples in each population*, as well as the number of *E. europaeus*, *E. roumanicus* and potential hybrid (i.e. admixed) individuals within each population [*Abbreviations: GER = Germany, AUT = Austria]

Population	# individuals	geographical range/background*	*E. europaeus/roumanicus* individuals
Iberian Peninsula (IBP)	8	Portugal + Spain	8/0
Shelter Mossautal (MT)	10	area of Mossautal, GER	10/0
Berlin (BER)	5	city of Berlin, GER	5/0
Bludenz (BLU)	31	Vorarlberg, AUT	31/0
Innsbruck (INS)	24	Tyrol, AUT	19/0/(5)
Salzburg (SBG)	1	Salzburg, AUT	0/1
Upper Austria West (UAUT_W)	20	Western Upper Austria, AUT	16/2/(2)
Upper Austria South (UAUT_S)	12	Southern Upper Austria, AUT	5/3/(4)
Upper Austria North (UAUT_N)	10	Norther Upper Austria, AUT	10/0
Linz (LINZ)	39	Linz, Upper Austria, AUT	30/8/(1)
Upper Austria East (UAUT_E)	47	Eastern Upper Austria, AUT	27/19/(1)
Lower Austria (LAUT)	2	Western Lower Austria, AUT	2/0
Czech Republic (CZE)	5	Southern Czech Republic	5/0
Poland (POL)	1	Southern Poland	1/0
Slovakia (SVK)	1	Western Slovakia	0/1
Eastern Austria and Hungary (EAUT + HU)	11	Eastern Lower Austria + Burgenland + Western Hungary	1/9/(1)
Carinthia (CAR)	30	Carinthia, AUT	0/30
Slovenia (SLO)	2	Central + Eastern Slovenia	0/2
Croatia (CRO)	1	Western Croatia	0/1
Greece and North Macedonia (GRE/NM)	4	Greece and North Macedonia	0/4

2.3 DNA Isolation

For DNA isolation of muscle tissue samples the "MM-Separator M96 P in house buffer system" used in Curto et al. (2019) was followed. Therefore a small piece of tissue was placed in 500µL lysis buffer (2% SDS, 2% PVP-40, 250mM NaCl, 200mM Tris HCl and 5mM EDTA at pH8), to which 16.67µL of Proteinase K [10mg/mL] were added, following an overnight or at least 2.5h incubation period at 56°C (600rpm for 20 seconds, every 10 min in a thermoblock). 16.67µL RNase [10mg/mL] were added after that, again followed by an incubation period of 20 min at 37°C (500rpm for 10 sec. every 1.5 min). After the second incubation 125µL of 3M KOAc (pH 4.7) were added, mixed by inverting and put on ice for 20 min. A series of centrifugation steps was then performed: 1.000rpm for one minute, 2.000rpm for one minute, 4.000rpm for one minute, 8.000rpm for one minute and 13.600rpm for eleven minutes. 400µL of the supernatant of each sample were then mixed with 10µL of MagSi-DNA beads (size 300nm, MagSi-DNA beads from MagnaMedics), as well as 400µL binding buffer

(2M GuHCl in 95% ethanol) and incubated at room temperature (five min.) for DNA purification purposes. To separate the resulting supernatant from the beads, the samples were placed on the magnetic separator SL-MagSep96 (Steinbrenner, Germany) for one minute. Two washing steps with 80% ethanol (600µL each) followed. After discarding the resulting supernatants the magnetic beads were air-dried at room temperature for at least ten minutes or in an oven for 30 seconds. Two elutions were acquired, each with 50µL of preheated (65°C) elution buffer (10mM TrisHCl at pH8), that was mixed with the beads and incubated for 10 minutes at room temperature, to allow separation of the genomic DNA from the magnetic beads. Tissue samples that were not treated as above, followed the DNA isolation as described in Curto et al. (2019).

DNA isolation of buccal swabs was conducted following the treatment described in Curto et al (2019).

To control for DNA isolation success and quality of the isolated genomic DNA a gel electrophoresis was conducted in a 0.8% gel, that was prepared with agarose, 1x TAE buffer and HDGreenplus DNA stain. 5µL of each sample were applied with 1µL of loading buffer (6x). 6µL of λEcoRI/HindIII ladder were used for comparison of fragment size. The program INTAS was used to survey the bands obtained within the gel electrophoresis process under UV light. Samples were expected to show bands with fragment sizes around 600bp (see Fig. 7). After control of genomic DNA isolation through gel electrophoresis it was decided whether to use the first or second elution of a sample for further preparation for multiplex PCR and sequence analysis, depending on the DNA isolation success.

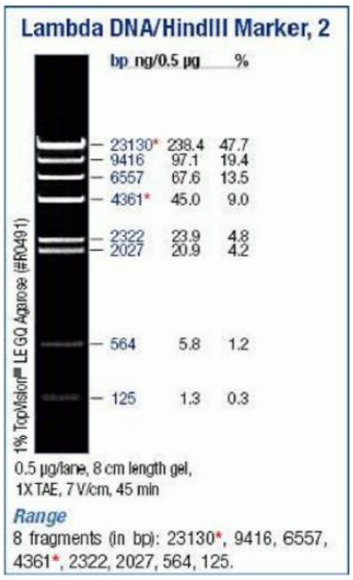

Fig. 7. lambda EcoRI/HindIII ladder used for comparison of fragment sizes of the isolated genomic DNA from hedgehog samples. Samples were expected to show fragments around 500-600bp size

2.4 Molecular Marker enrichment and amplification

2.4.1 Multiplex PCR

Short sequence repeats (SSR, i.e. microsatellites are "tandemly repeated DNA consisting of short sequences of 1 to 6 nucleotides repeated approximately 5 to 100 times" (Allendorf et al. 2013)) were used as molecular markers for amplification of DNA within the two observed hedgehog species (Curto et al. 2019). Amplification of those specific microsatellite regions within the genomes of *E. europaeus* and *E. roumanicus* was conducted using a multiplex PCR approach. In this concern multiplex PCR allows for the amplification of multiple loci, within a single PCR reaction (Allendorf et al. 2013: Genetic variation in natural populations: DNA, Single-copy nuclear loci, Microsatellites). 55 different primers were available for this approach, divided into the four primer mixes HH1, HH2 and HH3

(summing up to 30 different primers for amplifications within the genome of *E. roumanicus*) and WHH1 (containing 25 primer pairs for the use within *E. europaeus* genome). A list of all primers that have been used for initial Multiplex PCR can be found in Tab. S1 in the Appendix. These primer sets have been developed and improved by Curto et al. (2019). Amplification primers are build up by specific sequences that are elongated by Illumina P5/P7 sequences which correspond to the Illumina adapter. The forward primers were elongated with a part of the P5 motif (TCTTTCCCTACACGACGCTCTTCCGATCT) and the reverse primers with a part of the P7 motif (CTGGAGTTCAGACGTGTGCTCTTCCGATCT). The Multiplex PCR Kit from QIAGEN was used for performance of multiplex PCR. Therefore 1µL of template, i.e. genomic DNA, was added to a prepared mix of 5µL QIAGEN Multiplex PCR Master Mix (Qiagen, CA, U.S.A), 3.5µL of H_20 and 0.5µL of specific primer mix (1µM). Each sample was prepared for a PCR reaction with each primer mix. Tab. 2 shows the used program for the multiplex PCR.

Tab. 2. Multiplex PCR scheme for amplification of short sequence repeats within the genomes of *E. europaeus* and *E. roumanicus*. Shown are the individual cycle steps, as well as the temperature profile, time and number of cycles for each specific cycle step. Primes have been specifically designed to anneal at 55°C (Curto et al. 2019).

Cycle step	Temperature	Time	# cycles
1. Initial Denaturation	95°C	15 min	
2. Denaturation	95°C	30 sec	30
3. Annealing	55°C	1 min	30
4. Extension	72°C	1 min	30
5. Final Extension	72°C	10 min	

For evaluation of successful amplification and quality of the PCR products agarose gel electrophoresis was conducted to visualize PCR results (1.5% agarose gel with 0.79g of agarose, 52.2mL 1xTAE buffer and 0.7µL DNA stain). 3µL of loading buffer (4x) were mixed with 2µL of DNA and loaded onto the gel. For comparison of fragment size the "Gold"bp DNA ladder (Invitrogen) was used. Amplified fragments are expected to show a size of around 450bp. The program INTAS allowed for visualization of fragments under UV light.

2.4.2 Purification of Multiplex PCR products for Index PCR

Purification of PCR products in preparation of Index PCR followed the "Invers magnetic separator" protocol by Curto et al. (2019). In order to remove artefacts of the performed multiplex PCR (like unspecific amplification products, primer dimers, unused primers and buffer) before index PCR is started, the amplified fragments were purified using an inverse magnetic separator. Therefore all matching samples that had previously undergone separate amplification, with different primer mixes,

were now pooled (1.5µL of each sample x 4 different primer mixes = 6µL total sample volume). The total 6µL sample volume were mixed with 4.3µL of magnetic beads in a source plate/binding plate and incubated at room temperature for five minutes. A sample plate that had previously been attached to the magnetic separator was then inserted into the binding plate and moved in circles for two minutes, until a clear solution was obtained. The supernatant was discarded while the magnetic beads with the DNA stuck to the magnetic separator. The sample plate was then transferred to the first wash source plate, where the beads were washed with 200µL of 80% ethanol through circular movement for 45 seconds. This washing step was repeated under same conditions and after discarding the ethanol the magnetic particles were air-dried for at least five minutes. The sample plate was then put into a source elution plate with 17µL of preheated (65°C) elution buffer (10mM TrisHCl, pH 8) in each well. The magnetic separator was removed and mixing through circular movement for two minutes followed. The magnetic separator was then put on the sample plate again and this way magnetic beads were removed. The remaining solution contained the purified DNA fragments that could now be used for Index PCR.

2.4.3 Index PCR

Index PCR allows for the individual identification of pooled samples with specific forward and revers primers through specific assignment of indices. On the on hand the used primers help binding to the before amplified P5/P7 part of the primers used in Multiplex PCR. On the other hand they allow for binding to the flow cell in Illumina Sequencing and label the sample with an unique eight-bp index information, that helps assigning sequenced genotypes to single samples (P5: AATGATACGGCGACCACCGAGATCTACAC [Index] ACACTCTTTCCCTACACGACG; and P7: CAAGCAGAAGACGGCATACGAGAT [Index] GTGACTGGAGTTCAGACGTGT). For Index PCR 1µL of clean PCR product was mixed with 5µL of Multimix (QIAGEN), 2µL of specific P5 forward primer and 2µL of specific P7 reverse primer. The temperature profile that can be seen in Tab. 3 was used for Index PCR.

Tab. 3. Index PCR scheme for amplification and individual labelling of multiplex PCR products from *E. europaeus* and *E. roumanicus* samples. The process provides the before amplified products with specific indices for later assignment of specific genotypes to single samples and allows for the binding to an Illumina flow cell during sequencing. Shown are the individual cycle steps, as well as the temperature profile, time and number of cycles for each specific cycle step.

Cycle step	Temperature	Time	Number of cycles
1. Initial Denaturation	95°C	15 min	
2. Denaturation	95°C	30 sec	10
3. Annealing	58°C	60 sec	10
4. Extension	72°C	60 sec	10
5. Final Extension	72°C	5 min	

2.5 Illumina Sequencing

Following Index PCR samples were pooled and sent to the Biozentrum LMU in Munich, Germany, for sequencing in an Illumina MiSeq machine in both directions. Illumina Sequencing is a Next/Second Generation Sequencing approach (NGS), which operates through a sequencing by synthesis (SBS) concept. The prepared library, which has been sent in for sequencing, is in a first step loaded into an Illumina flow cell, where the fragments can hybridize to the surface through the before ligated P5/P7 parts of the primers. Through bridge amplification clonal clusters of each fragment are being generated. This step is followed by the actual SBS process, which operates through reversible terminator-bound dNTPs. Fluorescently labelled nucleotides are incorporated into the DNA template strands and the flow cell is imaged after each incorporation of a nucleotide, this way enabling the identification of each incorporated base, through emission wavelength and intensity. Sequencing in this NGS approach, however, is applied through paired-end sequencing, enabling to sequence both ends of library products and to align forward and reverse reads as read pairs (Illumina, Inc., downloaded 23.01.2020).

2.6 Sequence data analysis

The short sequence repeats genotyping by sequence (SSR-GBS) method used in this thesis follows Curto et al.´s (2019) methodology. Amplicon sequences are being used for determination of genotypes. In this context alleles are defined according to their length and the occurrence of single nucleotide polymorphisms (SNPs).

2.6.1 Sequence data extraction

Sequences resulting from the Illumina run were supplied in two FASTQ files (Read 1 and Read 2, resulting from forward and reverse strand, respectively), containing all sequences per index. The following processing and treatment of the samples was based on a combination of custom made scripts, as well as third party programs (see Curto et al. 2019). This includes the quality control of single bases, as well as each read, followed by trimming of low quality regions (Phred < 20, according to Curto et al. 2019). Sequences were aligned using the program PEAR (Zhang et al. 2014), meaning that paired reads (Read 1 and Read 2) were merged. The necessary overlapping range was set to a minimum of 10bp, with “a p-value below 0.01 for the highest observed expected alignment scores” (Zhang et al. 2014 in Curto et al. 2019). Markers have explicitly been designed to generate overlapping fragments of approx. 300 bp. Overlapping sequences below 250bp and non-overlapping reads were not considered during further steps. Through a “demultiplexing” step it was possible to identify primer sequences on each side of the merged reads and separate them per locus (in-house phyton script 1, Curto et al. 2019). As merged reads were supposed to begin with the forward primer

and end with the reverse primer sequence, based on library preparation. Finally sequences were sorted by locus and sample, resulting in corresponding files, that were used for further genotyping analysis (Curto et al. 2019).

2.6.2 Allele definition

Sequences with lengths under 300bp did not go into further genotyping analysis. Since amplicon construction was aiming at read lengths above 400bp, also markers below this threshold were deleted. Allele definition was largely based on two major steps – 1) sequence length, as well as 2) possible occurrence of SNPs in each separate length class (Curto et al. 2019).

Within each sample, each marker was examined for its most frequent sequence length class. This was again done through a custom-made/in house script (script 3, Curto et al. 2019) and manually controlled based on length histograms (resulting in "a graphical representation similar to traditional SSR chromatograms"). Loci were considered to be homozygous, if they comprised a certain length with a frequency equal to or above 90% among all reads for the respective marker. If a locus showed two lengths with a frequency greater than 90% of all reads (and those frequencies differed by less than 20%), the genotype was considered heterozygous. As well as the calling for alleles based on length frequencies, the employed script (script 3) checked for possible stutter within the selected alleles (Curto et al. 2019).

The various reads within the most frequent length class(es) of a (homozygous) locus were merged into one consensus sequence through script 5 (Curto et al. 2019). Therefore nucleotide positions were considered to be homozygous if they showed a frequency above 70% for a single position, and to be heterozygous if the frequency of a nucleotide within a single position was below 70%. Loci, within a specific sample, that had already been defined as homozygous based on their sequence length class, could be considered heterozygous based on the two most frequent nucleotides for a position (script 6, Curto et al. 2019). Nucleotide positions were considered to be linked, if more than on potential SNP occurred in a sequence. For samples already defined as heterozygous based on length class it was decided to choose the most frequent SNP combination (Curto et al. 2019).

Based on the called alleles from all samples a codominant matrix was set up (script 7, Curto et al. 2019), as the input for later population genetic analyses within different standard programs. This matrix consists of two specific numbers – corresponding to unique sequences (i.e. specific alleles) – for each investigated locus, of every sample.

2.7 Population genetic and phylogeographic analyses

The codominant matrix, that had been created with script 7 (Curto et al. 2019) after allele definition, was used as an input for the subsequently mentioned programs during population genetic analysis (in a phylogeographic context).

Before starting specific analyses some of the samples had to be excluded in consequence of usage restrictions, due to collaborations with several different institutions and other working groups, and because of sequence analysis resulting in too much missing data, within specific samples and markers. Concerning the latter, markers with more than 50% of missing data after initial sequence data analysis were excluded. As well as that samples with a threshold over 50% missing marker information have been excluded in a further step. A list of all excluded samples can be found in Tab. S3.

After the initial sequence analysis and exclusion of data 39 microsatellite markers were valid to be used (see Tab. S1) and 264 out of all samples were analysed in the course of population genetics and phylogeographic questions within this master thesis (see Tab. S2). 247 of them had already been defined as *E. europaeus* or *E. roumanicus* within previous analysis and on the basis of morphological assignment (Curto et al. 2019).

Specific analysis were conducted in two different approaches: 1) analysis of all 264 hedgehog samples; as well as 2) separate intraspecific analysis for the two respective species *E. europaeus* and *E. roumanicus*, after initial analysis of all samples and subsequent assignment to a specific species (assignment of each individual to *E. europaeus* or *E. roumanicus* on the basis of previous knowledge (Curto et al. 2019), previous morphological assignment, as well as PCoA results from GenAlEx analysis)

2.7.1 Population genetic and phylogeographic analysis within GenAlEx v.6.5

GenAlEx (Genetic Analysis in Excel, Peakall and Smouse 2006, 2012) is a software package designed for use in Excel, aiding users to analyse population genetic data in a simple manner.

Analyses within this master thesis were conducted 1) on the basis of all 264 hedgehog samples, as well as 2) for *E. europaeus* and *E. roumanicus* separately, after prior analysis of 1) and corresponding adjustment of data. Within each analysis 39 loci were available for investigation of various population genetics. Codominant genotypic data (codominant matrix with two columns per locus) was used as the input for analysis within GenAlEx 6.5 (Peakall and Smouse 2006, 2012).

2.7.1.1 Principal Coordinate Analysis (PCoA) within GenAlEx v.6.5

The Principal Coordinate Analysis (PCoA) is a multivariate technique, that assists with the investigation of "major patterns within a multivariate dataset", i.e. "multiple loci and multiple samples" (Blyton and Flanagan 2006, Peakall and Smouse 2009 a). This way it helps "visualizing the patterns of genetic relationship", through "the major axes of variation … located within a multidimensional data set" (Peakall and Smouse 2009 a).

The implemented PCoA is based on the calculation of genetic distance within GenAlEx 6.5. and the usage of a Tri Distance Matrix as input data type. For the PCoA method "covariance-standardized" was chosen (following Peakall and Smouse 2009 a).

For the 1) interspecific approach samples were divided into two different populations, i.e. *E. europaeus* and *E. roumanicus* (pre-assignment based on existing knowledge due to previous analysis (Curto et al. 2019), as well as morphological classification). The resulting PCoA was observed for consistency between samples and their assigned population, as well as for "intermediated" samples that might not show a clear pattern of membership/classification.

PCoA within 2) each separate species, i.e. *E. europaeus* and *E. roumanicus,* was based on the division of them into several sub-populations – 10 and 8 populations for West European and Northern White-breasted hedgehogs, respectively. For population set-up the before designed artificial populations (see Tab. 1) were used, with the exception of all samples belonging to the federal State of Upper Austria going into one single population. PCoA was conducted on the basis of Nei genetic distances between populations as well as on genetic distance tri matrix for all separate samples.

2.7.1.2 Isolation-by-distance analysis within GenAlEx v.6.5

To support the inference on population sub-structuring along a west-east-axis within each of the observed species, based on results potentially obtained within previous STRUCTURE analysis, an isolation-by-distance analysis within the Excel Add-In GenAlEx v.6.5 was conducted. Calculations were separated between the species *E. europaeus* and *E. roumanicus*.

"Isolation-by-Distance" hypothesis states that over larger geographical scales, genetic distances are likely to show a positive correlation with geographic distances, meaning that genetic differences between populations will be reflected. Within GenAlEx. v.6.5 the "Mantel test for Matrix Correspondences" allows for a statistical evaluation of this hypothesis. In this concern H0 states that no correlation between genetic and geographic distance is to be found (Rxy=0). H1, on the other hand, assumes a relationship between the both of them (Rxy>0) (Peakall and Smouse 2009 b).

Mantel test for isolation-by-distance calculations were conducted following Peakall and Smouse (2009 b, Ex 3.3): In a first step a pairwise geographic distance matrix (X matrix, see Tab. 6 and 8) was created, based on geographical coordinates in decimal Lat/Long for each inferred population (seven and five populations for *E. europaeus* and *E. roumanicus* respectively). The given coordinates for each population resemble an approximate central point of all samples within a population, since each population encompasses a wider geographic range due to sampling circumstances. Tab. 4 and 5 give an overview on the set up populations and corresponding values used for the conducted Mantel tests. Further on, the pairwise genetic distance matrix (Y matrix, see Tab. 7 and 9) was calculated. In this concern it was decided to work with a pairwise population matrix of Nei genetic distance (Peakall and Smouse 2009 a). Since working with Nei genetic distances requires a minimum population number of more than one individual, samples were grouped together and populations were re-arranged in comparison to the initial population set-up (see Tab. 1), as can be seen in Tab. 4 and 5.

Tab. 4. Parameters used for investigation of isolation-by-distance via Mantel test for *E. europaeus* populations. Given are the artificially created populations*, the number of individuals within each population, the geographic coordinates in decimal Lat/Long, as well as the corresponding region * [IBP = Iberian Peninsula, MT = Igelburg Mossautal e.V., BER = Berlin, BLU = Bludenz, INS = Innsbruck, UAUT = Upper Austria, CZE = Czech Republic]

Population	# individuals	POS N/latitude	POS E/longitude	region
IBP	8	39,74107778	-4,232027778	Portugal + Spain
MT	10	49,68759722	8,920125	Western Germany
BER	5	52,51971111	13,40490278	Northeastern Germany
BLU	31	47,16370833	9,806058333	Vorarlberg, AUT
INS	24	47,26383611	11,41428889	Tyrol, AUT
UAUT	93	48,19922222	14,38758611	Upper + Lower Austria, AUT
CZE	5	48,82941111	14,46251667	Southern Czech Republic

Tab. 5. Parameters used for investigation of isolation-by-distance via Mantel test for *E. roumanicus* populations. Given are the artificially created populations*, the number of individuals within each population, the geographic coordinates in decimal Lat/Long, as well as the corresponding region *[Abbreviations: UAUT = Upper Austria, EAUT + HU = Eastern Austria + Hungary, CAR = Carinthia, SLO/CRO = Slovenia/Croatia, GRE/NM = Greece/North Macedonia]

Population	# individuals	POS N/latitude	POS E/longitude	region
UAUT	33	48,19922222	14,38748611	Upper Austria, AUT
EAUT + HU	11	47,8618	16,98786944	Eastern Austria + Hungary
CAR	30	46,59824722	14,31148889	Carinthia, AUT
SLO/CRO	3	46,04690833	15,05567778	Slovenia + Croatia
GRE/NM	4	39,90357222	21,91797778	Greece + North Macedonia

2.7.2 Population genetic analysis within STRUCTURE v.2.3.4

2.7.2.1 Program STRUCTURE v.2.3.4

The program STRUCTURE (v.2.3.4), that has been developed in 2000 by Pritchard et al., is a "model-based" clustering method, which's application range spans from illustrating an underlying population structure within a set of samples, to the specific assignment of single samples to distinct populations and the investigation of potential hybrid ("admixed") individuals. This detection of underlying populations (structure) among a set of individuals in a genetic concern is based on the investigation of multilocus genotype data. Therefore STRUCTURE employs a Bayesian approach (Markov Chain Monte Carlo, MCMC), allowing for the computation of each individuals genetic proportion belonging to an inferred population (quantitative clustering method). This method operates through the assumption of K populations that differ in their allelic variants observed at specific loci (Pritchard et al. 2000; Sim downloaded on 28.1.2020).

2.7.2.2 Application of STRUCTURE v.2.3.4 on a codominant matrix for hedgehog population genetic analysis

Prior to loading the codominant matrix into STRUCTURE v.2.3.4 the corresponding excel file was adapted to conditions for input data in STRUCTURE, following Sim (downloaded on 28.01.2020) and Smith (2018). Analyses were conducted 1) on the basis of all 264 hedgehog samples (interspecific analysis), as well as 2) for *E. europaeus* and *E. roumanicus* separately. Within each analysis 39 loci were available for assignment to K populations. For both approaches the "Length of Burnin Period", as well as the "Number of MCMC Reps after Burnin" were given as 10.000. The "Ancestry Model" was chosen as "Use Admixture Model", the "Allele Frequency Model" as "Allele Frequencies Correlated". The chosen parameter set was run in a program with a range of K = 1 - 20 for the interspecific approach, K = 1 – 14 for *E. europaeus* intraspecific analysis and K = 1 – 11 for *E. roumanicus* intraspecific analysis. A random number was entered as seed starter, the "Number of Iterations" was given as 5.

After finishing the given job for all hedgehog samples (N=264) the K = 2 scenario (in assumption of two populations, i.e. *E. europaeus* and *E. roumanicus*) was bar-plotted within the STRUCTURE v.2.3.4 program and investigated for potential hybrid, i.e. admixed, individuals. This was conducted through observance of distribution between the two assumed populations, whereby individuals showing a probability value of less than 0.9 for one population, were considered to be admixed individuals (Bolfíková and Hulva 2012).

To choose the most likely value for all observed Ks and thereby determining the most likely number of populations (genetic groups) to be found within the underlying set of samples the "STRUCTURE

HARVESTER" (Earl and vonHoldt 2012) was used. STRUCTURE HARVESTER is a web portal (http://taylor0.biology.ucla.edu/structureHarvester/) or program that works through implementation of the Evanno method and thereby allows to calculate likelihood values to detect population numbers and estimate the probability of Ks in a simple manner (Earl and vonHoldt 2012).

To summarise all iterations of each K into one single summary output and graphically represent the results calculated within STRUCTURE v.2.3.4 the web portal CLUMPAK (Cluster Markov Packager Across K) (http://clumpak.tau.ac.il/), introduced by Kopelman et al. (2015), was used.

During latter evaluation results were assessed in context of the 20 artificially set up populations that are to be found within Tab. 1.

3. Results

3.1 Species delimitation and characterization of the contact zone

Out of the original 264 samples that underwent population genetic analysis 169 and 81 were assigned to the species *E. europaeus* and *E. roumanicus* respectively (see Tab. S1), on the basis of Bayesian clustering and PCoA. Fig. 8 shows the geographical distribution of West European and Northern White-breasted hedgehogs among all samples. *E. europaeus* individuals were found within the populations of the Iberian Peninsula (IBP), the area of Mossautal (MT), the area of Berlin (BER), within Vorarlberg (BLU), Tyrol (INS), Upper Austria (UAUT), Lower Austria (LAUT), Eastern Austria (EAUT), the Czech Republic (CZE) and Poland (POL). *E. roumanicus* individuals appeared in the populations of Salzburg (SBG), Upper Austria (UAUT), Eastern Austria and Hungary (EAUT + HU), Carinthia (CAR), Slovakia (SVK), Slovenia (SLO), Croatia (CRO) and Greece + North Macedonia (GRE + NM) (also see Tab. 1).

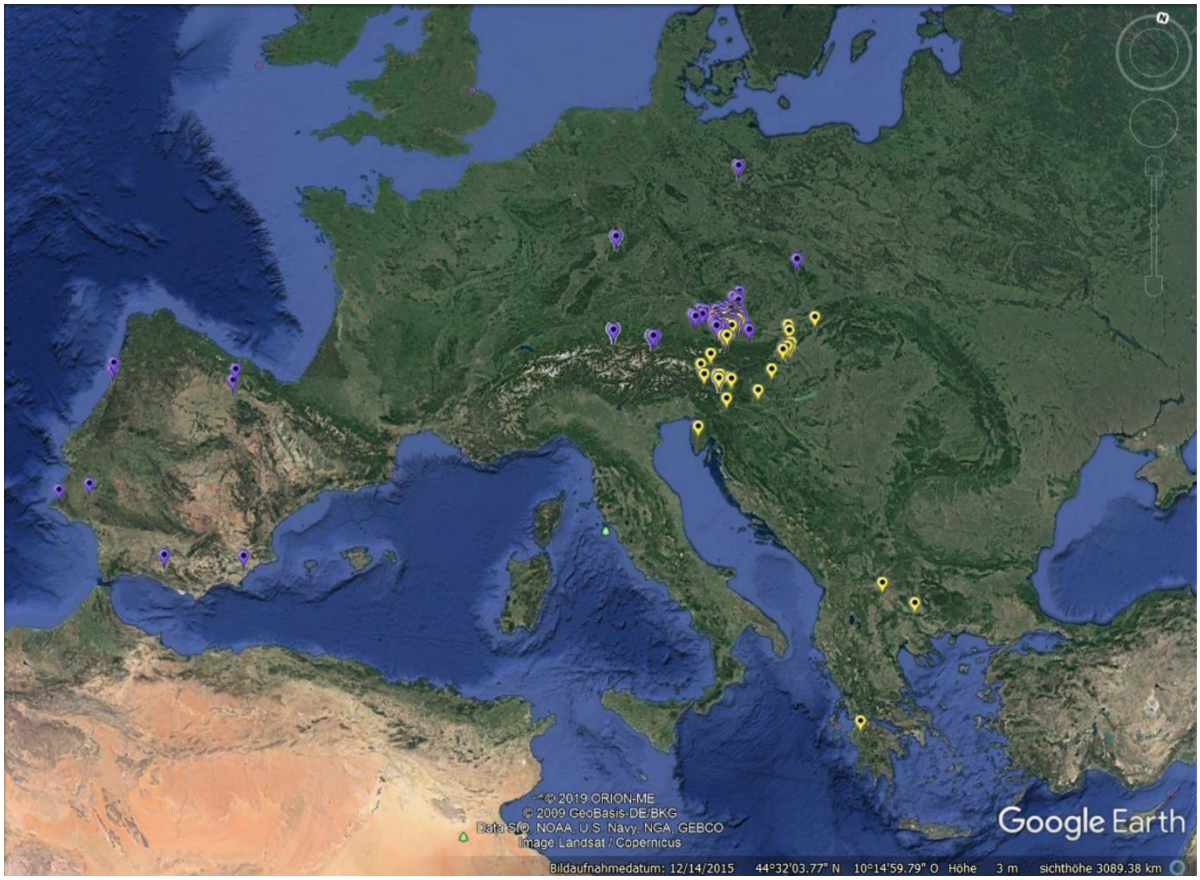

Fig. 8. Geographical distribution of all 264 West European and Northern White-breasted hedgehog individuals that had been analysed in a population genetic and phylogeographical context within the present master thesis. Colour-coding refers to the assignment of samples to *E. europaeus* (= blue) or *E. roumanicus* (= yellow) species. The map was created using Google Earth Pro Version 7.3.2.5776.

Fourteen samples did not allow for a clear assignment to either of the two species, *E. europaeus* and *E. roumanicus* on the basis of Bayesian clustering or PCoA analysis and are considered to be potential hybrid individuals. Their locations can be found in Fig. 9.

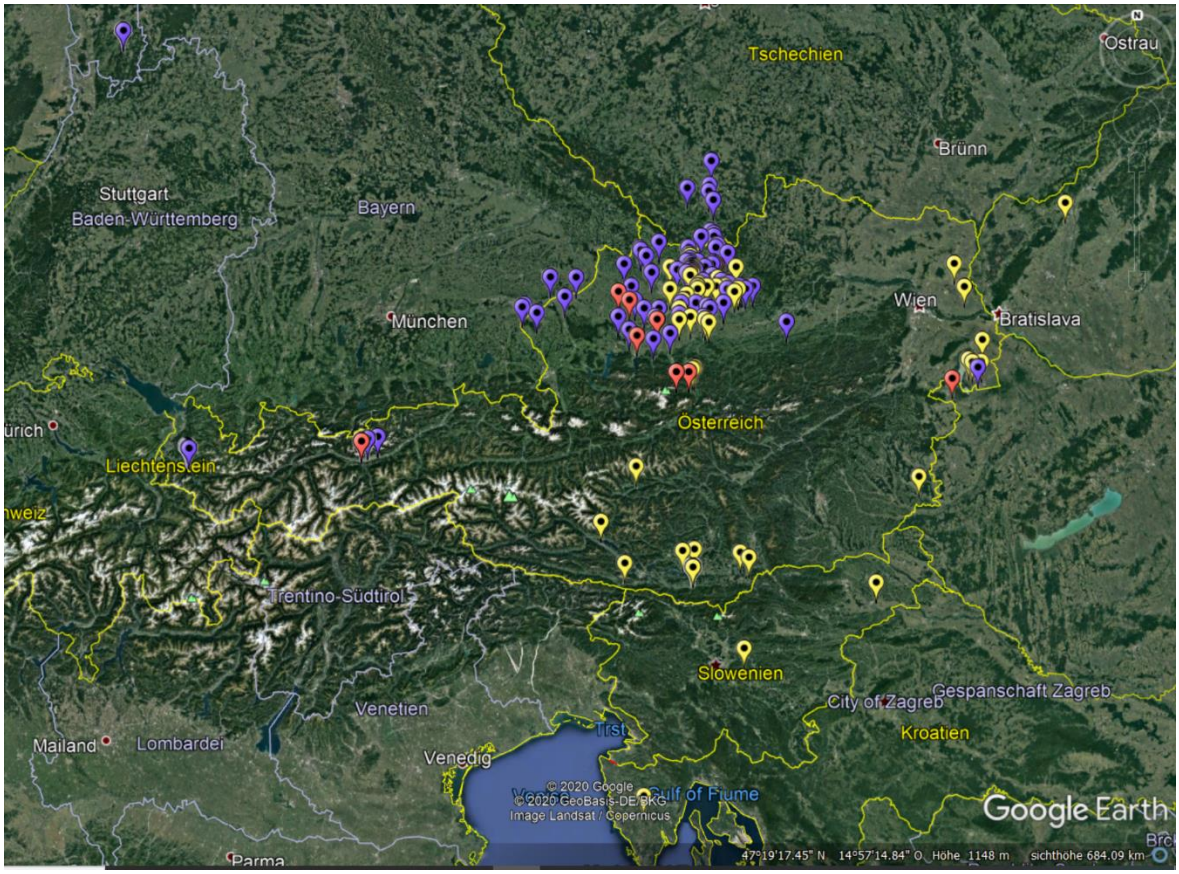

Fig. 9. Geographical distribution of potential *E. europaeus* x *E. roumanicus* hybrid individuals found within the area of Austria. Blue and yellow pins correspond to West European and Northern White-breasted hedgehog samples, respectively. Red pins indicate samples of admixed assignment (probability value below 0.9), considered as potential hybrid individuals. Assignment is based on Bayesian clustering within STRUCTURE (v. 2.3.4) analysis and Principal Coordinate Analysis (GenAlEx v. 6.5). The map was created using Google Earth Pro Version 7.3.2.5776.

Concerning further geographical investigations the most western *E. roumanicus* sample within the known zone of overlap was found in central Upper Austria (sample 2014581 and 2014838, coordinates: 48.333, 14.100 and 48.200, 14.100 respectively), while the actual most western *E. roumanicus* sample lies in Carinthia (sample KLF65 from Spittal a. d. Drau, no exact coordinates available). The most eastern *E. europaeus* sample, within Austria, was to be found amidst a number of Northern White-breasted hedgehog samples in the northern region of the Federal State of Burgenland in Eastern Austria (sample 2014584, coordinates 47.700, 16.883) (see Fig. 10). Hence, *E. europaeus* and *E. roumanicus* samples overlap in an area of approximately 215 km linear distance (calculated from the most western *E. roumanicus* sample (2014838) in the zone of overlap to the most eastern *E. europaeus* sample (2014584), calculated within Google Earth) (see Fig. 10).

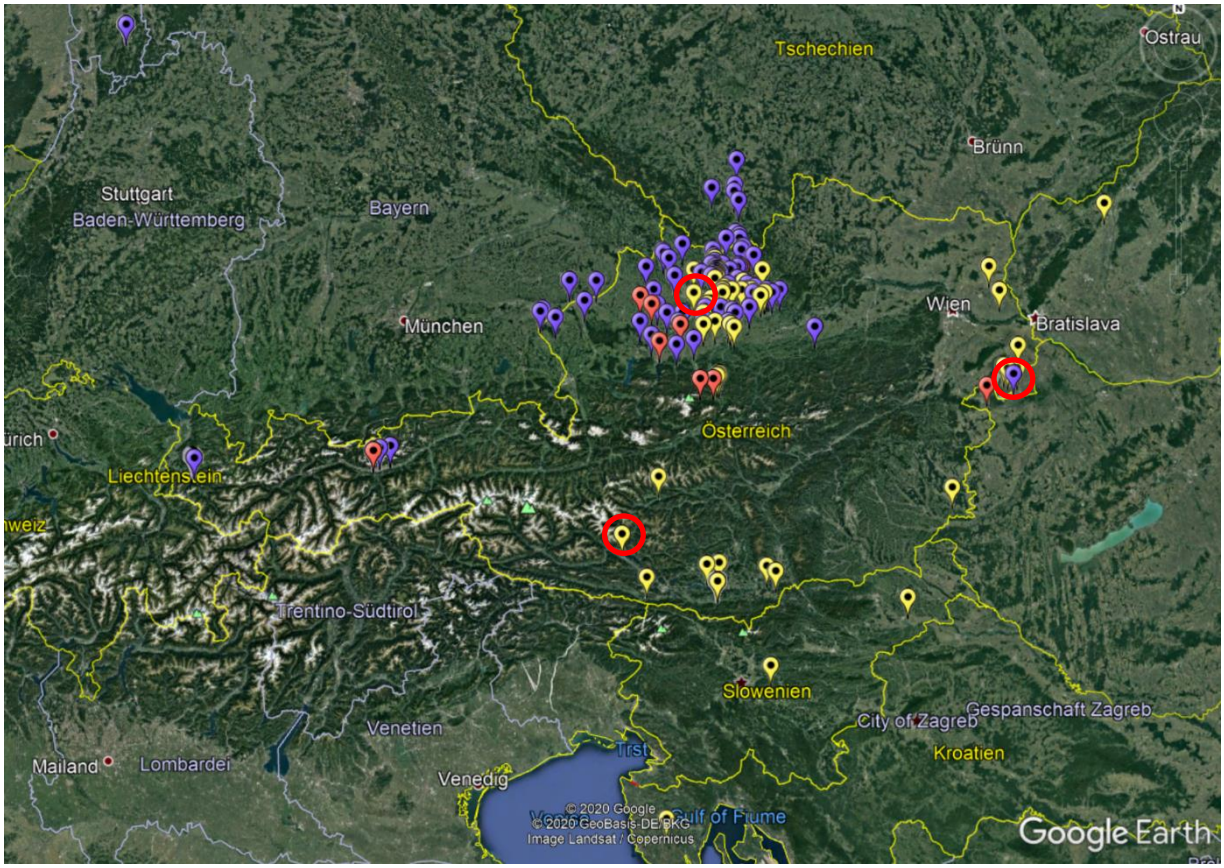

Fig. 10. Geographical locations of the most western *E. roumanicus* and most eastern *E. europaeus* individuals detected among a set of 226 hedgehog samples, observed in a population genetic and phylogeographic context within Austria. Red circles refer to the most eastern and western individuals of their geographical distribution range. Blue and yellow pins correspond to West European and Northern White-breasted hedgehog individuals respectively, red pins indicate potential hybrid, i.e. admixed, individuals (probability value below 0.9) investigated within STRUCTURE (v. 2.3.4) and Principal Coordinate Analysis (GenAlEx v. 6.5). The map was created using Google Earth Pro Version 7.3.2.5776 and the Microsoft Windows program Paint v. 1903

3.2 STRUCTURE analysis

3.2.1 Investigation of all European samples within STRUCTURE analysis

Calculations for most likely K from STRUCTURE analysis of all 264 hedgehog samples within the Structure Harvester led to a result of most likely K = 2 (delta K = 552,34; also see Fig. 11). Fig. 12 gives an overview on the single summary output of K = 2 - 6 created by CLUMPAK (Kopelman et al. 2015). K = 1 – 20 are given in the Appendix Fig. S1. Within the most likely K of 2 a clear restriction of West European hedgehogs to the samples from the Iberian peninsula, Germany, Western Austria, Poland and the Czech Republic can be seen. While the area of Upper Austria shows an intermixed pattern, populations from Eastern and Southern Austria (Carinthia), as well as from Slovakia, Slovenia, Croatia and Greece + North Macedonia represent a clear restriction of Northern White-breasted hedgehogs to the eastern parts of Europe. An exceptional case is formed by one *E. europaeus*

sample (2014583) that pertains to the population of Eastern Austria (and Hungary), indicated by the black arrow at K = 2 in Fig. 12.

When taking further substructuring into concern, an isolation of the Iberian peninsula at the K = 4 level can be seen. As well as that, a separation of the German (MT and BER) and western Austrian (BLU, INS) samples (K = 4 level) can be found, especially a distinction of the INS population from K = 5 onwards. Also within farther substructuring a zone of admixture within the area of Upper Austria can be seen. As it comes to the eastern part of the observed area, no such clear subdivision as within the west European region can be found (Fig. 12).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-36358.040000	7.033705	—	—	—
2	5	-27716.000000	13.214954	8642.040000	7299.200000	552.343973
3	5	-26373.160000	50.849907	1342.840000	656.060000	12.901892
4	5	-25686.380000	49.666508	686.780000	1817.360000	36.591258
5	5	-26816.960000	3125.728634	-1130.580000	2812.360000	0.899745
6	5	-25135.180000	325.594329	1681.780000	2869.960000	8.814527
7	5	-26323.360000	2108.620576	-1188.180000	1271.040000	0.602783
8	5	-26240.500000	1483.791355	82.860000	846.160000	0.570269
9	5	-25311.480000	716.867416	929.020000	1998.220000	2.787433
10	5	-26380.680000	706.807617	-1069.200000	898.840000	1.271690
11	5	-26551.040000	1150.396702	-170.360000	559.640000	0.486476
12	5	-27281.040000	1550.801031	-730.000000	934.280000	0.602450
13	5	-27076.760000	1424.322689	204.280000	508.200000	0.356801
14	5	-27380.680000	1878.341648	-303.920000	368.000000	0.195918
15	5	-28052.600000	1584.590412	-671.920000	42.320000	0.026707
16	5	-28682.200000	3339.376254	-629.600000	157.000000	0.047015
17	5	-29468.800000	2415.279654	-786.600000	1614.180000	0.668320
18	5	-28641.220000	3217.616491	827.580000	4412.680000	1.371413
19	5	-32226.320000	3500.749117	-3585.100000	6693.880000	1.912128
20	5	-29117.540000	3753.693097	3108.780000	—	—

Fig. 11. Evanno table from analysis of most likely K for 264 West European and Northern White-breasted hedgehog samples through STRUCTURE Harvester. Yellow line is indicating most likely K obtained by Evanno method.

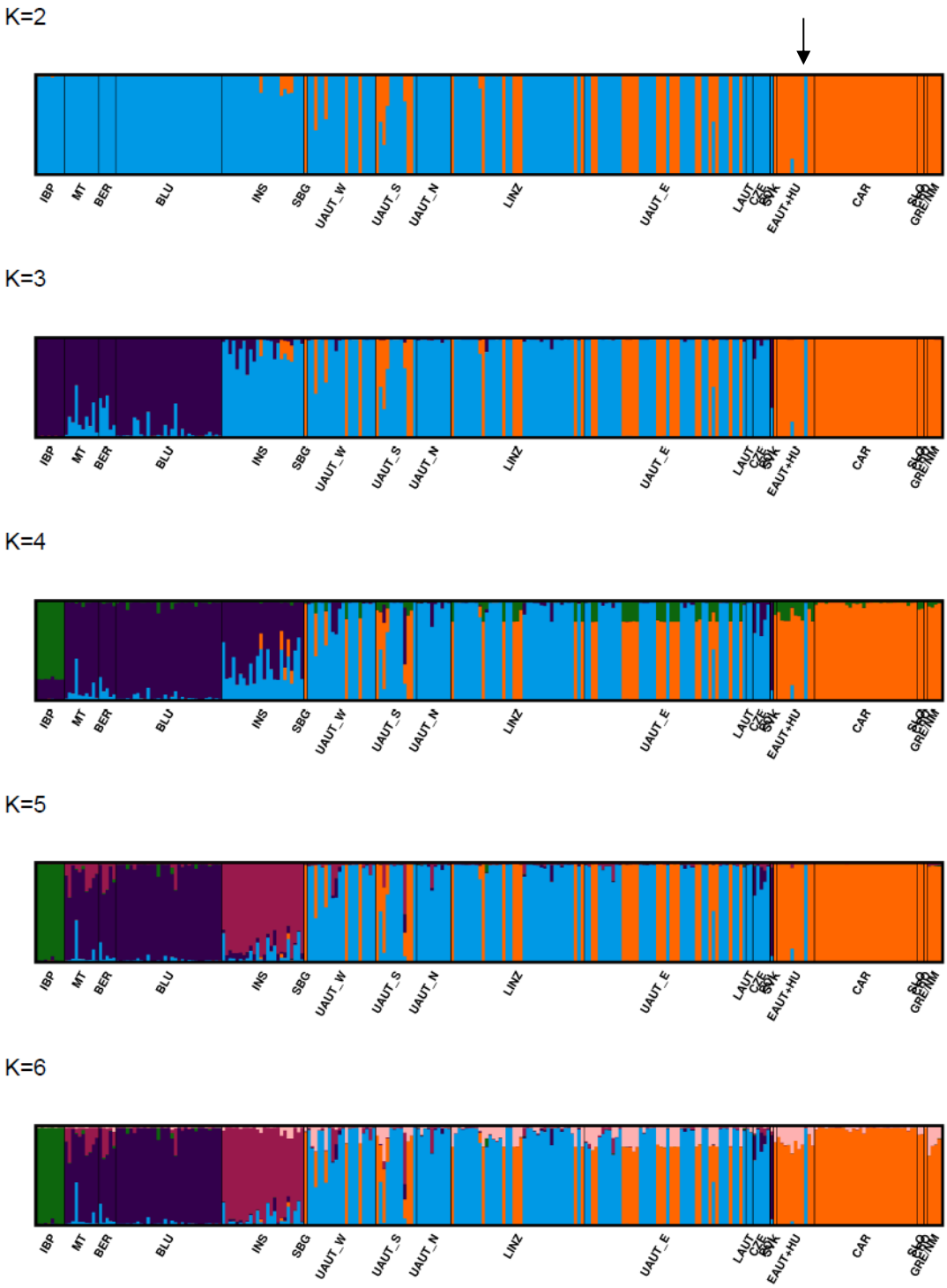

Fig. 12. Bayesian clustering of 264 West European and Northern White-breasted hedgehog samples based on genotypes at 39 microsatellite loci. The single summary output of K = 2 – 6 from STRUCTURE v2.3.4 (Pritchard et al. 2000) analysis is shown. Individuals are represented across the x-axis by a vertical bar. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: IBP = Iberian peninsula, MT = Igelburg Mossautal e.V., BER = Berlin, BLU = Bludenz, INS = Innsbruck, SBG = Salzburg, UAUT_W/S/N/E = Upper Austria West/South/North/East, LINZ = Linz, LAUT = Lower Austria, CZE = Czech Republic, POL = Poland, SVK = Slovakia, EAUT+HU = Eastern Austria + Hungary, CAR = Carinthia, SLO = Slovenia, CRO = Croatia, GRE/NM = Greece/North Macedonia

3.2.2 Investigation on potential hybrid individuals of *E. europaeus* and *E. roumanicus* within STRUCTURE analysis

Examination of potential hybrid individuals within STRUCTURE analysis at the K = 2 level revealed fourteen samples, that appeared to be of admixed origin (probability value below 0.9) (indicated by black arrows within Fig. 13). Those are namely “IBK2”, “IBK3”, “IBK4”, “IBK5”, “IBK6”, “2005613”, “2012159”, “2012156”, “2014419”, “2014427”, “2016388”, “200798”, “2015372” and “2014417” (see corresponding Tab. S2). They belong to the populations INS (5), UAUT_W (2), LINZ (5), UAUT_E (1) and EAUT+HU (1), respectively. Nine of these individuals correspond to the samples that were found within an intermediate state in the PCoA analysis (see Fig. 18).

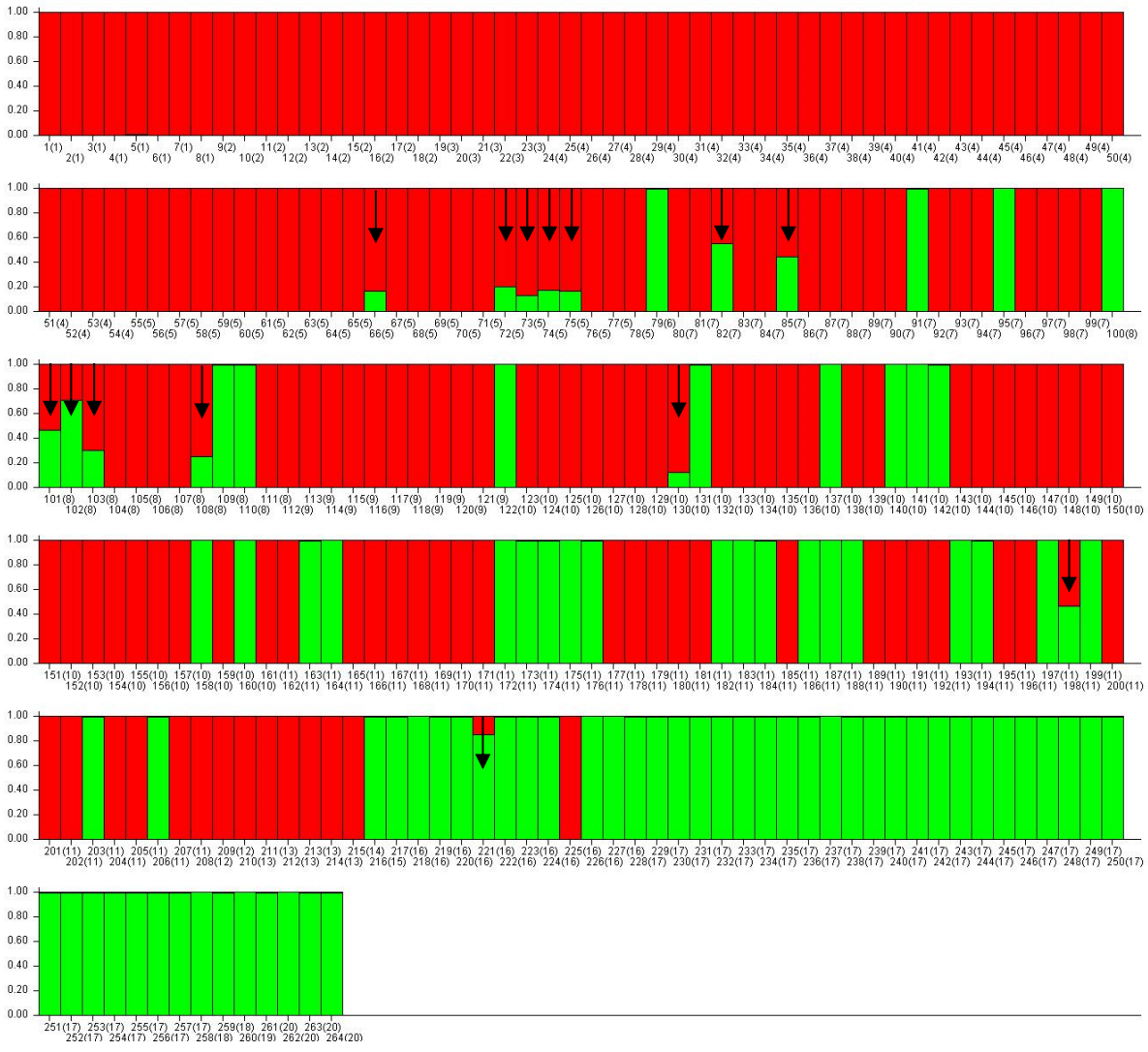

Fig. 13. Bayesian clustering of 264 West European and Northern White-breasted hedgehog samples analysed for their classification to *E. europaeus* and *E. roumanicus* species. The picture is showing the K = 2 scenario within the STRUCTURE clustering. Individuals are represented across the x-axis by a vertical bar that is divided into coloured segments that represent the individual´s probability of originating from *E. europaeus* (red) or *E. roumanicus* (green). Black arrows indicate samples of admixed origin, showing a probability value below 0.9

3.2.3 Intraspecific genetic divergence of *E. europaeus* analysed through Bayesian clustering

STRUCTURE analysis on a respective species level for *E. europaeus* showed similar results as the interspecific approach. Analysis within STRUCTURE Harvester resulted in a most likely K of 2 (delta K = 214,65; also see Fig. 14). At this level populations from the Iberian peninsula, Germany (MT and BER), the western parts of Austria (BLU and INS), as well as Poland showed a congruent assignment. As did populations surrounding the zone of secondary contact (Upper Austria West/South/North/East and Linz, Lower Austria, Eastern Austria and the Czech Republic) (Fig. 15). Again there is a substructuring of the Iberian peninsula (see IBP at K = 4 in Fig. 15), the German populations and western part of Austria (see MT + BER and BLU at K = 4 and K = 6 in Fig. 15) and the Innsbruck population (see INS at K = 3 in Fig. 15) at a further level of K to be found. K = 1 – 20 for this *E. europaeus* intraspecific analysis are given in the Appendix Fig. S2.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-19006.220000	2.370021	—	—	—
2	5	-17688.200000	3.250385	1318.020000	697.680000	214.645369
3	5	-17067.860000	6.921922	620.340000	48.940000	7.070291
4	5	-16496.460000	5.487531	571.400000	427.700000	77.940330
5	5	-16352.760000	29.460533	143.700000	348.980000	11.845678
6	5	-16558.040000	645.653094	-205.280000	567.220000	0.878521
7	5	-16196.100000	58.859876	361.940000	324.240000	5.508676
8	5	-16158.400000	162.371611	37.700000	79.680000	0.490726
9	5	-16200.380000	64.029774	-41.980000	165.680000	2.587546
10	5	-16408.040000	463.116328	-207.660000	76.100000	0.164322
11	5	-16539.600000	632.433087	-131.560000	995.000000	1.573289
12	5	-17666.160000	1480.825818	-1126.560000	1360.280000	0.918596
13	5	-17432.440000	1760.901971	233.720000	1177.140000	0.668487
14	5	-18375.860000	2098.610507	-943.420000	—	—

Fig. 14. Evanno table from analysis of most likely K for West European hedgehog samples through STRUCTURE Harvester. Yellow line is indicating most likely K obtained by Evanno method.

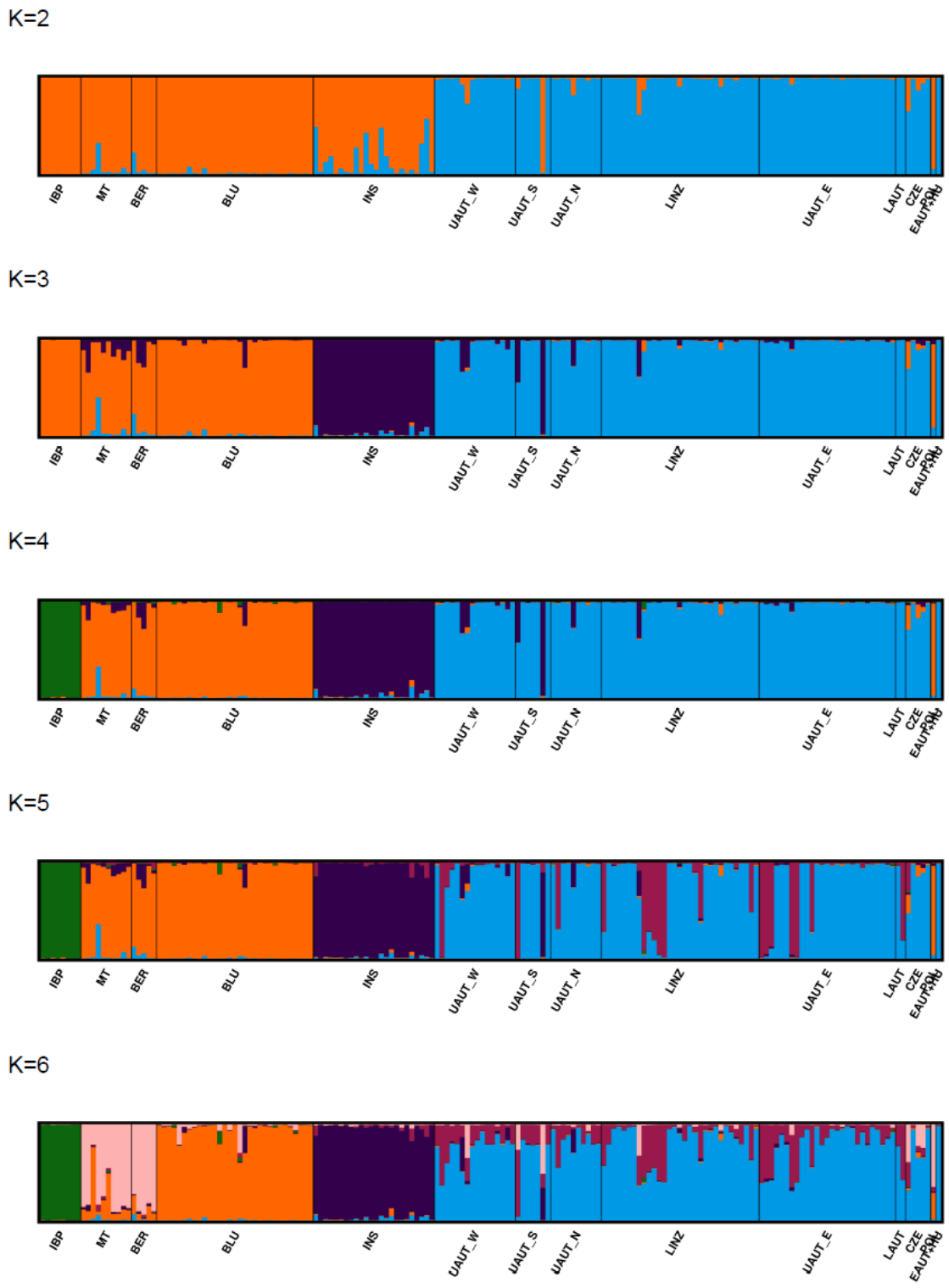

Fig. 15. Bayesian clustering of West European hedgehog samples observed for their population assignment based on genotypes at 39 microsatellite loci. The single summary output of K = 2 – 6 from STRUCTURE v2.3.4 (Pritchard et al. 2000) analysis is shown. Individuals are represented across the x-axis by a vertical bar. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: IBP = Iberian peninsula, MT = Igelburg Mossautal e.V., BER = Berlin, BLU = Bludenz, INS = Innsbruck, UAUT_W/S/N/E = Upper Austria West/South/North/East, LINZ = Linz, LAUT = Lower Austria, CZE = Czech Republic, POL = Poland, EAUT+HU = Eastern Austria + Hungary

3.2.4 Intraspecific genetic divergence of *E. roumanicus* analysed through Bayesian clustering

For *E. roumanicus* samples the most likely number of populations was calculated as K = 10 (delta K = 20,13), while the second most likely K was found to be K = 3 (delta K = 14.07; also see Fig. 16). No such clear substructuring as in *E. europaeus* was to be found within this analysis, but graphical representation largely showed a coherence between the populations of Slovenia, Croatia, Greece/North Macedonia and the southern parts of Austria (represented through Carinthia and Salzburg). Also samples from eastern Austria and parts from Upper Austria share genetic background with those (represented through blue colour in Fig. 17). K = 1 – 11 for this intraspecific analysis of *E. roumanicus* are given in the Appendix Fig. S3.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-7950.380000	4.714552	—	—	—
2	5	-7515.880000	36.683607	434.500000	237.340000	6.469920
3	5	-7318.720000	9.130827	197.160000	128.460000	14.068824
4	5	-7250.020000	41.684673	68.700000	97.360000	2.335631
5	5	-7278.680000	88.557112	-28.660000	83.240000	0.939958
6	5	-7390.580000	252.328292	-111.900000	230.120000	0.911987
7	5	-7272.360000	92.143383	118.220000	672.080000	7.293850
8	5	-7826.220000	686.648405	-553.860000	713.820000	1.039571
9	5	-7666.260000	332.797458	159.960000	284.080000	0.853612
10	5	-7222.220000	87.094443	444.040000	1752.920000	20.126657
11	5	-8531.100000	2162.158751	-1308.880000	—	—

Fig. 16. Evanno table from analysis of most likely K for Northern White-breasted hedgehog samples through STRUCTURE Harvester. Yellow line is indicating most likely K obtained by Evanno method.

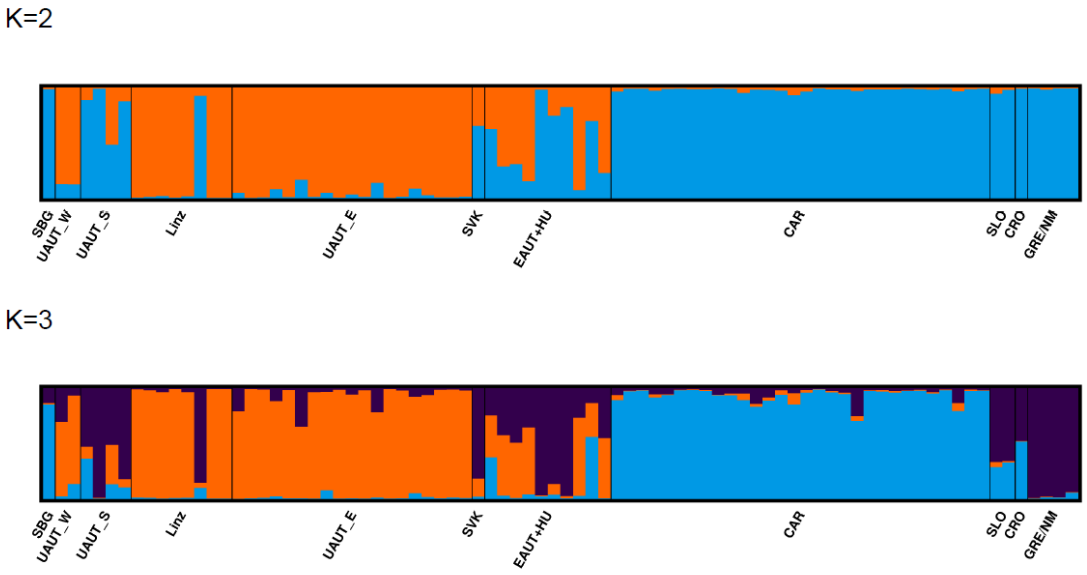

Fig. 17. Bayesian clustering of Northern White-breasted hedgehog samples observed for their population assignment based on genotypes at 39 microsatellite loci. The single summary output of K = 2 – 3 from STRUCTURE v.2.3.4 (Pritchard et al. 2000) analysis is shown. Individuals are represented across the x-axis by a vertical bar. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: SBG = Salzburg, UAUT_W/S/E = Upper Austria West/South/East, LINZ = Linz, SVK = Slovakia, EAUT + HU = Eastern Austria + Hungary, CAR = Carinthia, SLO = Slovenia, CRO = Croatia, GRE/NM = Greece/North Macedonia

3.3 GenAlEx analysis

3.3.1 Principal Coordinate Analysis (PCoA)

3.3.1.1 PCoA of all European hedgehog samples

Results of PCoA among all 264 European hedgehog individuals showed a clear division of *E. europaeus* and *E. roumanicus*, resulting in two major groups within the analysed samples (Fig. 18). Nine individuals showed an intermediate position between those two groups (indicated by the red circle in Fig. 18). These are namely “IBK2”, “IBK3”, “IBK4”, “IBK5”, “IBK6”, “2005613”, “2012159”, “2012156” and “2015372”, they belong to the populations of INS (5), UAUT_W (2), LINZ (1) and UAUT_E (1), respectively. Fig. 19 gives an overview on the percentage of variation explained by the first three axes of the PCoA. They sum up to 29.99% of explained variation.

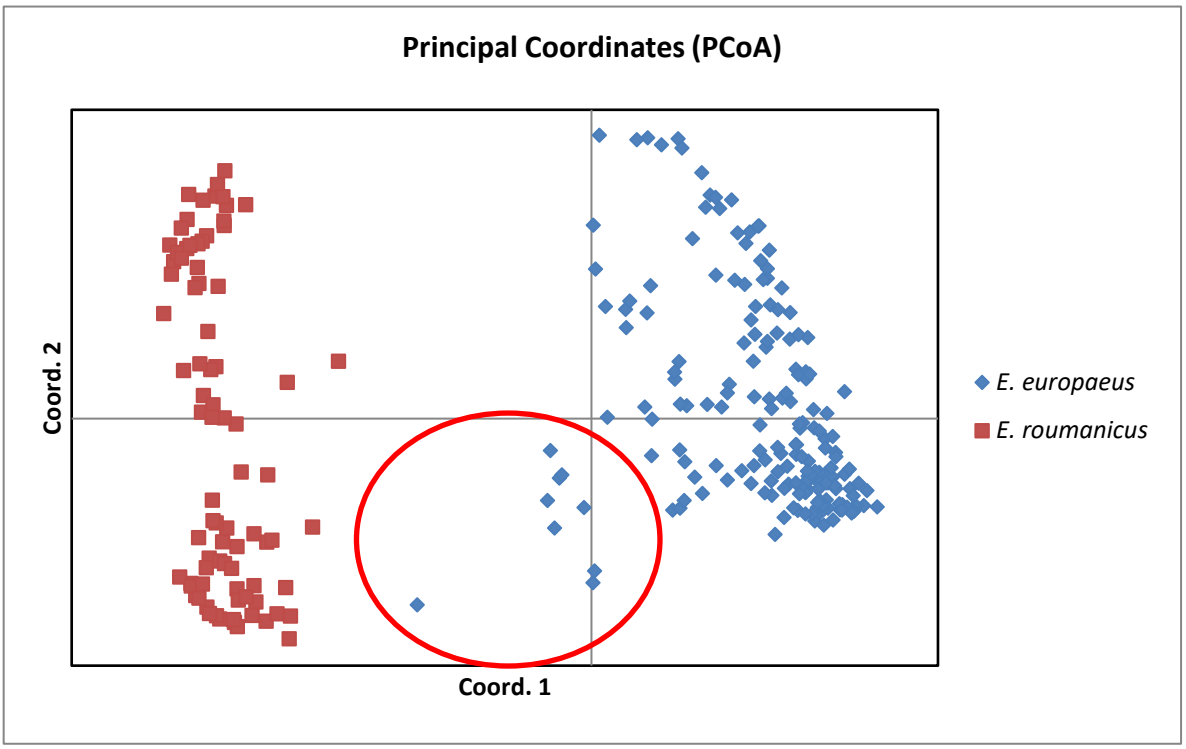

Fig. 18. Principal Coordinate Analysis of all 264 European hedgehog samples that have been analysed within a population genetic and phylogeographic context. Blue and red squares correspond to *E. europaeus* and *E. roumanicus* samples, respectively. The red circle is indicating potential hybrid, i.e. admixed, samples, which underwent latter verification within STRUCTURE analysis. PCoA was conducted through the Excel Add-In GenAlEx v.6.5.

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	20,32	5,89	3,78
Cum %	20,32	26,21	29,99

Fig. 19. Percentage of variation explained by the first three axes of Principal Coordinate Analysis for the investigation of all 264 European hedgehog samples. PCoA was conducted using the Excel Add-In GenAlEx v.6.5

3.3.1.2 PCoA of *E. europaeus* samples

Principal Coordinate Analysis for West European hedgehog populations based on Nei genetic distance matrices shows a clear distinction of the Iberian peninsula population. As well as that the populations surrounding the zone of secondary contact, i.e. Eastern Austria, Lower Austria, Upper Austria and the Czech Republic group closer together, as do populations in farther distance to this zone, i.e. Innsbruck, Bludenz, Mossautal and Berlin. The population of Poland, however, stands somewhat outside the others (see Fig. 20). Fig. 21 shows the values for the percentage of variation explained for the first three axes of this PCoA. They sum up to 68.47%.

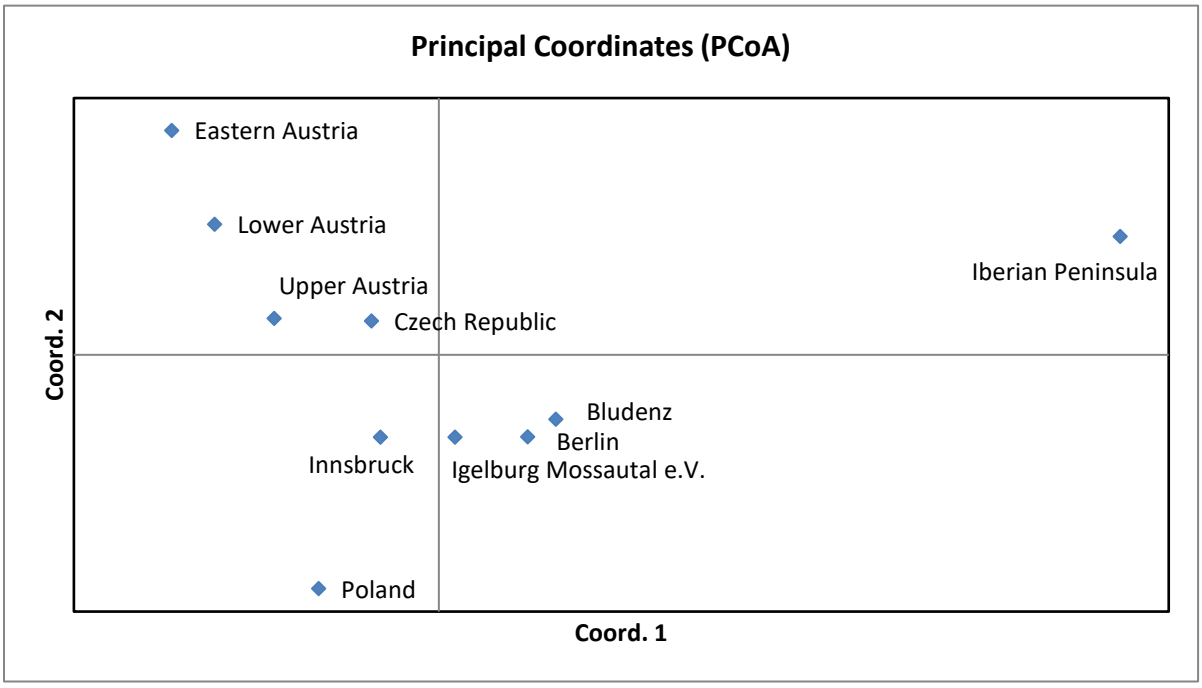

Fig. 20. Principal Coordinate Analysis of ten West European hedgehog populations that have been analysed within a population genetic and phylogeographic context. Blue squares correspond to respective populations. PCoA is based on a Nei genetic distance matrix and was conducted using the Excel Add-In GenAlEx v.6.5.

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	27,87	21,81	18,78
Cum %	27,87	49,69	68,47

Fig. 21. Percentage of variation explained by the first three axes of Principal Coordinate Analysis for the investigation of ten *E. europaeus* populations. PCoA was conducted based on a Nei genetic distance matrix, using the Excel Add-In GenAlEx v.6.5

Fig. 22 presents the PCoA of all *E. europaeus* samples, corresponding to ten respective populations. No such clear distinction as in Fig. 20 is to be found and the percentage of variation explained by the first three axes lies below 20% (Fig. 23). However, (inter)related samples generally group together more closely in their corresponding populations; with the exception of samples from the INS population, which is spreading across multiple other samples (Fig. 22).

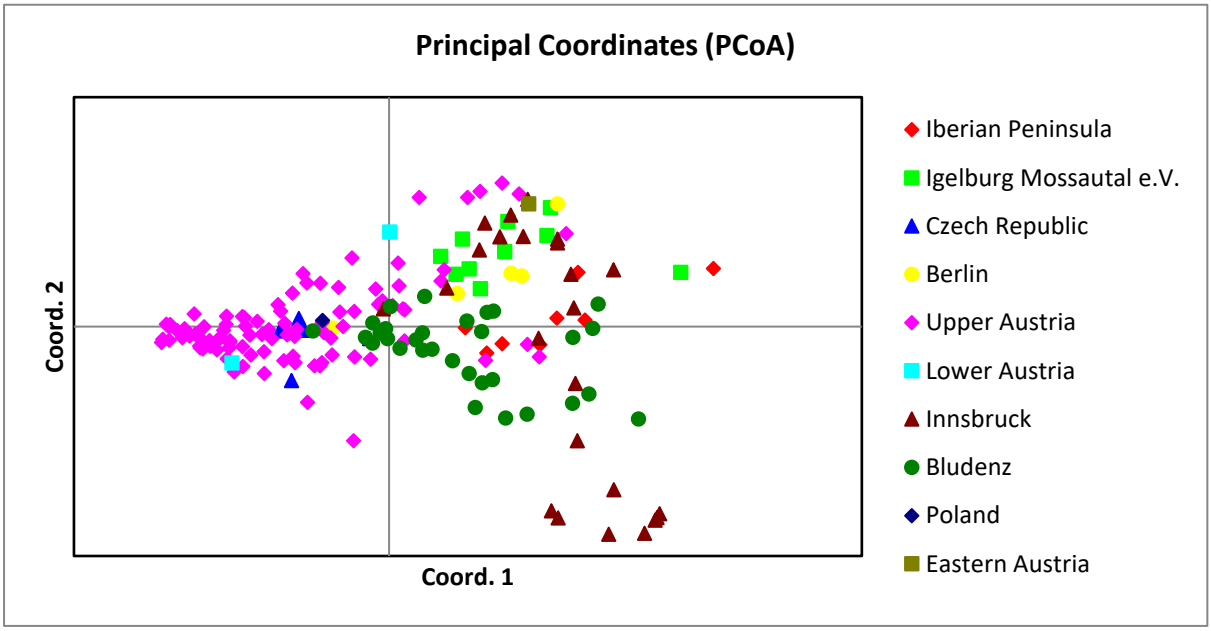

Fig. 22. Principal Coordinate Analysis of *E. europaeus* samples, analysed within a population genetic and phylogeographic context. Samples correspond to ten respective populations, indicated by various colours and symbols. PCoA was undertaken using the Excel Add-In GenAlEx v.6.5.

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	7,20	6,56	5,67
Cum %	7,20	13,76	19,43

Fig. 23. Percentage of variation explained by the first three axes of Principal Coordinate Analysis for the investigation of *E. europaeus* samples. PCoA was conducted using the Excel Add-In GenAlEx v.6.5

3.3.1.3 PCoA of *E. roumanicus* samples

Principal Coordinate Analysis for Norther White-breasted hedgehog populations based on Nei genetic distance matrices separates the populations of Greece/North Macedonia, Croatia, Slovenia and Carinthia from the populations of Upper Austria and Eastern Austria + Hungary. As well as that the populations of Slovakia and Salzburg are on the outer edges of the conducted analysis (Fig. 24). Fig. 25 shows the values for the percentage of variation explained for the first three axes of the PCoA. They sum up to 68.18%.

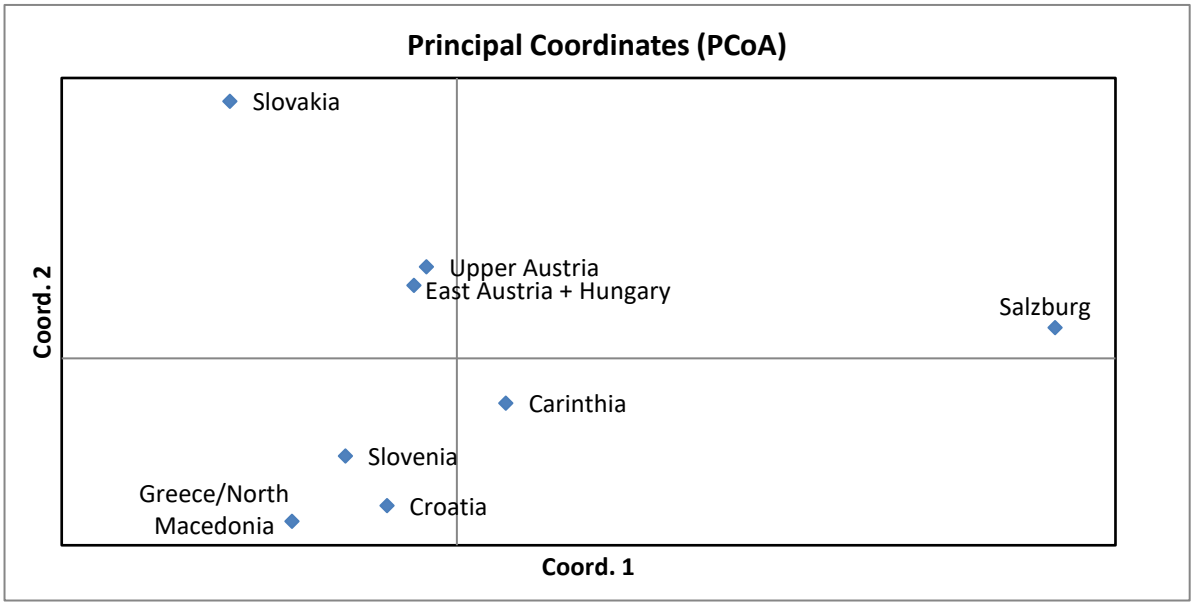

Fig. 24. Principal Coordinate Analysis of eight Northern White-breasted hedgehog populations that have been analysed within a population genetic and phylogeographic context. Blue squares correspond to respective populations. PCoA is based on a Nei genetic distance matrix and was conducted using the Excel Add-In GenAlEx v.6.5.

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	32,90	20,00	15,28
Cum %	32,90	52,90	68,18

Fig. 25. Percentage of variation explained by the first three axes of Principal Coordinate Analysis for the investigation of eight *E. roumanicus* populations. PCoA was conducted based on a Nei genetic distance matrix, using the Excel Add-In GenAlEx v.6.5

Fig. 26 gives an overview on the PCoA of all *E. roumanicus* samples, corresponding to eight respective populations. No such clear distinction as in Fig. 24 is to be found and the percentage of variation explained by the first three axes lies by 22.27% (Fig. 27). Carinthian samples largely part from other populations and Balkan populations, i.e. Slovenia, Croatia and Greece/North Macedonia group closer together. The populations of Salzburg and Slovakia are amidst other samples within this analysis. Samples from Upper Austria are generally closer to East Austrian and Hungarian samples than others (Fig. 26).

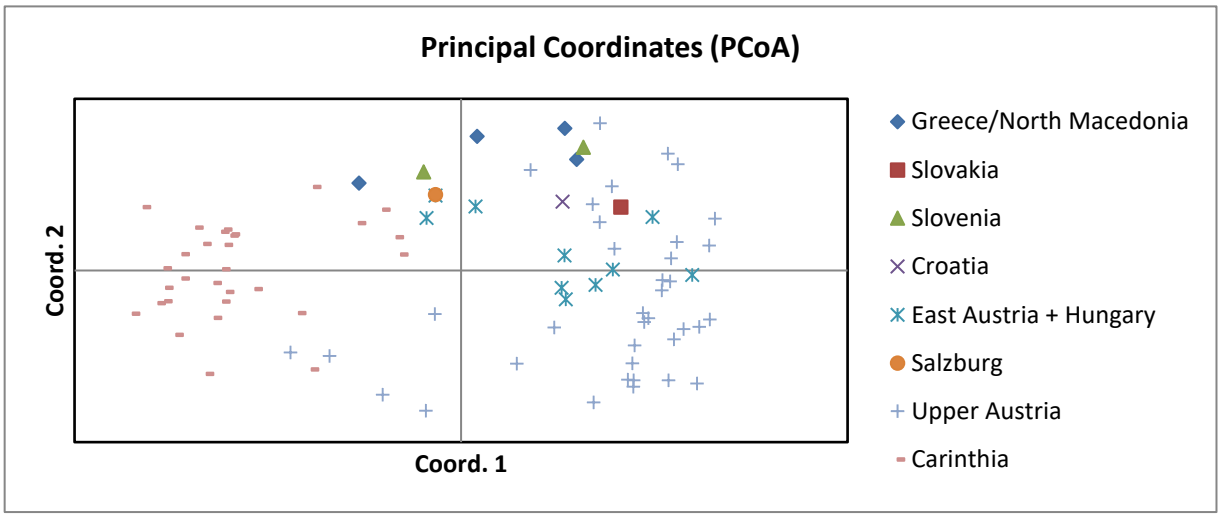

Fig. 26. Principal Coordinate Analysis of *E. roumanicus* samples, analysed within a population genetic and phylogeographic context. Samples correspond to eight respective populations, indicated by various colours and symbols. PCoA was undertaken using the Excel Add-In GenAlEx v.6.5.

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	13,93	4,30	4,04
Cum %	13,93	18,23	22,27

Fig. 27. Percentage of variation explained by the first three axes of Principal Coordinate Analysis for the investigation of 86 *E. roumanicus* samples. PCoA was conducted using the Excel Add-In GenAlEx v.6.5

3.3.2 Isolation-by-distance analysis

3.3.2.1 Isolation-by-distance analysis of *E. europaeus* populations

Isolation-by-distance analysis through Mantel test revealed a clearly positive relation between the geographical and genetic distance of West European hedgehog populations based on Nei genetic distance matrix (Rxy = 0.947; p = 0.020), as can be seen in Fig. 28.

The geographic distance matrix, as well as the pairwise population matrix of Nei genetic distance for calculation of isolation-by-distance for *E. europaeus* populations can be found in Tab. 6 and 7, respectively.

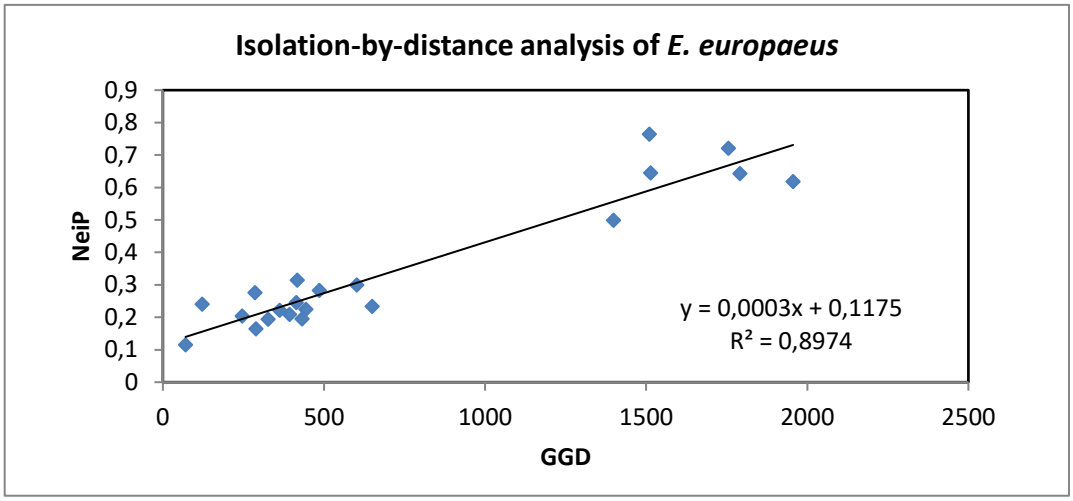

Fig. 28. Isolation-by-distance analysis of seven *E. europaeus* populations. Analysis was conducted using a Mantel test within the Excel Add-In GenAlEx v.6.5. Genetic distance values are based on a pairwise population matrix of Nei genetic distance (NeiP values), pairwise geographic distance matrix was set up based on decimal Lat/Long coordinates (GGD values).

Tab. 6. Pairwise geographic distance matrix for calculation of isolation-by-distance among *E. europaeus* populations. Seven different populations have been set up on West European hedgehog samples. Data is based on geographic coordinates, provided in decimal Lat/Long and implemented through the Excel Add-In GenAlEx v. 6.5. The used coordinates for each population resemble an approximate central point of all samples within a population, since each population encompasses a wider geographic range due to sampling circumstances. Due to calculation/program induced limitations not all samples could be included in the population set up and some populations that had been treated separately in earlier analyses were grouped together.

IBP	MT	BER	BLU	INS	UAUT	CZE	
0,000							IBP
1513,351	0,000						MT
1956,014	443,938	0,000					BER
1398,436	288,151	648,866	0,000				BLU
1509,654	326,208	601,509	121,978	0,000			INS
1755,025	432,114	485,435	361,727	245,473	0,000		UAUT
1791,150	413,276	417,043	392,784	285,708	70,291	0,000	CZE

Tab. 7. Pairwise population matrix of Nei genetic distance for calculation of isolation-by-distance among *E. europaeus* populations. Seven artificial populations have been set up on West European hedgehog samples. Data is based on 39 different microsatellite loci, implemented through a codominant matrix within the Excel Add-In GenAlEx v. 6. 5. Due to calculation/program induced limitations not all samples could be included in the population set up and some populations that had been treated separately in earlier analyses were grouped together.

IBP	MT	BER	BLU	INS	UAUT	CZE	
0,000							IBP
0,645	0,000						MT
0,619	0,225	0,000					BER
0,499	0,165	0,233	0,000				BLU
0,765	0,194	0,299	0,240	0,000			INS
0,721	0,195	0,284	0,222	0,204	0,000		UAUT
0,643	0,245	0,315	0,209	0,276	0,115	0,000	CZE

3.3.2.2 Isolation-by-distance analysis of *E. roumanicus* populations

Also Northern White-breasted hedgehog populations showed a positive relation between geographical and genetic distance investigated within an isolation-by-distance analysis through Mantel test (Rxy = 0.880; p = 0.010), as can be seen in Fig. 29.

The geographic distance matrix and pairwise population matrix of Nei genetic distance for calculation of isolation-by-distance for *E. roumanicus* populations can be found in Tab. 8 and 9, respectively.

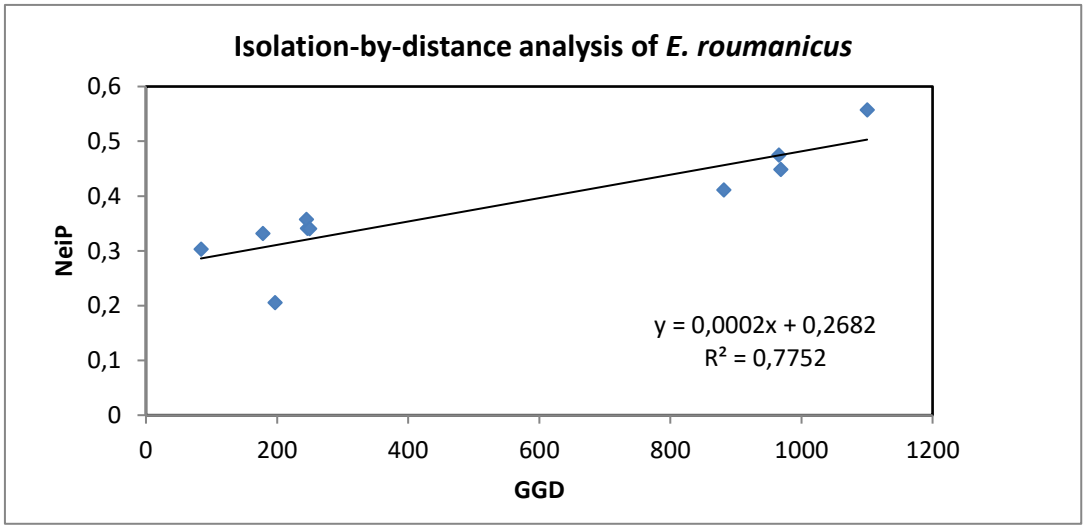

Fig. 29. Isolation-by-distance analysis of five *E. roumanicus* populations. Analysis was conducted using a Mantel test within the Excel Add-In GenAlEx v.6.5. Genetic distance values are based on a pairwise population matrix of Nei genetic distance (NeiP values), pairwise geographic distance matrix was set up based on decimal Lat/Long coordinates (GGD values).

Tab. 8: Pairwise geographic distance matrix for calculation of isolation-by-distance among *E. roumanicus* populations. Five different populations have been set up on Northern White-breasted hedgehog samples. Data is based on geographic coordinates, provided in decimal Lat/Long and implemented through the Excel Add-In GenAlEx v. 6.5..The used coordinates for each population resemble an approximate central point of all samples within a population, since each population encompasses a wider geographic range due to sampling circumstances. Due to calculation/program induced limitations not all samples could be included in the population set up and some populations that had been treated separately in earlier analyses were grouped together.

UAUT	EAUT + HU	CAR	SLO/CRO	GRE/NM	
0,000					UAUT
196,960	0,000				EAUT + HU
178,112	246,106	0,000			CAR
244,605	249,447	83,810	0,000		SLO/CRO
1100,185	968,608	965,312	881,516	0,000	GRE/NM

Tab. 9: Pairwise population matrix of Nei genetic distance for calculation of isolation-by-distance among *E. roumanicus* populations. Five artificial populations have been set up on Northern White-breasted hedgehog samples. Data is based on 39 different microsatellite loci, implemented through a codominant matrix within the Excel Add-In GenAlEx v. 6.5. Due to calculation/program induced limitations not all samples could be included in the population set up and some populations that had been treated separately in earlier analyses were grouped together.

UAUT	EAUT + HU	CAR	SLO/CRO	GRE/NM	
0,000					UAUT
0,205	0,000				EAUT + HU
0,332	0,342	0,000			CAR
0,358	0,341	0,303	0,000		SLO/CRO
0,558	0,449	0,475	0,411	0,000	GRE/NM

4. Discussion

The aim of this master thesis was to investigate potential hybrid zone dynamics of the two hedgehog species *E. europaeus* and *E. roumanicus* within their Austrian contact zone and infer on potential phylogeographic influences. The results support a clear delimitation of the two species, with some potential hybridization. Additionally, their zone of secondary contact may be broader than previously assumed. The data allowed for phylogeographic inferences on factors shaping the genetic diversity of Austrian hedgehog populations, to a certain extent. More precise explanation can be found in the following sections.

4.1 Phylogeography and genetic differentiation of *E. europaeus* and *E. roumanicus*

As expected, and in concordance with previous studies on hedgehog genetic divergence and phylogeography (Santucci et al. 1998; Seddon et al. 2001) West European hedgehog samples are to be found from the Iberian Peninsula over to the known mid-European contact zone. This includes the for this thesis established and examined populations of IBP (Iberian Peninsula), MT and BER (German samples), BLU and INS (western part of Austria), CZE (Czech Republic), POL (Poland), LAUT (Lower Austria) and partly the population of Upper Austria (UAUT and LINZ). Also one exceptional and comparatively unexpected sample from Eastern Austria (EAUT + HU) was identified as *E. europaeus* (but for further information see 4.4 Investigations on *E. europaeus* x *E. roumanicus* hybrid individuals within Austria). The Northern White-breasted hedgehog was detected within the investigated areas and corresponding populations of Slovakia (SVK), Eastern Austria and Hungary (EAUT+HU), Carinthia (CAR), Salzburg (SBG), Slovenia (SLO), Croatia (CRO), Greece and North Macedonia (GRE/NM) and again partly Upper Austria (UAUT). Thereby spanning its range from the central European contact zone over to the Balkans.

Concerning the species of *E. europaeus*, already within the analysis of all European hedgehog samples an underlying subdivision on higher levels of K was detectable. While IBP, MT, BER and BLU formed a uniform cluster within K=3, samples from Portugal and Spain (forming the Iberian Peninsula and representing the corresponding glacial southern refugium (Hewitt 2000)) grouped together from K=4 on. Samples from the INS population, however, even though lying in proximity to BLU, separated into a distinct group as of K=5. Further stages of K revealed no clearer or more distinct patterns of substructuring. Populations of *E. roumanicus* individuals showed no such (clear) further subdivision, as was found in the distribution area of *E. europaeus*. Thereby raising suggestion that only one lineage of *E. roumanicus* emerged from the southern refugium of the Balkan Peninsula (Hewitt 2000), which had already been deduced from the results of Santucci et al. (1998) and Seddon et al. (2001). Upper Austria, representative for the potential hybridisation zone within Austria, is

populated by both species, i.e. individuals comprising genetical membership to either species were found within the interspecific STRUCTURE analysis. This matter of fact, however, is acting as prerequisite for the anticipation of hybrid occurrence (see Filippucci and Lapini 1988; Suchentrunk et al. 1998; Bogdanov et al. 2009).

4.2 Phylogeography and intraspecific genetic divergence of *E. europaeus*

Bayesian clustering revealed a most likely K=2 for *E. europaeus* populations and individuals within Western and Central Europe. This would refer to an existence of two clades or lineages within *E. europaeus* (as indicated by Santucci et al. 1998), based on analysis of nuclear markers. One of these seems to be originating from ancestral hedgehogs, surviving Pleistocene glaciations within the southern refugia of the Iberian Peninsula (Hewitt 1999, 2000) to eventually expand northwards into France (from which samples were not available for this thesis) and further into Germany and (probably) western Austria (constituted by the population BLU). Our IBP population in this concern, refers to the E2 clade observed by Seddon et al. (2001), spreading from Portugal and Spain, into France and the Netherlands, as well as Germany and Switzerland, over to the UK and Ireland. This is further supported by analysis from Santucci et al. 1998, who found that haplotypes from Spain, France and UK are closer, and distinct from Italian and German haplotypes. Thereby, indicating "an east-west geographical partitioning down central Europe between France and Germany" as it comes to *E. europaeus* sub-division. They, however, did not find any hedgehogs of the E2 clade within Austria, which still could be attributed to a low sampling number or differing in sampling regions. Due to its proximity to Switzerland and Germany, one could propose a possible existence of their E2 clade within Austria´s most western range (BLU). This is furthermore strengthened by the congruent assignment of IBP, MT, GER and BLU into one group within STRUCTURE analysis (K=3), despite their relative geographic distance and the genetic distinctiveness of the INS population, despite its relative geographic proximity.

The second *E. europaeus* lineage in this master thesis is constituted by populations from North-Eastern Austria and the Czech Republic, resembling parts of the potential zone of hybridisation. This separation of Upper Austrian and Czech Samples (on a K=2 level) might be explained on their proximity to and coverage of the zone of secondary contact and underlying genetic divergence or introgression (Bolfíková et al. 2017). Their genetic set-up, might be established on possible introgression with *E. roumanicus* individuals, as they are situated in the potential hybridisation zone. A possible influence of individuals from the secondary contact zone on analysis of genetic divergence within the species has already been indicated by Bolfíková et al. (2017). They too recognized their *Erinaceus* population from the contact zone to be different from the remaining study area, which they attributed to "processes acting at … parapatric range edges". Also PCoA groups the populations of

Eastern, Lower and Upper Austria, as well as the Czech Republic more closely together, than with other populations. Bolfíková and Hulva (2012) found an indication of only one *E. europaeus* lineage (most likely K=1) within their analysis of the central European suture zone, represented by Czech and Slovakian samples. This, however, might relate to our calculated Structure assignment of Upper Austrian, Lower Austrian, East Austrian and Czech samples within one group in the most likely case of K=2.

A second lineage of *E. europaeus* (E1) was also proposed by Santucci et al. (1998) and Seddon et al. (2001), who proved post-glacial expansion out of the Italian Peninsula "northwards through Austria, Switzerland, Germany, the Netherlands, Scandinavia and Estonia" (Seddon et al. 2001). The coherence of our (1) Polish sample to samples and populations that lie on the western range of the sampled area (German and western Austria), might be based on the already observed expansion route of *E. europaeus* out from the Italian peninsula through Austria and Germany over to southern Scandinavia and Poland into Russia (Santucci et al. 1998; Hewitt 2000, also see Fig 3; Seddon et al. 2001). In contrast to many other European species hedgehogs have been shown to overcome the Alpine Mountains barrier and colonize Europe out from Italy (Hewitt 1999, 2000).

The lack of Italian individuals within this thesis constitutes a major issue, since no refugial samples are available for comparison to the populations in Austria and Germany. A lack of samples from this refugial area might lead to the finding of more diversity within central Europe, that is not to be found anywhere else. Still the distinct pattern of the INS population, that can be found as of K=3, might be explained by their potential Italian origin. The pattern INS samples reveal is also striking within PCoA of all *E. europaeus* samples (Fig. 22). While samples from one population generally group together more closely, INS samples are roughly distributed in two groups, possibly indicating two-fold origin of Iberian, as well as Italian refugia. The percentage of explained variation by the first three axes, however, lies below twenty percent. PCoA among *E. europaeus* hedgehog populations, however, groups the populations of Germany and western Austria (including INS!) rather closely together and shows a percentage of explained variation by the first three axes of nearly 70%. This again might strengthen a possible Italian origin, since the IBP population is indicated to be very distinct from all other *europaeus* populations.

Separation of IBP from a higher level of K, as of K=4, might be in concordance with Filippucci and Simson's (1996) observation of the sub-species *E. hispanicus* and is in agreement with the general isolation-by-distance observed among *E. europaeus* populations. Further support in this concern, is given by the PCoA result, which indicates the population of IBP to be very different from all other populations.

Basing on the assumption of two lineages for the *E. europaeus* species and the result of a most likely K=2, it could be concluded that the populations of IBP, MT, BER, BLU, POL and INS originated from the Iberian Peninsula, while populations from Upper, Lower and Eastern Austria, as well as CZE

are tracing back to the Italian Peninsula. This is based on the underlying genetical sub-structuring detected with STRUCTURE v.2.3.4 and existing knowledge on hedgehog glacial refugia (Hewitt 1999, 2000). However, the lack of Italian (and French) samples and a possible introgression within the potential hybridisation zone of *E. europaeus* and *E. roumanicus* do pose substantial issues in this conclusion. Additionally, the congruent, but rather distinct and unusual behaviour of the INS samples, raises further questions. A more detailed and complementary examination within haplotype analysis and under consideration of further samples will be necessary.

4.3 Phylogeography and intraspecific genetic divergence of *E. roumanicus*

One of the clades (C1, representing *E. roumanicus* samples) Seddon et al. (2001) detected, when examining *E. roumanicus* and *E. concolor* within Europe, corresponds to our (subset) of *E. roumanicus* populations and samples. C1 is found within a broad area of eastern Europe, encompassing Greece, Austria as well as Hungary and Estonia (Seddon et al. 2001). While their C1 clade does show further sub-division into lineages spreading from the Balkans to Austria or Poland and from Austria to Estonia and northwest Russia, *E. roumanicus* from Poland or Estonia are missing within this study. However, expansion from the Balkan Peninsula to Austria is well represented within the results of this thesis. Also Santucci et al. (1998), who found *E. roumanicus* to be deeply diverged from *E. concolor*, detected this *E. roumanicus* clade to be formed by hedgehogs from the Balkans, Greece and north-eastern Italy, again being in concordance with our *E. roumanicus* samples.

As already discussed a further sub-structuring of *E. roumanicus* within the interspecies approach was not indicated, but intraspecific species analysis provided evidence for such. Most likely K was calculated to 10, which is assumed to be a calculation issue, since it does not help identifying a clear structure. When examining K=2 no clear pattern in its sub-division is to be found. While populations from Slovenia, Croatia and Greece, the southern Austrian population (CAR) and the Salzburg sample, as well as some of the Upper and Eastern Austria samples, are assigned to one group, the other is constituted by the remaining Upper and Eastern Austria samples. Slovakia is showing an intermediate pattern. One would expect that the Balkan populations, having been colonized out of the southern glacial refugium of the Balkan peninsula (Hewitt 1999, 2000), would form a set. They partly do, in the K=2 scenario, but within further sub-division they do not stay in a uniform pattern. The observed ´missing structure´ within Bayesian clustering of *E. roumanicus* populations, however, is not in concordance with the results from Bolfíková et al. (2017), who found a clear differentiation into three populations, and subsequent within-population division at higher levels of K. They merely indicate slight admixture within specific clusters (Bolfíková et al. 2017). It is assumed, that again the potential hybrid zone of Upper (and Eastern) Austria, poses an impact on the genetic set-up of individuals found within this area, as already described in the above section concerning *E. europaeus* (conclusion drawn

based on results from Bolfíková et al. 2017).

In summary, there is an unusual pattern within Bayesian clustering of *E. roumanicus*, since Balkan populations (Greece, Croatia and Slovenia) are closer to some individuals within Upper and Eastern Austria, than to south Austrian (CAR) individuals, as of K=3. This stands in contrast to expected isolation-by-distance, but Bolfíková et al. (2017) already showed, that physical barriers like the Alps and Dinarids or climatic variations have the potential to lead to a "local parapatric evolutionary process", resulting in limited gene flow, which they found within the Slovenian population they investigated. This might as well hold true for our southern Austrian population (CAR).

PCoA of the eight populations constituted by Northern White-breasted hedgehogs reveals a grouping of "Balkan populations", i.e. Greece, Croatia and Slovenia together, with proximity to the population of CAR (southern Austria), as well as Upper and Eastern Austria populations. The populations of Slovakia and Salzburg (both of them build up by only one individual!), however, stand separately. These findings are partly in concordance with the observed genetic structure in the K=2 scenario of intraspecific *E. roumanicus* Bayesian clustering and also the proven isolation-by-distance among *E. roumanicus* populations, which is furthermore in accordance with isolation-by-distance analysis of Bolfíková et al. (2017) and the deduced post-glacial recolonization (Seddon et al 2001).

When comparing results on *E. roumanicus* intraspecific genetic differentiation, it can be seen that a combination of both, individual-based methods (like STRUCUTRE v2.3.4) and population-based methods (like PCoA in GenAlEx), are needed to create a holistic picture, because one alone might lead to incomplete and uncertain results (Allendorf et al. 2013: Units of conservation, genetic relationships within species, individual-based methods).

4.4 Investigations on *E. europaeus* x *E. roumanicus* hybrid individuals within Austria

14 out of the originally 264 *Erinaceus* hedgehog samples, analysed through Bayesian clustering showed no ´purebred´ assignment to one of the two species, *E. europaeus* and *E. roumanicus* (probability value below 0.9 in the (most likely) K=2 scenario) and are therefore considered as potential hybrid individuals. This includes samples from the area of Upper Austria, where both species have been detected before (Spitzenberger 2001 on a morphological and palaeontological basis; as well as Curto et al. 2019 through SSR usage), but also one sample from the area of Eastern Austria and five samples from the INS population, located within Tyrol, which are thought to be solely inhabited by *E. roumanicus* and *E. europaeus*, respectively (Spitzenberger 2001). Already Seiter (2018) indicated "possible hybrids in Tyrol as explanation for a lower amount of private alleles" within the results for her Tyrol population.

Central Europe is a crucial place for hybridisation events, triggered by post-Pleistocene range expansions (Hewitt 2004; Avise 2009). Contact zones with hybrid occurrence have been documented

for a variety of species, from mammals like the house mouse (*Mus domesticus* and *M. musculus*, Ferris et al. 1983; Boursot et al. 1996), over to birds (hooded and carrion crow (*Corvus cornix* and *C. corone*; Mayr 1963), as well as an assortment of amphibians and reptiles (e.g. the yellow- and fire-bellied toad (*Bombina variegata* and *B. bombina*, Szymura 1993). This, of course, includes the area of Austria, especially the federal states of Upper and Lower Austria, which have been indicated to host both of the central European *Erinaceus* species, *E. europaeus* and *E. roumanicus* (Spitzenberger 2010).

The most western found *E. roumanicus* individual in this thesis was detected in the CAR population in southern Austria, known to be inhabited by *E. roumanicus* (Spitzenberger 2001). The most eastern *E. europaeus* individual, however, was found in an area where, besides, only Northern White-breasted hedgehog samples had been detected – the population of Eastern Austria, close to the Hungarian border. Basing on the most western *E. roumanicus* individual within the known contact zone in Upper Austria, this indicates a potential zone of overlap with a magnitude of (at least) 215 km linear distance.

Samples from Styria and the centre of Lower Austria, as well as Vienna would be helpful for a more precise differentiation and indication of the two species occurrence in their sympatric distribution range. This need for a "more detailed sampling in adjacent regions" was already indicated by Bolfíková and Hulva (2012). Spitzenberger (2001), however, gives an extensive overview on the distribution ranges of *E. europaeus* and *E. roumanicus* in Austria, based on morphological (and palaeontological) assessment. Based on her distribution maps *E. europaeus* is distributed throughout Vorarlberg and Tyrol, where *E. roumanicus* is not resident. The majority of Salzburg, except for the most south-eastern part (where also our data indicate an *E. roumanicus* individual!), as well as the whole of Upper Austria, the western edge of Lower Austria and the north-western region of Styria are populated by the West European hedgehog. The most eastern individuals, therein, to be found within the western area of Lower Austria. Northern White-breasted hedgehogs within Austria are to be found in the areas of Carinthia, the majority of Styria, Burgenland, Vienna and Lower Austria, as well as the eastern range of Upper Austria (Spitzenberger 2001). The overlapping region of both species can therefore be indicated to lie within the boundaries of eastern Upper and western Lower Austria. Our *E. europaeus* sample found within the region of Northern Burgenland (Eastern Austria), constitutes an exceptional case and indicates a broader zone of overlap between the two *Erinaceus* hedgehog species present in Austria than known by now. A subsequently conducted haplotype analysis, within the already mentioned more comprehensive hedgehog study at the INF, bears further prove of the above mentioned EAUT sample being an *E. europaeus* individual (results not shown). Furthermore, one of the other Eastern Austria samples showed an *europaeus* haplotype in the same analysis (results not shown), besides its purebred *roumanicus* pattern in nuclear DNA analysis. This additionally confirms the broader than expected zone of overlap and potential hybridisation.

Palaeontological data indicate an occurrence of the West European hedgehog further east than its current Austrian distribution range (Holocene distribution and evidence at the southern border of

Lower Austria and northern border of Styria), according to Spitzenberger (2001). There is consensus on the displacement and replacement of *E. europaeus* by *E. roumanicus* within the area of the Northern Alpine foothills from the Neolithic period onwards (Spitzenberger 2001). Bolfíková and Hulva (2012), however, speak about a lack of reliability, as it comes to palaeontological data supporting phylogeographic assessments.

Distribution patterns of *E. europaeus* and *E. roumanicus* within their sympatrically shared range to the north and south of the Alps are dominated by their ecological potential of adaption (Spitzenberger 2001). The Northern White-breasted hedgehog has been detected to preferably inhabit regions of lower altitude (Bolfíková and Hulva 2012), with a warmer climate and less forested region (Spitzenberger 2001), which poses limitations to potential areas of hybridisation within the central European contact zone. However, "distribution in Central Europe cannot be considered microallopatry", as proven by Bolfíková and Hulva (2012), who found "localities with syntopic occurrence of both species". This is as well in accordance with some of our samples (samples 200695 *E. europaeus* and 2008184 *E. roumanicus*, both within Linz).

Translocations within this concern must not be neglected, since they can pose substantial influence on the (artificially enhanced) distribution of both *Erinaceus* species within Austria. In times of animal shelters and public awareness concerning conservation issues, potential rescue missions distort and skew actual distribution ranges of (sub-)populations, especially in the zone of sympatry and potential hybridisation.

E. europaeus x *E. roumanicus* hybrids had rarely been detected in the wild before. The first genetical evidence of a hybrid individual was yielded by Bogdanov et al. (2009). They, however, were studying the Eastern European contact zone (in Russia), which Bolfíková and Hulva (2012) assumed to be in a state of incomplete reproductive isolation, based on its later establishment (Seddon et al. 2001). This is also supposed to explain the rather high percentage of hybrids they had found (1 out of 5) (Bogdanov et al. 2009; Bolfíková and Hulva 2012).

Already in 2012 Bolfíková and Hulva showed that both species, *E. europaeus* and *E. roumanicus*, are distributed throughout the Czech Republic, thereby acting as a north(eastern)wards elongation of our indicated broad zone of overlap. Their findings indicate a broad overlapping in CZE, while the distribution in AUT so far has been indicated to be rather distinct (see Spitzenberger 2001: overlapping within the area of eastern Upper and western Lower Austria). The Alps thereby posing a substantial barrier (Hewitt 1996; Avise 2009) to the east- and westwards expansion of *E. europaeus* and *E. roumanicus*, respectively, concerning the federal states of Tyrol, Salzburg and Carinthia (Spitzenberger 2001). Also in North-eastern Italy both species have been shown to occur sympatrically, while no evidence of hybridisation was detected (Filippucci and Lapini 1988).

Bolfíková and Hulva (2012) were not able to detect a hybrid individual or potential

introgression within their 2012 study, in which they thoroughly investigated the whole area of the Czech Republic (n=202), as well as parts of Slovakia (n=12), for a possible hybrid occurrence. This fact seems to delimit the possibility for detection of hybrids or potential introgression within the central European contact zone. In 2017, however, Bolfíková et al. themselves found the first *E. europaeus* x *E. roumanicus* hybrid individual within the area of the Central European contact zone (Slovakia). Their discovery of a hybrid individual within the state of Slovakia, however, is not only supporting our potential hybrid findings, but also our detection of an *E. europaeus* individual within Eastern Austria (close to the Slovakian border). Bolfíková et al. (2017) hypothesize that reinforcement (i.e. "the process by which natural selection increases reproductive isolation" (Ridley 2004)) led to reproductive isolation between the two *Erinaceus* species after an initial phase of hybridisation in the secondary contact zone, since occurrence of hybrid individuals based on their studies seems to be rare. They indicated that hybridisation of *E. europaeus* and *E. roumanicus* within the mid-European contact zone might have a need for the analysis of whole genome data, since "introgression modes may be complex". They as well urged for studies on the potential hybrid zone running through Italy and Slovenia (Bolfíková et al. 2017).

Our results, indicate the presence of eight potential hybrid individuals within the known Central European contact zone in Austria (Upper Austrian population), as well as five samples from the INS population and one from the Eastern Austrian population that appear to be of admixed origin, based on Bayesian clustering analysis within STRUCTURE v.2.3.4 (Pritchard et al. 2000) and PCoA within GenAlEx 6.5 (Peakall and Smouse 2006, 2012) using nuclear genetic markers. While all INS samples that constituted an admixed pattern, showed at least an 80% probability of belonging to *E. europaeus* and also the one Eastern Austrian sampled exhibited a probability value of 0.85, four of the Upper Austrian samples possessed an assignment in the range of [0.4; 0.6]. Hitherto conducted analysis of mitochondrial data, however, bear evidence of further hybrid occurrence within the investigated area, separately from those that had already been indicated (results not shown).

Evaluation as well as verification of the observed results through mitochondrial DNA analysis (in progress) and within programs using statistical methods that directly detect hybrid individuals (like the NewHybrids 1.1 program (Anderson and Thompson 2002)), will be necessary to draw further conclusions on the extent of hybrid occurrence within the Central European contact zone in Austria.

4.5 Comparison of methodological approaches used within phylogeographic analyses

Analyses within this master thesis were solely conducted using nuclear markers, 39 different loci precisely. Mammalian phylogenetic and population genetic studies generally refer to a lower number of microsatellite markers, like in Bolfíková and Hulva (2012), as well as Bolfíková et al. (2017), who applied nine microsatellite markers developed for *E. europaeus* (Becher and Griffiths 1997; Henderson et al. 2000) within their studies on hedgehog inter- and intraspecific genetic diversity.

Curto et al. (2019), however, developed a different "approach to study the genetic structure patterns of … *E. europaeus* and *E. roumanicus*", using amplicon sequences for determination of genotypes, defining alleles based on their lengths and the existence of potential SNPs (resulting in complete sequence information) (Curto et al. 2019; see as well section 2. Material and Methods). This method was proven to help with a more precise discrimination between species and allowed for evidence of higher genetic diversity, compared to sole usage of length information (Curto et al. 2019). They rely on amplicon sequencing because of its prominent role in the analysis of short sequence repeats (de Barba et al. 2017; Farrell et al. 2016; Vartia et al. 2016), while microsatellites´ relevance in population genetics is based on a high statistical power due to the coverage of multiple alleles per locus (Schlötterer 2000; Ellegren 2004). Cost effectiveness and easy implementation complement this strategy (Hodel et al. 2016; Hodel et al. 2017). The usage of a high number of markers is supposed to strengthen statistical power within the conducted research (de Barba et al. 2017; Tibihika et al. 2019; Vartia et al. 2016).

Additionally, Curto et al. (2019) developed markers from both species, *E. europaeus* and *E. roumanicus* and reviewed their reliability for amplification of cross-species markers. It was the first time microsatellites had been developed for *E. roumanicus*. Ascertainment bias (i.e. "the selection of loci for marker development from an unrepresentative sample of individuals" (Allendorf et al. 2013)), originating from the selection of specific markers based on their variation (high heterozygosity, for example) (Brandström and Ellegren 2008) and resulting in an "increased information content despite limited numbers of markers" is a crucial point within this regard (Curto et al. 2019). Studies on the genetic variation between two closely related species, may be biased when using microsatellite markers that had been developed solely based on selection criteria within one of these two species (Morin et al. 2004 in Allendorf et al. 2013) The implementation of marker sets corresponding to both species is supposed to limit this ascertainment bias. Furthermore the usage of such a vast number of markers is supposed to help with questions concerning the comparison between the two species (Curto et al. 2019). A corresponding increase in information content of observed loci shall as well be of use for detection of possible hybridization events (Corander and Marttinen 2006; Ryman et al. 2006 in Curto et al. 2019).

Even though our data indicate a potential and most likely subdivision of the *E. europaeus* species into two divergent clades, this result has to be treated with caution. A limited and different resolution resulting of nuclear marker usage has been shown in various studies (Seddon et al. 2001; Berggren et al. 2005). Seddon et al. (2001) were not able to detect the three clades of *E. europaeus*, which they had detected through haplotype analysis, when using nuclear markers for a similar observation. The deep genetic divergence between *E. concolor* and *E. roumanicus* species, however, was indicated by the nuclear markers. This lack of congruence between mtDNA and nuclear DNA was confirmed by Berggren et al. (2005), studying *Erinaceus* hedgehog genetic diversity through usage of markers for the MHC complex. They explain the missing concordance by "historical differences in the refugial

population size of *europaeus* and *concolor*" (Seddon et al. 2001). Mitochondrial DNA, being more sensitive to bottlenecks affecting (effective) population size, because of its haploid state, shows genetical structuring sooner, than nuclear DNA, being less sensitive to recent demographic processes (Seddon et al. 2001; Bolfíková and Hulva 2012). Assuming that (the founding population of) *E. concolor* underwent a more severe bottleneck than its western counterpart *E. europaeus*, this is thought to be evident in both mitochondrial, as well as nuclear DNA (Seddon et al. 2001). They exclude "male biased dispersal" as possible explanation within this concern, due to a lack in data on sex-biased dispersal differences. Seddon et al. (2001), however, employed the widely used nested clade analysis (NCPA, Templeton 1998) for their assessment of mitotype distribution, which is under criticism as it "might provide the correct inference about phylogeographic history, but we cannot easily quantify the probability of it being correct" (Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography). A broad range of shortcomings within this concern has been pointed out by Beaumont et al. (2010), who set it in comparison to and recommends model-based concepts (Allendorf et al. 2013: Units of conservation, genetic relationships within species, phylogeography).

Resulting in the need for further verification of the observed intraspecific genetic substructuring and divergence within this master thesis through haplotype analysis, which´s application for phylogeographic studies is broadly distributed (Taberlet 1998; Hewitt 1999; in Berggren 2005). Avise (2009) even speaks of a "prerequisite for phylogeographic analyses", when it comes to the rapid evolution of nucleotide sequences in mtDNA.

The conclusion of these results, however, is that sole usage of either mitochondrial or nuclear markers is not valid for an informative statement on hedgehog intraspecific phylogeographic pattern and divergence (Berggren 2005). Already Hewitt (1996), as well as Avise (2009) recommended the implementation of mitochondrial, as well as nuclear data, when conducting phylogeographic analyses. Also Bolfíková and Hulva (2012) conducted their studies on (European) hedgehog genetic diversity and corresponding examination of their sympatric zone, based on nuclear, as well as mtDNA markers. In order to "employ the specific features of particular genetic pools", "compare the genetic integrity of populations living in allopatry and sympatry" and differentiate between "female- and male-mediated gene-flow" (Bolfíková and Hulva 2012).

The importance of examining both, nuclear, as well as mtDNA, for determination of inter- and intraspecific genetic relationships, was well shown in a case study of polar and brown bears. Those were thought to have a paraphyletic relationship, based on mtDNA assessments (Talbot and Shield 1996), while substitutional analysis with nuclear markers showed "that polar bears are monophyletic and diverged from brown bears" (Hailer et al. 2012). Past hybridisation and introgression is suspected to be responsible for polar bears carrying brown bear mtDNA (Allendorf et al. 2013: Units of conservation, phylogeny reconstruction, gene trees and species trees, mtDNA gene tree versus species tree).

Among the 264 valid sample, used within this master thesis, 226 stem from the region of Austria. Although this constitutes a vital and essential contribution to the observation of the Central European contact zone within Austria, it as well poses a major issue concerning the phylogeographic observation of the two examined European hedgehog species. The existing restriction in sample numbers of particular populations, as well as the uneven distribution of sampled area is the resultant of the usage of a mass of *Erinaceus* hedgehog samples available after sampling for divers other master thesis, as well as a forthcoming, more comprehensive research project (Curto et al. 2019). Hewitt (2000) stated that a "wide-range coverage is important to produce a full phylogeography" and the need for further sampling of regions still missing for a full (European) coverage within this phylogeographic study, is evident. Samples from Italy will be needed to cover the third glacial refugium of European hedgehogs (Hewitt 1999, 2000) within this phylogeographic study and allow for conclusions on intraspecific genetic divergence of *E. europaeus* (within Austria), in the course of postglacial expansion. A lack in Italian samples, as third glacial refugium, might lead to a detection of more diversity within central Europe, that cannot be found elsewhere within the sampled area, as indicated by the INS population. As well as that, French and Swiss samples are necessary for further comprehension within this concern. Also Balkan populations are underrepresented in terms of numbers of individuals per population and coverage of the studied geographic area , requiring further sampling. Populations only consisting of one (1) individual cannot statistically be relied on. The circumstance of a limited number of samples within several populations in this thesis leads to a sampling error, in terms of a non-representative set of individuals being used. If only a few individuals (or sometimes just one sample!) are being used for representing a wide geographic area, this might result in the detection of only a small subset of existing lineages. (Allendorf et al. 2013: Units of conservation, phylogeny reconstruction, gene trees and species trees, lineage sorting and sampling error).

It would also be interesting to examine if a mere observation of populations that do not lie in proximity to the central European contact zone gives rise to a different pattern of potential or even existent subdivision within *E. europaeus*. After all, populations within this study, that show connection to the potential hybridisation zone and central European contact zone display a similar genetic pattern and therefore assignment to one group within the (most likely) K=2 scenario. It would be important and informative to know whether additional samples from the Italian Peninsula, France and Switzerland would show concise assignment.

Missing coordinates for samples from shelter individuals (e.g. BLU, INS, CAR) may violate population set-up to a certain degree, but shelter animals usually originate from the close surroundings of a shelter, since people invest only a certain effort in the rescue of wild animals, and wildlife sanctuaries and animal shelters are distributed throughout Austria (personal knowledge based on internships conducted at the "Verein für kleine Wildtiere in großer Not", located in Graz). As well as that, it is known that shelter samples stem from no further than 100 km around the corresponding shelters (Curto et al. 2019), which would pose problems in the detected potential area of 215 km

overlap *of E. europaeus* and *E. roumanicus*, but should not account for the western and southern Austrian populations of BLU, INS, CAR, as well as the German population of MT.

5. Conclusion

Investigations on potential hybrid zone dynamics within Austria, on the basis of dense sampling and the usage of multiple markers, clearly allowed for rejection of the hypothesis "No hybrid occurrence expected". Fourteen samples revealed signals of admixture between *E. europaeus* and *E. roumanicus*. Characterization of both species´ contact zone within Austria, resulted in the rejection of the second hypothesis within this concern "A narrow zone of overlap between eastern Upper and western Lower Austria is expected". *E. europaeus* and *E. roumanicus* overlap in an area of approximately 215 km linear distance, reaching from Upper Austria to Eastern Austria.

Investigations on the phylogeography of *E. europaeus* and *E. roumanicus* within Central Europe revealed significant isolation-by-distance for both species – leading to the rejection of the hypothesis "No isolation-by-distance is expected". Bayesian clustering, as well as PCoA allowed for a clear differentiation between West European and Northern White-breasted hedgehogs. Intraspecific phylogeographic analyses resulted in a most likely K of two for *E. europaeus* substructuring, separating Western and Central European populations from samples originating from the contact zone. Within *E. roumanicus* intraspecific analyses Balkan and southern Austrian populations largely differed from samples corresponding to the potential hybrid zone.

Evaluation and verification of the observed potential hybrid individuals within the Austrian zone of secondary contact through, inter alia, haplotype analysis, is intended. The implementation of statistical methods directly detecting hybrid individuals will be necessary to draw further conclusions on the extent of hybrid occurrence within the Central European contact zone in Austria. Moreover, a denser sampling concerning the third glacial refugium (Italy) within Europe will be required, to draw further conclusions about potential phylogeographic factors influencing genetic diversity within Austrian hedgehog populations.

Additional analysis, solely focusing on hedgehog populations within animal shelters, are a further future objective. Investigations on those shall allow for a deeper insight into potential implications of human-mediated translocations on hedgehog genetic diversity and potential hybridization.

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7. Appendix

Tab. S1. List of all primers used in the multiplex PCR approach for sequence analysis of *E. europaeus* and *E. roumanicus*. The table shows the primer name – including the repetition motif, the number of repeats of the motif in the original sequence, the primer sequence (forward and reverse), the allele length variation (Amplicon length variation) and the corresponding primer mix. Primer mixes HH1, HH2 and HH3 are supposed to amplify loci within *E. roumanicus*, whereas primer mix WHH1 contains primers that were designed from *E. europaeus*. The missing data column indicates the amount of missing data over all loci after initial sequence analysis. Primers that are mentioned underneath the boundary line did not go into further population genetic analysis as they showed over 50% missing data among all analysed loci (primer development by Curto et al. 2019).

Number	Primer name	Nr. Repeats	Forward	Reverse	Amplicon length variation	Primer mix	missing data [%]
1	HH1_AC	16	TCATGCTAGGCACTGCTATT	AAGTGCAATCAGACCAGTGA	454-486	HH1	31,75
3	HH11_AAAG	7	ACGTTCCTCTCTGGGGAATA	TTCAAGACCCTGTTCTCCAC	428-460	HH1	12,74
4	HH13_AAGG	11	AGGTAGAAGGCAGACAATGG	TTGAAACACTGACGTAGGCT	428-476	HH3	12,31
5	HH16_AAGAA	12	CACTAGGCAGAAAACACACG	ACCACAGATGCTGTAGACAG	425-480	HH2	9,50
6	HH18_ATTTT	9	TAGCCTGGGGGAAAATCAAG	GCAATTTCCAGTAGAGGGGA	438-475	HH1	10,37
7	HH19_GAAAT	12	CCTTGCTTGTTTCCTAAGCC	ATCACTGGGACTCCCTCAAT	472-527	HH3	12,96
8	HH2_AC	16	TGGGTAGCAGCTAAAGGAAG	GACAAAATCCTCCCTGGCTA	448-482	HH2	15,33
9	HH20_TATTC	11	TGGATGGATGGAAAACTGAGA	GGGTGCTGATTCATCTACCT	477-522	HH3	35,21
10	HH22_AG	15	ACGGAAGGAAATACTGCCAA	CCTTCCCCTTTGTGAGAACT	470-502	HH3	10,58
11	HH24_GCA	7	TTCTGAGGTCTCATCTGTGC	CTGTCTGTGGTCCAGGAAAG	452-452	HH3	11,02
13	HH26_CAA	10	TTAAGGAACTCAGGGTTGGG	GTGTCAATGGAAGCAAAGCT	487-502	HH1	27,21
14	HH27_TTG	9	AGATGCTCAAGGGAAACTGA	TCACAGCATACTTAGGAGCC	452-477	HH2	9,94
15	HH28_TTG	8	CCTAGTGGTAGCTTCTCACA	TTGGCTCCAGTCAAGTTCTC	440-452	HH3	14,04
16	HH29_TCAA	7	CTTGTGCACTGTGATGTGAG	ACGAAGTTTCCAGGAAGCTC	486-494	HH1	25,05
17	HH3_CA	17	GGCAGACTGTCTAGTTCACA	GGTCTAGGACTGCACCATTT	476-512	HH3	40,39
18	HH30_TCAC	8	AGCGTTAAATACATCCGGCT	AACCCATTGACTCTCTGACA	430-458	HH2	7,13
19	HH31_AACA	7	GGAAGCGCCTTCATTATAGC	CTCCTGTCACTAGCCAGAAG	476-484	HH1	12,96
21	HH34_TCTA	10	AGACCACAGTGTCCAAGTTT	GATTTCCCCTGTGTAGGTGA	428-466	HH2	10,58
22	HH35_AAAAT	6	TGGGTTGTAGATAACACTCA	ACTGCAGGTGGAGATATGTG	464-482	HH2	33,91
23	HH36_GAAAG	9	ACAGTGAAGACAGGGAAGC	CTTAAAATGGCTAAGGTGGT	452-517	HH1	12,96
24	HH37_CTTTT	7	CTGCAGTTTGCTCTGGATTC	AAGAAAGAAGCCCTTGTCCA	446-480	HH2	8,86
25	HH4_CT	13	TCAAGGAGTGTGTTGACCAG	ATCCCTTTGCTCAGCCAAT	452-462	HH1	14,90

26	HH5_GA	17	TTTCTTGCTCAGAACCCTGA	CAGGGGGAATGCTTTTCAAG	446-480	HH2	32,18
29	HH8_ATT	8	CCTCCAGGAGAGATTTTGCT	CAAATGAGTGGAAGCCATGC	489-498	HH3	33,91
30	HH9_ATT	13	GTTGACACTCTTTGCTGCTT	CAAGTCCTCACTAAGCCTGT	425-444	HH1	25,27
32	WHH10_AAAAC	7	ATAGCTGGATAGTGGTCTGG	ACATCTTTTCTTCCTCACAGT	398-433	WHH1	11,66
33	WHH11_CTTC	10	AGTCACCATTCTCCACTTTC	ACCCTGAGTGAAGAAGGATA	413-435	WHH1	42,76
34	WHH12_GAAA	8	AACTCAAATTACAAGGGGCC	TCCAATAACTAGGGGTTTAAGT	386-474	WHH1	13,39
36	WHH14_ATAG	10	AAAAGGACCTAAATGGGAGG	ACAGGGAACAAAGATGCTTA	376-408	WHH1	10,80
38	WHH16_TTAA	7	GTGTAAAGCAGTATGTTGCC	AATACAGTGTACAAGGACGC	407-419	WHH1	31,10
40	WHH19_TTCT	13	AGAGATCAGACTAACGTTTTT	GGGGAGAATTTGGTACTGTA	402-443	WHH1	35,21
43	WHH23_TGGA	13	TCTTCCCTTAAGCTACTGGA	TCTCAATTGTTTAGACATTGAGT	386-414	WHH1	15,77
46	WHH29_CT	15	CATTACCGTGCACACAGA	GTTTGATCCCCACCACTTAA	406-422	WHH1	28,08
48	WHH30_CT	17	TCTCATTGGATAGTGCACTG	TGCCTAATAGCAAATACACA	405-441	WHH1	30,45
49	WHH32_GT	13	CAGTCAATGCATTCCCAATC	TGTGTGGTACAGGGAATAGA	415-451	WHH1	46,22
51	WHH5_AAAAT	8	CACCAGGTTAAGCGTACATA	AAAAGTGCTACTAGGGAAGC	NA	WHH1	39,96
53	WHH7_TCTTT	9	TTAGCTTGGTTTTCACAGGT	GAGTGGCAGTCTTCAAGTAG	384-419	WHH1	15,12
54	WHH8_TTCCT	10	ATAGGAGGACTGGCGATC	AATGGAGGGAGTAGATGGG	364-424	WHH1	9,29
55	WHH9_TTTCT	10	TTCAATCTCAAGTACCACATT	GATGCACCTGGTTGAGAG	384-414	WHH1	43,63
2	HH10_AAAG	11	AAGCACAACAACAATGGCAA	ACGTACTGAGCCTTTCAAGA	437-545	HH2	69,11
12	HH25_TAC	9	TGTTATCATGCCTGAGGACC	CTGGTTGGGAAGAGAAACCT	NA	HH1	94,60
20	HH32_ATCT	7	TGACAGTGTGTGGTTGACTT	TTCACCATCGCAGAGAACAT	NA	HH3	98,06
27	HH6_AAT	16	CTCTTGGTGTGCATGACAAG	CTGTGACCCGTGTAGTTGG	NA	HH1	97,62
28	HH7_AAT	10	ACCATAGCTTTGTAATCTCCT	AGGATGATGGCCCTTTGAAA	445-463	HH2	65,44
31	WHH1_AAAAT	7	GGGTAAAACAGGTCTGATGT	AAACTTGTCAGGAAGCAGTT	382-407	WHH1	64,15
35	WHH13_TTTA	7	TTTCACTCTGGGTTACTGTG	AAGTGGTGCAACTCTAAGAC	386-395	WHH1	88,77
37	WHH15_ATAA	8	ATACTCCCAGCCTGTTTCTA	ACCTCCCAAGAACTCTATCA	367-390	WHH1	76,67
39	WHH18_AATA	8	ACTCAAAAGTTTTCCACCCT	TTTTAGGCTCTGCTCTTCTG	403-411	WHH1	77,97
41	WHH20_TAGA	8	TGCACATTACAATGTTCAAGG	TACATCAGGGAGAGTACAGG	NA	WHH1	100,00
42	WHH21_TTTA	7	ACTTCACTATCACCCTTCAA	ACTTGATTTGTTTATGGGGTG	395-403	WHH1	57,02
44	WHH24_ATA	13	GCAATAATAACAAGAAGGGCA	AAGAAGTGACTGGTTTGGAG	NA	WHH1	94,38
45	WHH26_TAT	15	TTTCCAGAAGATGTGGTCAG	TACAAATCTCAGCACCACTC	NA	WHH1	98,06
47	WHH3_AAAGA	6	GAAGAAGTTTCCTCCTCTGG	GGTGGACTGAACCATTTCTT	NA	WHH1	98,92

50	WHH33_CA	11	AGAAAAGACCTCAGGAGACT	CCTGGAGAGTGGAAAAGTTA	424-456	WHH1	50,11
52	WHH6_TTATT	7	AGGAGTTCTCAGTGATGAGA	AATACAGGCTCTGGGATAGT	378-404	WHH1	96,76

Tab. S2. Samples used for population genetic and phylogeographical analysis of *E. europaeus* and *E. roumanicus* within Europe. Given are the sample name, the artificially designed population a sample has been assigned to*, the assigned species (*E. europaeus*, *E. roumanicus* or potential hybrid) based on Bayesian clustering and PCoA, sampling region, sampling coordinates (in decimal Lat/Long; NA = not available, mainly for shelter individuals), sampled material (tissue, saliva or DNA isolate/extract), material origin (institutions or shelters that provided samples)*, percentage of missing data for each sample after initial sequence analysis [%]. [*Abbreviations: IBP = Iberian Peninsula, MT = shelter Igelburg Mossautal e.V., BER = Berlin, BLU = shelter Bludenz, INS = shelter Innsbruck, SBG = Salzburg, UAUT_W/S/N/E = Upper Austria West/South/North/East, LINZ = region of Linz, LAUT = Lower Austria, CZE = Czech Republic, POL = Poland, SVK = Slovakia, EAUT + HU = Eastern Austria and Hungary, CAR = shelter Carinthia, SLO = Slovenia, CRO = Croatia, GRE/NM = Greece and North Macedonia; CIBIO = Research Centre in Biodiversity and Genetic Resources; IWZ = Leibniz Institute for Zoo and Wildlife Research; INF = Institute for Integrative Nature Conservation Research]

sample name	assigned population	*species*	sample region	Coordinates	Sampled material	Material origin	missing data per sample [%]
720	IBP	*E. europaeus*	Portugal, Douro Litoral	41.333, -8.666	DNA	CIBIO	0,00
782	IBP	*E. europaeus*	Portugal, Minho	41.927, -8.587	DNA	CIBIO	2,56
942	IBP	*E. europaeus*	Spain, Castilla-León	42.436, -3,72	tissue	CIBIO	28,21
1012	IBP	*E. europaeus*	Spain, Andaluzia	37.133, -3.666	tissue	CIBIO	20,51
1142	IBP	*E. europaeus*	Spain, Castilla-León	42.745, -3.806	DNA	CIBIO	2,56
2801	IBP	*E. europaeus*	Portugal, Alentejo	37.922, -7.507	DNA	CIBIO	7,69
2805	IBP	*E. europaeus*	Portugal, Algarve	37.29, -8.592	DNA	CIBIO	7,69
3848	IBP	*E. europaeus*	Spain, Alicante	38.083, -0.839	DNA	CIBIO	2,56
MT-1	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	15,38
MT-10	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	15,38
MT-11	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	30,77
MT-2	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	15,38
MT-4	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	10,26
MT-5	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	15,38
MT-6	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	5,13
MT-7	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	15,38
MT-8	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	7,69
MT-9	MT	*E. europaeus*	Germany, Mossautal	NA	saliva	Igelburg Mossautal e.V.	48,72
36516	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	2,56
371-16-2	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	12,82
372-16-1	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	7,69
373-16-2	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	20,51
374-16-1	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	12,82
VA25	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA26	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	5,13
VA27	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	17,95
VA28	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA29	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA30	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA31	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	7,69

VA-32-1	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	35,90
VA33	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	0,00
VA34	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	10,26
VA-35-1	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA36	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	10,26
VA37	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	0,00
VA38	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	0,00
VA39	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	0,00
VA40	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	5,13
VA41	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	30,77
VA42	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	15,38
VA43	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	33,33
VA44	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	17,95
VA45	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	5,13
VA46	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	15,38
VA47	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	17,95
VA48	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	12,82
VA49	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	7,69
VA50	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	7,69
VA51	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	7,69
VA52	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	12,82
VA53	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	12,82
VA54	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	2,56
VA-55-1	BLU	*E. europaeus*	Bludenz	NA	saliva	shelter Bludenz	23,08
IBK1	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	38,46
IBK10	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	15,38
IBK11	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	2,56
IBK12	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	41,03
IBK13	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	12,82
IBK14	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	28,21
IBK-15-1	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	28,21
IBK16	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	20,51
IBK17	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	17,95
IBK18	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	25,64
IBK19	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	20,51
IBK2	INS	potential Hybrid	Innsbruck	NA	saliva	shelter Innsbruck	33,33
IBK20	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	17,95
IBK21	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	10,26
IBK22	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	25,64
IBK23	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	20,51
IBK24	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	28,21
IBK3	INS	potential Hybrid	Innsbruck	NA	saliva	shelter Innsbruck	33,33

IBK4	INS	potential Hybrid	Innsbruck	NA	saliva	shelter Innsbruck	33,33
IBK5	INS	potential Hybrid	Innsbruck	NA	saliva	shelter Innsbruck	35,90
IBK6	INS	potential Hybrid	Innsbruck, Pradl	NA	saliva	shelter Innsbruck	35,90
IBK7	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	33,33
IBK8	INS	*E. europaeus*	Hall in Tirol	NA	saliva	shelter Innsbruck	33,33
IBK9	INS	*E. europaeus*	Innsbruck	NA	saliva	shelter Innsbruck	5,13
2014819	SBG	*E. roumanicus*	Salzburg, Wölting	47.133, 13.800	tissue	Biologiezentrum Linz	28,21
200697	UAUT_W	*E. europaeus*	Upper Austria, Hadermarkt	48.083, 12.767	tissue	Biologiezentrum Linz	0,00
200698	UAUT_W	*E. europaeus*	Upper Austria, Franking	48.050, 12.900	tissue	Biologiezentrum Linz	38,46
2005613	UAUT_W	potential Hybrid	Upper Austria, Haag am Hausruck	48.183, 13.633	tissue	Biologiezentrum Linz	7,69
2006604	UAUT_W	*E. europaeus*	Upper Austria, Weng	48.217, 13.750	tissue	Biologiezentrum Linz	5,13
2007199	UAUT_W	*E. europaeus*	Upper Austria, Durchham	48.267, 13.250	tissue	Biologiezentrum Linz	12,82
2012159	UAUT_W	potential Hybrid	Upper Austria, Gaspoltshofen	48.133, 13.733	tissue	Biologiezentrum Linz	10,26
2012160	UAUT_W	*E. europaeus*	Upper Austria, Obereinwald	48.033, 13.633	tissue	Biologiezentrum Linz	0,00
2014429	UAUT_W	*E. europaeus*	Germany, eastern Bavaria	48.267, 13.017	tissue	Biologiezentrum Linz	5,13
2014430	UAUT_W	*E. europaeus*	Germany, eastern Bavaria	48.267. 13.017	tissue	Biologiezentrum Linz	7,69
2014434	UAUT_W	*E. europaeus*	Upper Austria, Hadermarkt	48.083, 12.767	tissue	Biologiezentrum Linz	0,00
2014440	UAUT_W	*E. europaeus*	Upper Austria, Gschnarret	48.300, 13.933	tissue	Biologiezentrum Linz	10,26
2014581	UAUT_W	*E. roumanicus*	Upper Austria, Feldkirchen a. d. Donau	48.333, 14.100	tissue	Biologiezentrum Linz	17,95
2014681	UAUT_W	*E. europaeus*	Upper Austria, Hinzenbach	48.300, 13.933	tissue	Biologiezentrum Linz	35,90
2014683	UAUT_W	*E. europaeus*	Upper Austria, Wilhering	48.317, 14.167	tissue	Biologiezentrum Linz	12,82
2014822	UAUT_W	*E. europaeus*	Upper Austria, Pessenlittring	48.083, 14.000	tissue	Biologiezentrum Linz	23,08
2014838	UAUT_W	*E. roumanicus*	Upper Austria, Marchtrenk	48.200, 14.100	tissue	Biologiezentrum Linz	7,69
2015371	UAUT_W	*E. europaeus*	Upper Austria, Helpfau-Uttendorf	48.150, 13.150	tissue	Biologiezentrum Linz	12,82
2015734	UAUT_W	*E. europaeus*	Upper Austria, Meggenhofen	48.133, 13.783	tissue	Biologiezentrum Linz	2,56
2016173	UAUT_W	*E. europaeus*	Upper Austria, Lambach	48.083, 13.867	tissue	Biologiezentrum Linz	12,82
28INF-eu-2	UAUT_W	*E. europaeus*	Upper Austria, Regau	47.955, 13.733	saliva	Igelhof Aurachtal	2,56
200787	UAUT_S	*E. roumanicus*	Upper Austria, Pichl	47.717, 14.300	tissue	Biologiezentrum Linz	2,56
2012156	UAUT_S	potential Hybrid	Upper Austria, Gmunden	47.917, 13.800	tissue	Biologiezentrum Linz	2,56
2014419	UAUT_S	potential Hybrid	Upper Austria, Hinterstoder	47.683, 14.150	tissue	Biologiezentrum Linz	20,51
2014427	UAUT_S	potential Hybrid	Upper Austria, Eberstalzell	48.017, 13.983	tissue	Biologiezentrum Linz	12,82
2014428	UAUT_S	*E. europaeus*	Upper Austria, Eberstalzell	48.017, 13.983	tissue	Biologiezentrum Linz	7,69
2014443	UAUT_S	*E. europaeus*	Upper Austria, Eberstalzell	48.033, 13.967	tissue	Biologiezentrum Linz	0,00
2014454	UAUT_S	*E. europaeus*	Upper Austria, Viechtwang	47.900, 13.950	tissue	Biologiezentrum Linz	0,00
2014664	UAUT_S	*E. europaeus*	Upper Austria, Schlierbach	47.933, 14.100	tissue	Biologiezentrum Linz	5,13
2016388	UAUT_S	potential Hybrid	Upper Austria, Roßleithen	47.700, 14.267	tissue	Biologiezentrum Linz	2,56
2004-347-2	UAUT_S	*E. roumanicus*	Upper Austria, Windischgarsten	47.717, 14.317	tissue	Biologiezentrum Linz	43,59
2005-612-2	UAUT_S	*E. roumanicus*	Upper Austria, Windischgarsten	47.717, 14.317	tissue	Biologiezentrum Linz	15,38
2012-157-2	UAUT_S	*E. europaeus*	Upper Austria, Gmunden	47.917, 13.800	tissue	Biologiezentrum Linz	2,56
200791	UAUT_N	*E. europaeus*	Upper Austria, Zwettl	48.450, 14.267	tissue	Biologiezentrum Linz	7,69
2005609	UAUT_N	*E. europaeus*	Upper Austria, Thierberg	48.517, 14.383	tissue	Biologiezentrum Linz	17,95
2007100	UAUT_N	*E. europaeus*	Upper Austria, St.Willibald	48.350, 13.683	tissue	Biologiezentrum Linz	0,00
2012126	UAUT_N	*E. europaeus*	Upper Austria, Freistadt	48.517, 14.500	tissue	Biologiezentrum Linz	20,51

2014436	UAUT_N	*E. europaeus*	Upper Austria, Unternberg	48.483, 14.000	tissue	Biologiezentrum Linz	0,00
2014444	UAUT_N	*E. europaeus*	Upper Austria, Rainbach im Mühlkreis	48.550, 14.533	tissue	Biologiezentrum Linz	2,56
2015370	UAUT_N	*E. europaeus*	Upper Austria, Waldkirchen a. Wesen	48.433, 13.833	tissue	Biologiezentrum Linz	12,82
2004-248-2	UAUT_N	*E. europaeus*	Upper Austria, Vierzehn	48.533, 14.500	tissue	Biologiezentrum Linz	23,08
2009-390-2	UAUT_N	*E. europaeus*	Upper Austria, St.Agatha	48.400, 13.883	tissue	Biologiezentrum Linz	33,33
39INF-1	UAUT_N	*E. europaeus*	Upper Austria, Wesenufer	48.445, 13.827	tissue	Biologiezentrum Linz	38,46
200669	LINZ	*E. roumanicus*	Upper Austria, Linz	48.300, 14.300	tissue	Biologiezentrum Linz	10,26
200695	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	5,13
200696	LINZ	*E. europaeus*	Upper Austria, Puchenau	48.300, 14.250	tissue	Biologiezentrum Linz	23,08
200788	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	0,00
200789	LINZ	*E. europaeus*	Upper Austria, Linz	48.367, 14.283	tissue	Biologiezentrum Linz	5,13
200790	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	5,13
200792	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	10,26
200797	LINZ	*E. europaeus*	Upper Austria, Altenberg bei Linz	48.367, 14.350	tissue	Biologiezentrum Linz	23,08
200798	LINZ	potential Hybrid	Upper Austria, Linz	48.300, 14.283	tissue	Biologiezentrum Linz	0,00
201465	LINZ	*E. roumanicus*	Upper Austria, Kirchschlag bei Linz	48.400, 14.283	tissue	Biologiezentrum Linz	38,46
2002243	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	12,82
2005181	LINZ	*E. europaeus*	Upper Austria, Linz	48.367, 14.350	tissue	Biologiezentrum Linz	17,95
2005607	LINZ	*E. europaeus*	Upper Austria, Linz	48.383, 14.283	tissue	Biologiezentrum Linz	38,46
2005614	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.283	tissue	Biologiezentrum Linz	12,82
2006432	LINZ	*E. europaeus*	Upper Austria, Linz	48.317, 14.300	tissue	Biologiezentrum Linz	38,46
2006613	LINZ	*E. roumanicus*	Upper Austria, Linz	48.283, 14.283	tissue	Biologiezentrum Linz	10,26
2007596	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.283	tissue	Biologiezentrum Linz	0,00
2007626	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	0,00
2008184	LINZ	*E. roumanicus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	5,13
2008214	LINZ	*E. roumanicus*	Upper Austria, Linz	48.233, 14.367	tissue	Biologiezentrum Linz	7,69
2014425	LINZ	*E. roumanicus*	Upper Austria, Linz	48.317, 14.317	tissue	Biologiezentrum Linz	25,64
2014431	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	12,82
2014438	LINZ	*E. europaeus*	Upper Austria, Linz	48.250, 14.400	tissue	Biologiezentrum Linz	0,00
2014439	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	0,00
2014441	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.283	tissue	Biologiezentrum Linz	7,69
2014453	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.283	tissue	Biologiezentrum Linz	10,26
2014455	LINZ	*E. europaeus*	Upper Austria, Linz	48.300, 14.267	tissue	Biologiezentrum Linz	0,00
2014456	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	2,56
2014623	LINZ	*E. europaeus*	Upper Austria, Linz	48.300, 14.250	tissue	Biologiezentrum Linz	25,64
2014665	LINZ	*E. europaeus*	Upper Austria, Linz	48.317, 14.283	tissue	Biologiezentrum Linz	10,26
2014684	LINZ	*E. europaeus*	Upper Austria, Steyregg	48.317, 14.333	tissue	Biologiezentrum Linz	17,95
2014839	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	7,69
2015786	LINZ	*E. europaeus*	Upper Austria, Linz	48.333, 14.300	tissue	Biologiezentrum Linz	0,00
2016169	LINZ	*E. europaeus*	Upper Austria, Linz	48.300, 14.267	tissue	Biologiezentrum Linz	0,00
2016551	LINZ	*E. europaeus*	Upper Austria, Linz	48.400, 14.267	tissue	Biologiezentrum Linz	7,69
20111166	LINZ	*E. europaeus*	Upper Austria, Linz	48.400, 14.283	tissue	Biologiezentrum Linz	7,69
2013-296-2	LINZ	*E. roumanicus*	Upper Austria, Linz	48.300, 14.283	tissue	Biologiezentrum Linz	10,26

2015-787-2	LINZ	*E. europaeus*	Upper Austria, Lichtenberg	48.367, 14.250	tissue	Biologiezentrum Linz	17,95
42INF-1	LINZ	*E. roumanicus*	Upper Austria, Linz	48.317, 14.307	tissue	Biologiezentrum Linz	15,38
200663	UAUT_E	*E. europaeus*	Upper Austria, Obervisnitz	48.350, 14.483	tissue	Biologiezentrum Linz	12,82
200664	UAUT_E	*E. europaeus*	Upper Austria, Deiming	48.217, 14.733	tissue	Biologiezentrum Linz	7,69
200674	UAUT_E	*E. roumanicus*	Upper Austria, Naarn im Machlande	48.217, 14.600	tissue	Biologiezentrum Linz	17,95
200675	UAUT_E	*E. roumanicus*	Upper Austria, Steyregg	48.267, 14,533	tissue	Biologiezentrum Linz	10,26
200693	UAUT_E	*E. europaeus*	Upper Austria, Baumgartenberg	48.217, 14.750	tissue	Biologiezentrum Linz	5,13
200725	UAUT_E	*E. europaeus*	Upper Austria, Dorf a. d. Enns	48.083, 14.467	tissue	Biologiezentrum Linz	0,00
200793	UAUT_E	*E. europaeus*	Upper Austria, Stadlkirchen	48.100, 14.433	tissue	Biologiezentrum Linz	0,00
200794	UAUT_E	*E. europaeus*	Upper Austria, Rohrbach	48.217, 14.350	tissue	Biologiezentrum Linz	0,00
2004247	UAUT_E	*E. europaeus*	Upper Austria, Bad Zell	48.333, 14.700	tissue	Biologiezentrum Linz	17,95
2005615	UAUT_E	*E. europaeus*	Upper Austria, Grein	48.217, 14.850	tissue	Biologiezentrum Linz	5,13
2006180	UAUT_E	*E. europaeus*	Upper Austria, Standorf	48.283, 14.967	tissue	Biologiezentrum Linz	0,00
2006182	UAUT_E	*E. roumanicus*	Upper Austria, Sierning	48.033, 14.283	tissue	Biologiezentrum Linz	12,82
2006183	UAUT_E	*E. roumanicus*	Upper Austria, St.Ulrich bei Steyr	48.000, 14.450	tissue	Biologiezentrum Linz	33,33
2006184	UAUT_E	*E. roumanicus*	Upper Austria, St.Ulrich bei Steyr	48.017, 14.417	tissue	Biologiezentrum Linz	23,08
2006185	UAUT_E	*E. roumanicus*	Upper Austria, St.Ulrich bei Steyr	48.017, 14.417	tissue	Biologiezentrum Linz	7,69
2006187	UAUT_E	*E. roumanicus*	Upper Austria, Baumgartenberg	48.200, 14.717	tissue	Biologiezentrum Linz	17,95
2006605	UAUT_E	*E. europaeus*	Upper Austria, Straß	48.200, 14.617	tissue	Biologiezentrum Linz	0,00
2006606	UAUT_E	*E. europaeus*	Upper Austria, Friensdorf	48.350, 14.500	tissue	Biologiezentrum Linz	7,69
2007101	UAUT_E	*E. europaeus*	Upper Austria, Perg	48.233, 14.633	tissue	Biologiezentrum Linz	0,00
2007102	UAUT_E	*E. europaeus*	Upper Austria, Dorf a. d. Enns	48.083, 14.467	tissue	Biologiezentrum Linz	2,56
2007103	UAUT_E	*E. europaeus*	Upper Austria, Bad Hall	48.050, 14.183	tissue	Biologiezentrum Linz	0,00
2008185	UAUT_E	*E. roumanicus*	Upper Austria, Feyregg	48.017, 14.183	tissue	Biologiezentrum Linz	5,13
2008186	UAUT_E	*E. roumanicus*	Upper Austria, Kematen	48.233, 14.183	tissue	Biologiezentrum Linz	12,82
2008215	UAUT_E	*E. roumanicus*	Upper Austria, Neuhofen a. d. Krems	48.133, 14.217	tissue	Biologiezentrum Linz	35,90
2008219	UAUT_E	*E. europaeus*	Upper Austria, Obervisnitz	48.350, 14.483	tissue	Biologiezentrum Linz	35,90
2009167	UAUT_E	*E. roumanicus*	Upper Austria, Bad Zell	48.333, 14.700	tissue	Biologiezentrum Linz	2,56
2010212	UAUT_E	*E. roumanicus*	Upper Austria, Markt St. Florian	48.200, 14.350	tissue	Biologiezentrum Linz	20,51
2014418	UAUT_E	*E. roumanicus*	Upper Austria, Markt St. Florian	48.217, 14.350	tissue	Biologiezentrum Linz	12,82
2014433	UAUT_E	*E. europaeus*	Upper Austria, Saxen	48.200. 14.783	tissue	Biologiezentrum Linz	2,56
2014435	UAUT_E	*E. europaeus*	Upper Austria, Aisting	48.250, 14.567	tissue	Biologiezentrum Linz	2,56
2014437	UAUT_E	*E. europaeus*	Upper Austria, Lasberg	48.450, 14.517	tissue	Biologiezentrum Linz	0,00
2014442	UAUT_E	*E. europaeus*	Upper Austria, Lasberg	48.467, 14.533	tissue	Biologiezentrum Linz	5,13
2014565	UAUT_E	*E. roumanicus*	Upper Austria, Furth	48.250, 14.583	tissue	Biologiezentrum Linz	10,26
2014582	UAUT_E	*E. roumanicus*	Upper Austria, Enns	48.217, 14.433	tissue	Biologiezentrum Linz	10,26
2014821	UAUT_E	*E. europaeus*	Upper Austria, Neuhofen a. d. Krems	48.117, 14.200	tissue	Biologiezentrum Linz	10,26
2014823	UAUT_E	*E. europaeus*	Upper Austria, Engerwitzdorf	48.333, 14.417	tissue	Biologiezentrum Linz	5,13
2014837	UAUT_E	*E. roumanicus*	Upper Austria, Pyburg	48.217, 14.517	tissue	Biologiezentrum Linz	7,69
2015372	UAUT_E	potential Hybrid	Upper Austria, Pregarten	48.350, 14.517	tissue	Biologiezentrum Linz	0,00
2016168	UAUT_E	*E. roumanicus*	Upper Austria, Mitterkirchen	48.183, 14.683	tissue	Biologiezentrum Linz	2,56
2016170	UAUT_E	*E. europaeus*	Upper Austria, Hainbach	48.117, 14.333	tissue	Biologiezentrum Linz	0,00
2016171	UAUT_E	*E. europaeus*	Upper Austria, Wartberg o. d. Aist	48.350, 14.483	tissue	Biologiezentrum Linz	0,00

2016172	UAUT_E	*E. europaeus*	Upper Austria, Engerwitzdorf	48.333, 14.417	tissue	Biologiezentrum Linz	0,00
2005-605-2	UAUT_E	*E. roumanicus*	Upper Austria, Nöstlbach	48.167, 14.250	tissue	Biologiezentrum Linz	28,21
2005-616-2	UAUT_E	*E. europaeus*	Upper Austria, Steyr	48.033, 14.400	tissue	Biologiezentrum Linz	5,13
2006-92-2	UAUT_E	*E. europaeus*	Upper Austria, Schwertberg	48.250, 14.583	tissue	Biologiezentrum Linz	10,26
2014-424-2	UAUT_E	*E. roumanicus*	Upper Austria, Markt St. Florian	48.217, 14.383	tissue	Biologiezentrum Linz	5,13
2017Gutau	UAUT_E	*E. europaeus*	Upper Austria, Gutau	48.418, 14.624	tissue	Biologiezentrum Linz	35,90
2014432	LAUT	*E. europaeus*	Lower Austria, Scheibbs	48.000, 15.150	tissue	Biologiezentrum Linz	20,51
2014445	LAUT	*E. europaeus*	Lower Austria, Haindorf	48.117, 14.583	tissue	Biologiezentrum Linz	5,13
200689	CZE	*E. europaeus*	Czech Republic, Ceske Budejovice	48.972, 14.473	tissue	Biologiezentrum Linz	5,13
2006603	CZE	*E. europaeus*	Czech Republic, Kaplice	48.733, 14.483	tissue	Biologiezentrum Linz	2,56
2008188	CZE	*E. europaeus*	Czech Republic, Kajov	48.815, 14.284	tissue	Biologiezentrum Linz	0,00
20111185	CZE	*E. europaeus*	Czech Republic, Velesin	48.846, 14.456	tissue	Biologiezentrum Linz	12,82
20111186	CZE	*E. europaeus*	Czech Republic, Netrebice	48.786, 14.456	tissue	Biologiezentrum Linz	7,69
2005168	POL	*E. europaeus*	Poland, Domaszkow	50.200, 16.667	tissue	Biologiezentrum Linz	0,00
2008174	SVK	*E. roumanicus*	Slovakia, Castkovce	48.683, 17.767	tissue	Biologiezentrum Linz	5,13
2008182	EAUT+HU	*E. roumanicus*	Burgenland, Mönchhof	47.867, 16.933	tissue	Biologiezentrum Linz	17,95
2012154	EAUT+HU	*E. roumanicus*	Lower Austria, Gänserndorf	48.333, 16.700	tissue	Biologiezentrum Linz	12,82
2012155	EAUT+HU	*E. roumanicus*	Lower Austria, Gänserndorf	48.333, 16.700	tissue	Biologiezentrum Linz	7,69
2013168	EAUT+HU	*E. roumanicus*	BurgenlandPamhagen	47.733, 16.917	tissue	Biologiezentrum Linz	15,38
2014417	EAUT+HU	potential Hybrid	Hungary, Kóphaza	47.617, 16.617	tissue	Biologiezentrum Linz	20,51
2014421	EAUT+HU	*E. roumanicus*	Burgenland, Illmitz	47.750, 16.800	tissue	Biologiezentrum Linz	7,69
2014422	EAUT+HU	*E. roumanicus*	Burgenland, Illmitz	47.750, 16.800	tissue	Biologiezentrum Linz	17,95
2014423	EAUT+HU	*E. roumanicus*	Burgenland, Apetlon	47.733, 16.833	tissue	Biologiezentrum Linz	35,90
2014583	EAUT+HU	*E. europaeus*	Burgenland, Pamhagen	47.700, 16.883	tissue	Biologiezentrum Linz	43,59
2015109	EAUT+HU	*E. roumanicus*	Burgenland, Güssing	47.050, 16.317	tissue	Biologiezentrum Linz	15,38
04NHMro	EAUT+HU	*E. roumanicus*	Lower Austria, Haringsee	NA	tissue	Natural History Museum Vienna	2,56
KLF-56-1	CAR	*E. roumanicus*	Carinthia, Klagenfurt	NA	saliva	shelter Carinthia	35,90
KLF-57-1	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	20,51
KLF-58-1	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	25,64
KLF59	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	41,03
KLF60	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	35,90
KLF-61-1	CAR	*E. roumanicus*	Carinthia, Krumpendorf	NA	saliva	shelter Carinthia	30,77
KLF-62-1	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	35,90
KLF-63-1	CAR	*E. roumanicus*	Carinthia, Arnoldstein	NA	saliva	shelter Carinthia	33,33
KLF65	CAR	*E. roumanicus*	Carinthia, Spittal	NA	saliva	shelter Carinthia	43,59
KLF66	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	17,95
KLF67	CAR	*E. roumanicus*	Carinthia, Bleiburg	NA	saliva	shelter Carinthia	28,21
KLF68	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	43,59
KLF69	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	41,03
KLF70	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	30,77
KLF-71-1	CAR	*E. roumanicus*	Carinthia, Klagenfurt	NA	saliva	shelter Carinthia	28,21
KLF72	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	38,46

KLF73	CAR	*E. roumanicus*	Carinthia, Mittlern	NA	saliva	shelter Carinthia	30,77
KLF74	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	43,59
KLF75	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	46,15
KLF76	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	38,46
KLF77	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	35,90
KLF78	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	43,59
KLF-79-1	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	30,77
KLF80	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	33,33
KLF81	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	41,03
KLF82	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	35,90
KLF83	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	15,38
KLF84	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	30,77
KLF85	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	46,15
KLF86	CAR	*E. roumanicus*	Carinthia, Ferlach	NA	saliva	shelter Carinthia	35,90
2008176	SLO	*E. roumanicus*	Slovenia, Podvinci	46.417, 15.917	tissue	Biologiezentrum Linz	5,13
43INF-1	SLO	*E. roumanicus*	Slovenia	NA	NA	INF	15,38
2014420	CRO	*E. roumanicus*	Croatia, Zminj	45.133, 13.883	tissue	Biologiezentrum Linz	5,13
2824	GRE/NM	*E. roumanicus*	Greece, Kedriki Makedhonía	41.237, 23.086	DNA	CIBIO	7,69
2825	GRE/NM	*E. roumanicus*	Greece, Kedriki Makedhonía	41.258, 23.095	DNA	CIBIO	7,69
38INF-1	GRE/NM	*E. roumanicus*	Greece, Karpeta, Pelepones	37.856, 21.648	tissue	INF	15,38
201463	GRE/NM	*E. roumanicus*	North Macedonia, Veles	41.733, 21.75	tissue	Biologiezentrum Linz	23,08

Tab. S3. Samples excluded from population genetic and phylogeographical analysis of *E. europaeus* and *E. roumanicus* within Europe. Reasons for sample exclusion in given order: too much missing data (>50%) (block 1); no information on coordinates and sampling region (block 2); other than investigated species (*E. concolor*) (block 3). Given are the sample name, the artificially designed population a sample has been assigned to* (none, if sample location is unknown or species is not investigated), the assigned species (*E. europaeus*, *E. roumanicus* or *Erinaceus sp.*, if unknown) based on morphological assessment and previous genetic analysis, sampling region (NA = not available), sampling coordinates (in decimal Lat/Long; NA = not available,), sampled material (tissue, saliva or DNA isolate/extract), material origin (institutions or shelters that provided samples)*, percentage of missing data for each sample after initial sequence analysis [%]. [*Abbreviations: IBP = Iberian Peninsula, BER = Berlin, UAUT = Upper Austria, LAUT = Lower Austria, CZE = Czech Republic, SVK = Slovakia, EAUT + HU = Eastern Austria and Hungary, RO = Romania, BH = Bosnia-Herzegovina, GRE/NM = Greece and North Macedonia; CIBIO = Research Centre in Biodiversity and Genetic Resources; IWZ = Leibniz Institute for Zoo and Wildlife Research; INF = Institute for Integrative Nature Conservation Research; TUM = Technical University of Munich; CZU = Czech University of Life Sciences Prague; shelter (MF) = shelter sampled by Marilene Fuhrmann; shelter (LF) = shelter sampled by Lea Ficker]

sample name	assigned population	species	sample region	Coordinates	Sampled material	Material origin	missing data per sample [%]
781	IBP	*E. europaeus*	Spain, Castilla-León	42.502, -5.598	tissue	CIBIO	92,31
1017	IBP	*E. europaeus*	Spain, Andaluzia	37.368, -2.754	spike	CIBIO	100,00
36516single	BER	*E. europaeus*	Berlin	NA	tissue	IWZ	66,67
200694	UAUT	*E. europaeus*	Upper Austria, Steyregg	48.16, 14.22	tissue	Biologiezentrum Linz	84,62
2014682	UAUT	*E. europaeus*	Upper Austria, Wilhering, Ufer	48.19, 14.10	tissue	Biologiezentrum Linz	97,44
12INF	UAUT	*E. europaeus*	Upper Austria, Wolfshütte	48.055, 13.669	tissue, spines, hair	INF	64,10
2008218	UAUT	*E. europaeus*	Upper Austria, Wartberg o.d. Aist, Scheiben	48.21, 14.29	tissue	Biologiezentrum Linz	76,92
41INF-1	UAUT	*E. europaeus*	Upper Austria, Ottnang a. Hausruck	NA	tissue	INF	92,31
09INF	LAUT	*Erinaceus sp.*	Lower Austria, Theiß	NA	tissue, spines	INF	66,67
2008217	CZE	*E. europaeus*	Czech Republic, Skoronice	48.42, 14.28	tissue	Biologiezentrum Linz	56,41
2008173	SVK	*E. roumanicus*	Slovakia, Branc	48.12, 18.08	tissue	Biologiezentrum Linz	64,10
2008-175-2	SVK	*E. roumanicus*	Slovakia, Jablonov nad Turnou	48.41, 20.41	tissue	Biologiezentrum Linz	100,00
07INF	EAUT+HU	*Erinaceus sp.*	Lower Austria, Seibersdorf	47.958, 16.527	tissue, spines, hair	INF	66,67
08INF	EAUT+HU	*Erinaceus sp.*	Lower Austria, Mannersdorf	47.976, 16.605	tissue, spines, hair	INF	66,67
40INF-1	EAUT+HU	*E. roumanicus*	Burgenland, Heiligenbrunn	47.059, 16.359	tissue	INF	51,28
10INFsingle	RO	*E. roumanicus*	Romania, Transsylvania, Craciunelu de Jos	46.175, 23.817	tissue	INF	64,10
201464	BH	*E. roumanicus*	Bosnia-Herzegovina, Republika Srbska, Vrabaska	45.06, 17.10	tissue	Biologiezentrum Linz	94,87
37INF-1	GRE/NM	*E. roumanicus*	Greece, Pelepones, Lakonia, Skala	36.813, 22.636	tissue	INF	100,00
i10-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00

i11-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i13-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	97,44
i14-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i15-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i18-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i21-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	64,10
i22-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i27-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	79,49
i39-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	94,87
i41-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i52-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i54-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	79,49
i55-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i7-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i8-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
i8-2	none	*Erinaceus sp.*	NA	NA	DNA	TUM	100,00
18	none	*Erinaceus sp.*	NA	NA	DNA	CZU	100,00
20	none	*Erinaceus sp.*	NA	NA	DNA	CZU	51,28
30	none	*Erinaceus sp.*	NA	NA	DNA	CZU	71,79
48	none	*Erinaceus sp.*	NA	NA	DNA	CZU	64,10
53	none	*Erinaceus sp.*	NA	NA	DNA	CZU	94,87
56	none	*Erinaceus sp.*	NA	NA	DNA	CZU	74,36
65	none	*Erinaceus sp.*	NA	NA	DNA	CZU	100,00
75	none	*Erinaceus sp.*	NA	NA	DNA	CZU	69,23
76	none	*Erinaceus sp.*	NA	NA	DNA	CZU	92,31
80	none	*Erinaceus sp.*	NA	NA	DNA	CZU	92,31
82	none	*Erinaceus sp.*	NA	NA	DNA	CZU	87,18
99	none	*Erinaceus sp.*	NA	NA	DNA	CZU	89,74
108	none	*Erinaceus sp.*	NA	NA	DNA	CZU	69,23
52B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	92,31
6B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	64,10

81B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	84,62
MF-17	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	64,10
i1-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	17,95
i1-2	none	*Erinaceus sp.*	NA	NA	DNA	TUM	51,28
i12-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	5,13
i16-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	20,51
i17-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	38,46
i19-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	5,13
i20-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	0,00
i2-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	5,13
i33-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	2,56
i34-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	23,08
i35-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	0,00
i36-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	5,13
i37-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	7,69
i38-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	7,69
i40-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	12,82
i42-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	33,33
i43-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	10,26
i44-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	5,13
i45-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	12,82
i46-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	2,56
i47-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	0,00
i51-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	0,00
i53-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	0,00
i9-1	none	*Erinaceus sp.*	NA	NA	DNA	TUM	23,08
2	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
8	none	*Erinaceus sp.*	NA	NA	DNA	CZU	5,13
10	none	*Erinaceus sp.*	NA	NA	DNA	CZU	10,26
12	none	*Erinaceus sp.*	NA	NA	DNA	CZU	2,56
13	none	*Erinaceus sp.*	NA	NA	DNA	CZU	2,56

14	none	*Erinaceus sp.*	NA	NA	DNA	CZU	7,69
16	none	*Erinaceus sp.*	NA	NA	DNA	CZU	33,33
17	none	*Erinaceus sp.*	NA	NA	DNA	CZU	46,15
19	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
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27	none	*Erinaceus sp.*	NA	NA	DNA	CZU	7,69
28	none	*Erinaceus sp.*	NA	NA	DNA	CZU	2,56
29	none	*Erinaceus sp.*	NA	NA	DNA	CZU	5,13
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41	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
42	none	*Erinaceus sp.*	NA	NA	DNA	CZU	35,90
43	none	*Erinaceus sp.*	NA	NA	DNA	CZU	7,69
44	none	*Erinaceus sp.*	NA	NA	DNA	CZU	12,82
45	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
47	none	*Erinaceus sp.*	NA	NA	DNA	CZU	7,69
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23A	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
25A	none	*Erinaceus sp.*	NA	NA	DNA	CZU	5,13
33B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	5,13
35B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	20,51
37A	none	*Erinaceus sp.*	NA	NA	DNA	CZU	2,56
3B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	2,56
4B	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
55A	none	*Erinaceus sp.*	NA	NA	DNA	CZU	0,00
7A	none	*Erinaceus sp.*	NA	NA	DNA	CZU	5,13
BM-1	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	5,13
BM-10	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	5,13
BM-11	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
BM-12	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	10,26
BM-13	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	2,56
BM-14	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	2,56
BM-15-1	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
BM-15-2	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	10,26
BM-17	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
BM-18	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	2,56
BM-19	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	30,77
BM-2	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	0,00
BM-20	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
BM-3	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	0,00
BM-4	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	7,69
BM-5	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	0,00
BM-6	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	2,56
BM-7	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82

BM-8	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
BM-9	none	*Erinaceus sp.*	NA	NA	saliva	shelter (LF)	12,82
MF-1	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	35,90
MF-10	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	46,15
MF-11	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	35,90
MF-12	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	30,77
MF-13	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	30,77
MF-14	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	38,46
MF-15	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	41,03
MF-16	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	46,15
MF-18	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	30,77
MF-19	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	41,03
MF-2	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	41,03
MF-20	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	41,03
MF-3	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	7,69
MF-4	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	23,08
MF-5	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	12,82
MF-6	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	23,08
MF-7	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	35,90
MF-8	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	25,64
MF-9	none	*Erinaceus sp.*	NA	NA	saliva	shelter (MF)	25,64
1468	none	*E. concolor*	Turkey, Firat	38.211, 35.865	DNA	CIBIO	5,13
2823	none	*E. concolor*	Armenia, Caucaso	39.000, 46.500	DNA	CIBIO	12,82

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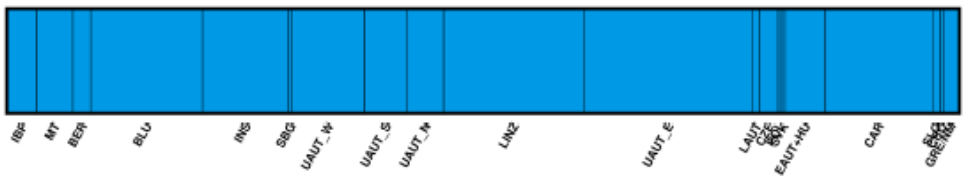

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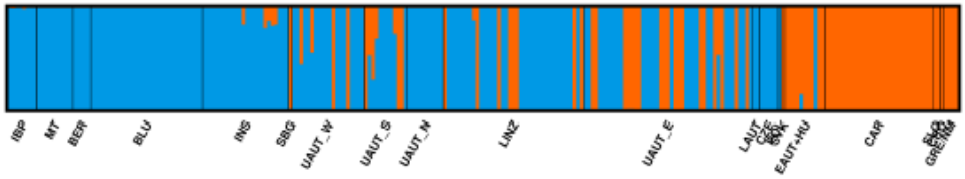

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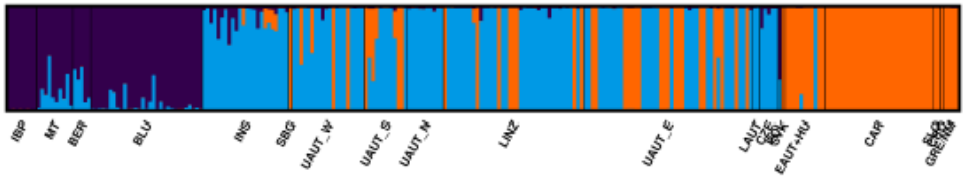

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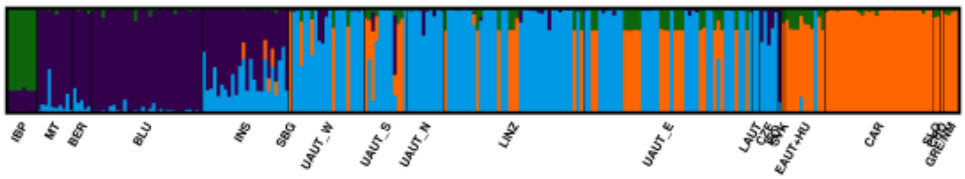

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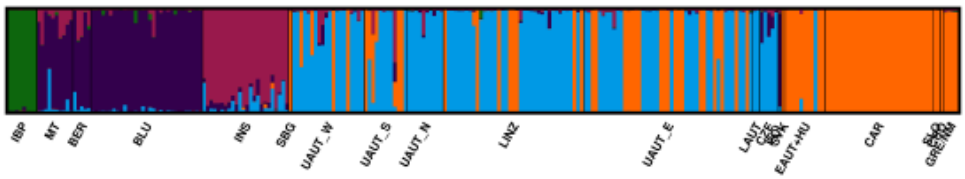

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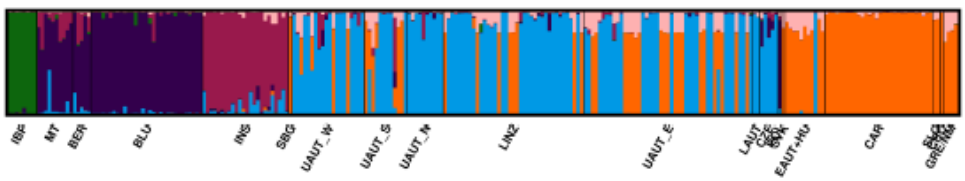

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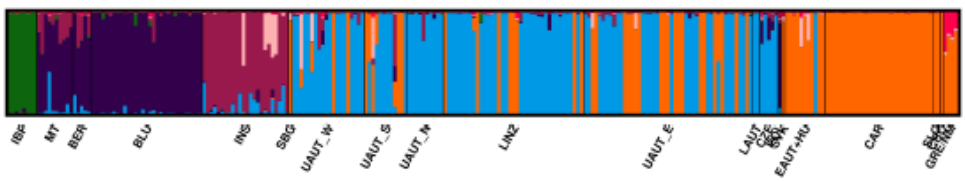

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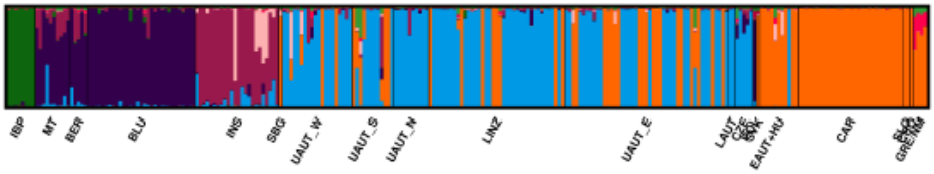

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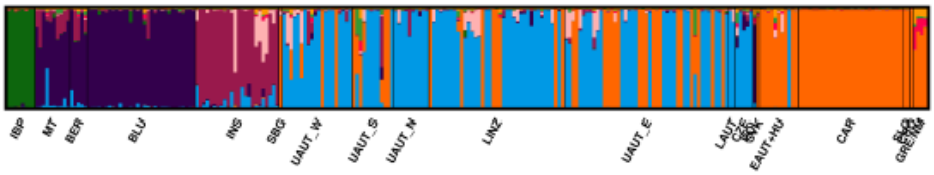

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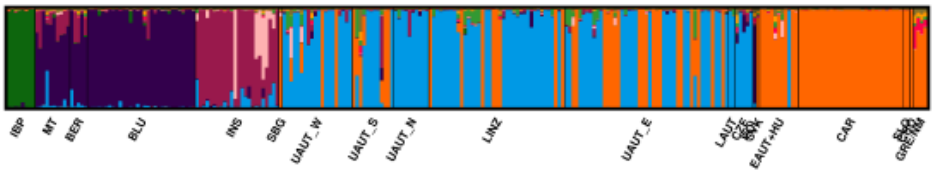

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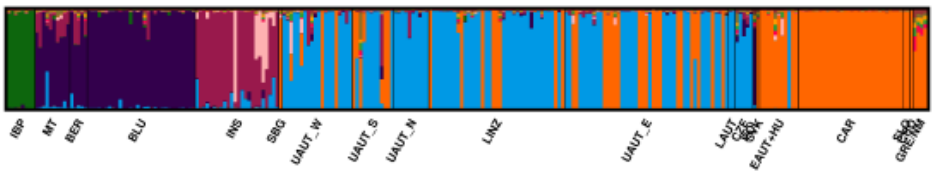
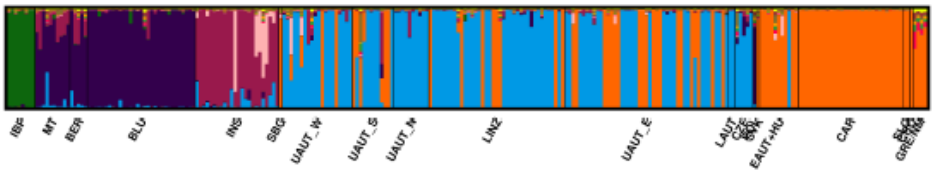
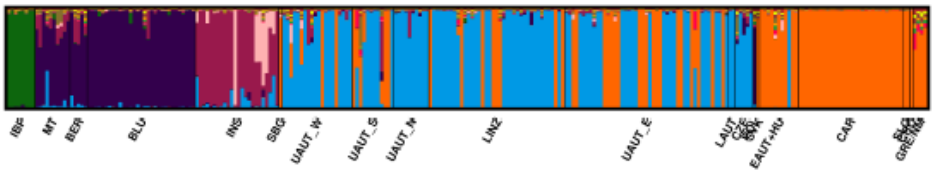


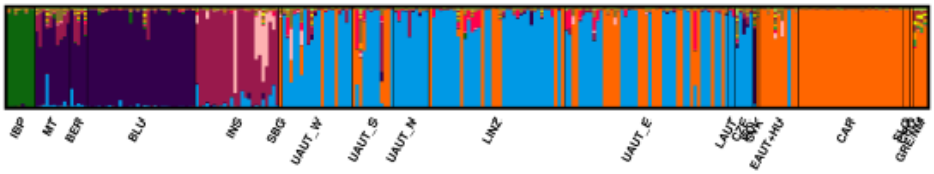
K=12



K=13



K=14



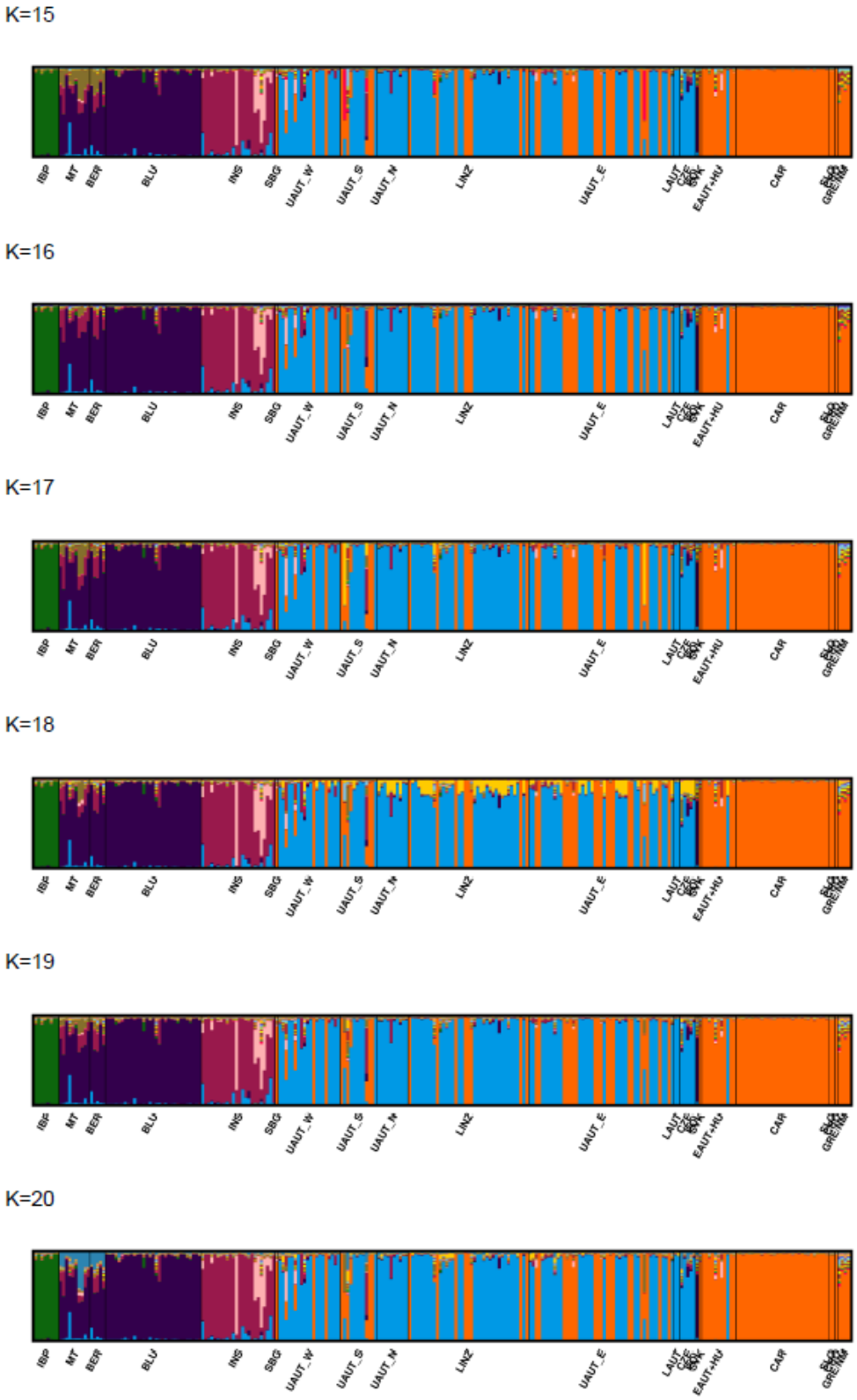
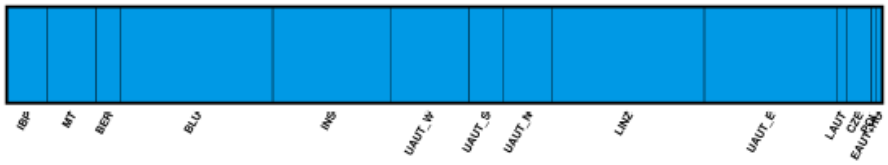
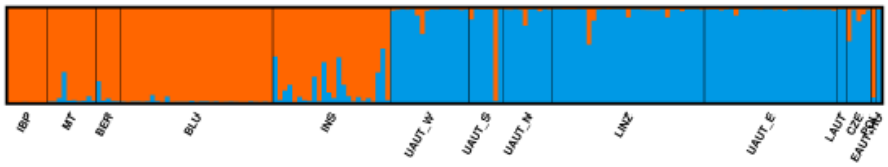


Fig. S1. Single summary output of K = 1 -20 from STRUCTURE analysis of 264 West European and Northern White-breasted hedgehog samples. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: IBP = Iberian peninsula, MT = Igelburg Mossautal e.V., BER = Berlin, BLU = Bludenz, INS = Innsbruck, SBG = Salzburg, UAUT_W/S/N/E = Upper Austria West/South/North/East, LINZ = Linz, LAUT = Lower Austria, CZE = Czech Republic, POL = Poland, SVK = Slovakia, EAUT+HU = Eastern Austria + Hungary, CAR = Carinthia, SLO = Slovenia, CRO = Croatia, GRE/NM = Greece/North Macedonia

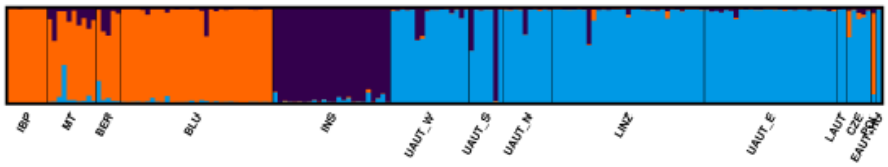
K=1



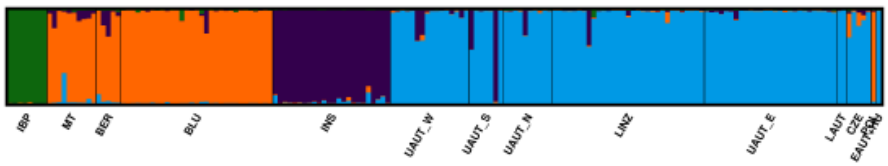
K=2



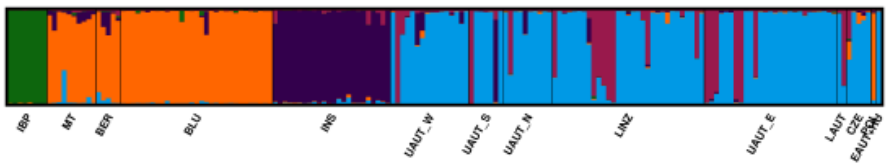
K=3



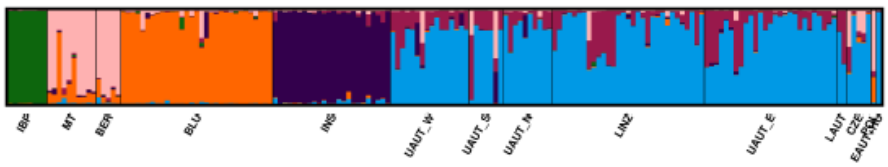
K=4



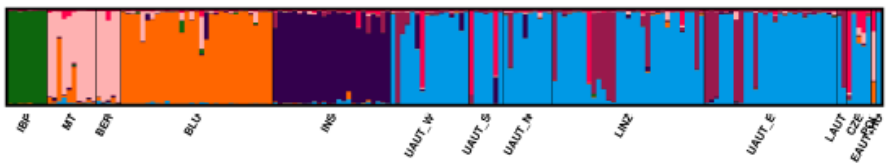
K=5



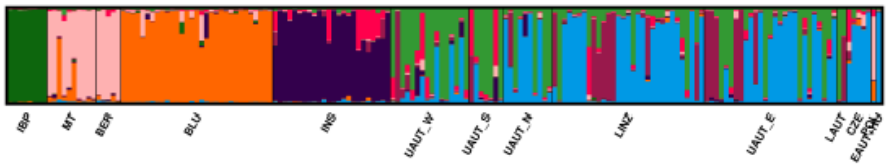
K=6



K=7



K=8



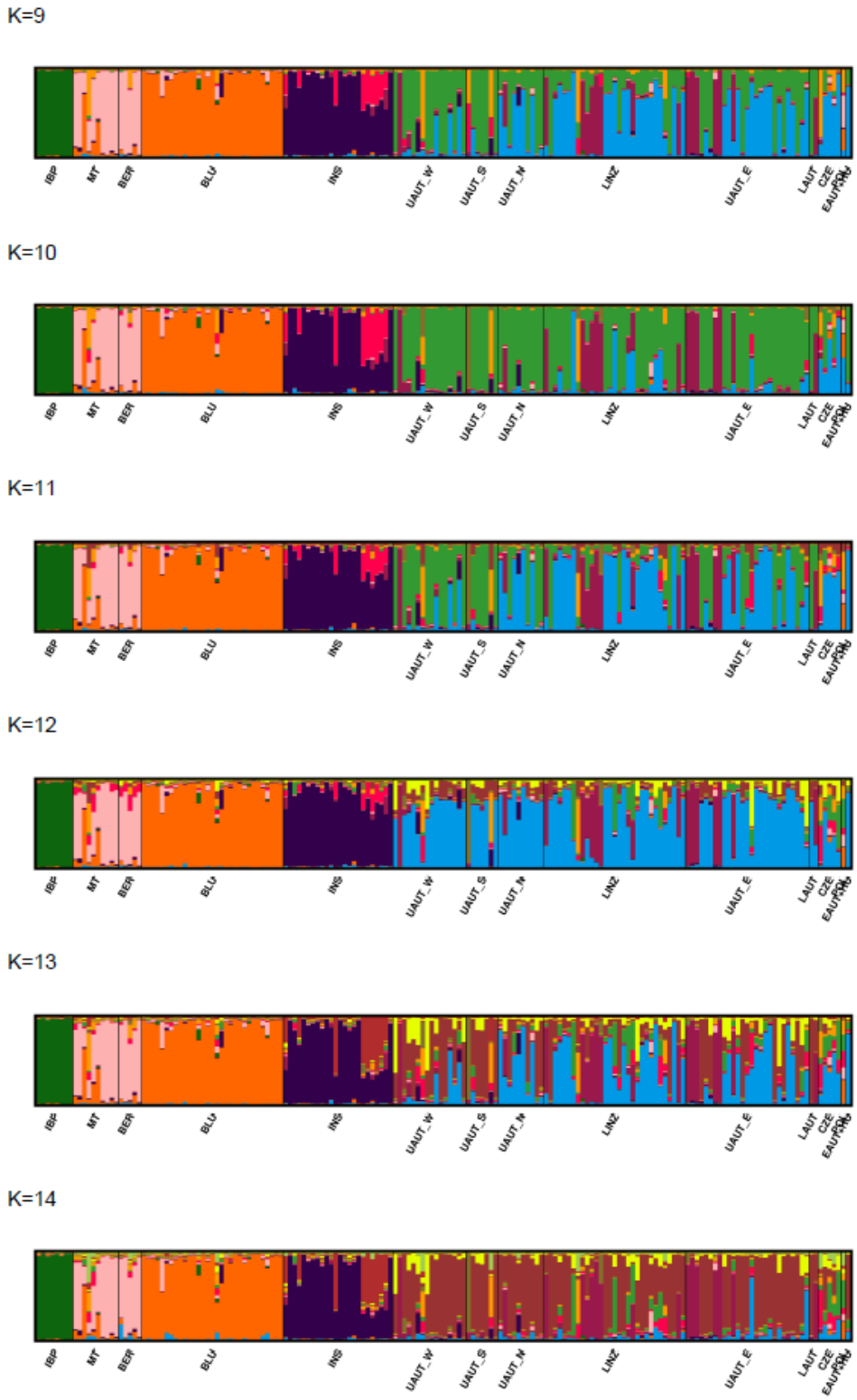
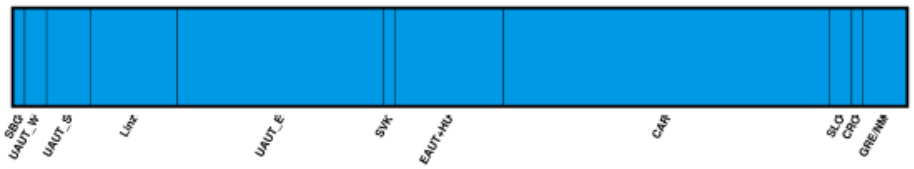
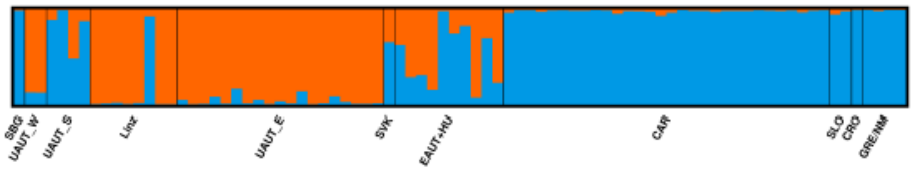


Fig. S2: Single summary output of K = 1 -14 from STRUCTURE analysis of all West European hedgehog samples. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: IBP = Iberian peninsula, MT = Igelburg Mossautal e.V., BER = Berlin, BLU = Bludenz, INS = Innsbruck, UAUT_W/S/N/E = Upper Austria West/South/North/East, LINZ = Linz, LAUT = Lower Austria, CZE = Czech Republic, POL = Poland, EAUT+HU = Eastern Austria + Hungary

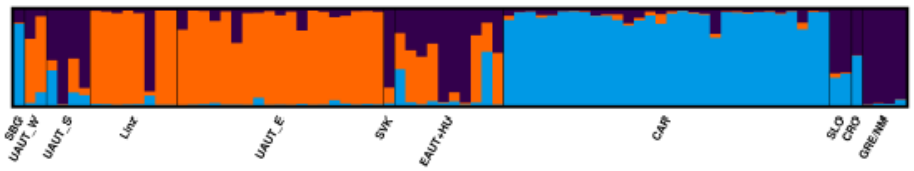
K=1



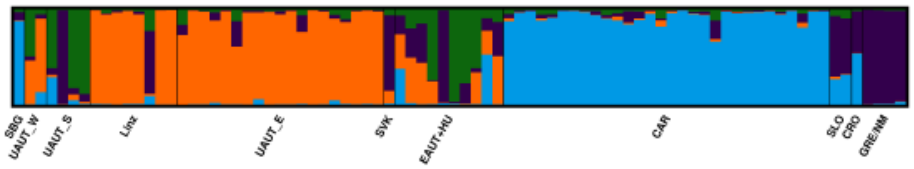
K=2



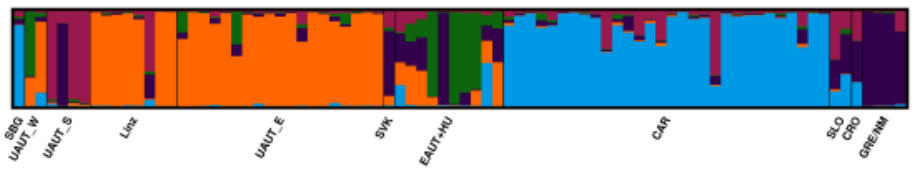
K=3



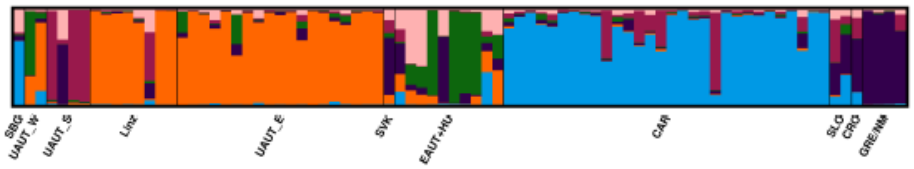
K=4



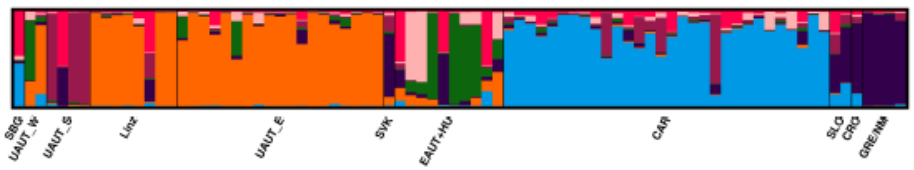
K=5



K=6



K=7



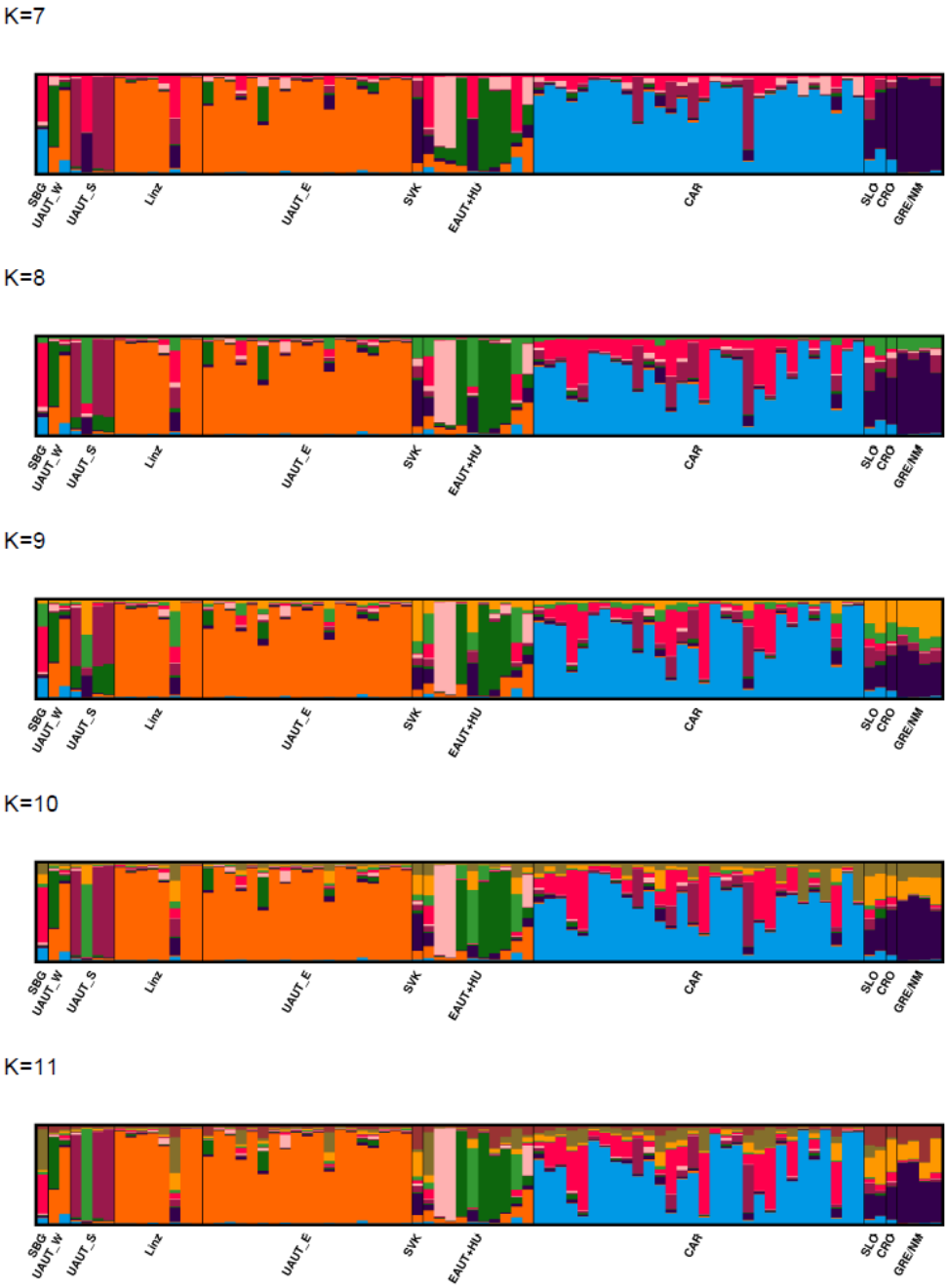


Fig. S3: Single summary output of K = 1 -11 from STRUCTURE analysis of all Northern White-breasted hedgehog samples. The web portal CLUMPAK was used to summarise all iterations of each K into one single output and graphically represent them. Population abbreviations: SBG = Salzburg, UAUT_W/S/E = Upper Austria West/South/East, LINZ = Linz, SVK = Slovakia, EAUT+HU = Eastern Austria + Hungary, CAR = Carinthia, SLO = Slovenia, CRO = Croatia, GRE/NM = Greece/North Macedonia]