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Inter-species differences within Central European hedgehogs

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Inter-species differences within Central European hedgehogs independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 27. 4. 2018

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Anna Nováková

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Abstract

Hedgehogs from *Erinaceus* genus are suitable models for research of Quaternary fluctuations and subsequent processes connected with secondary contact of the two previously diverged species. This thesis aimed to follow up the previous studies made about transect of the contact zone located in Central Europe, attempted to map the rest of the overlap in this area and to evaluate the outputs with formerly published results. We isolated DNA from 51 new samples from Germany, Poland and Austria and performed genetic analyses with the newly obtained samples and with the older datasets from previous studies using both mitochondrial control region and microsatellite markers.

The results of the mtDNA and nuclear data analyses yielded distinctive results; in *E. roumanicus*, nuclear data showed higher variability over mitochondrial data and *E. europaeus* indicated more complex structure in the analyses of mtDNA. Discordance between results of mitochondrial and nuclear data suggests complex evolutionary history of the species and indicates processes, which shaped the population in the recent past. We did not detect any presence of hybrids in the new dataset; one individual did show signs of cytonuclear incompatibility from the area of Germany on the margin of distribution of *E. roumanicus.* This area should be addressed with closer investigation in future research. Recent expansion of populations was indicated in both species. We did find differentiations in outputs of the analyses in comparison with the original research but the evaluation of the dynamics of the contact zone should require a larger amount of data, what should be assessed in further research. However, outputs of this thesis did bring a fresh insight to the population structure of this intriguing species from the view of postglacial processes connected with recolonization of Europe and to the consequences it carries along.

Key words: *Erinaceus*, inter-species interactions, population genetics, secondary contact, speciation

Contents

List of tables

List of figures

List of the abbreviations used in the thesis

Mya – million years ago

Kya – thousand years ago

LGM – Last Glacial Maximum

e.g. – exempli gratia - for example

mtDNA – mitochondrial DNA

PCR – Polymerase Chain Reaction

MCMC – Markov chain Monte Carlo

HWE – Hardy–Weinberg equilibrium

spp. – species

1. Introduction and Literature Review

1.1. Quaternary; processes determining nowadays species distribution

Quaternary, the most recent period of the Cenozoic Era, dated since 2.58 Mya, which continues until now (Berger et al. 2016), is known by many changes of climatic conditions, which influenced the distribution of many plant and animal species on Earth (Hewitt 2004).

The most significant Quaternary events, influencing the landscape, were series of ice ages, colder, drier periods with extensive ice cover (glacials), cyclically appearing, interfered by warmer interglacial periods (Berger et al. 2016). Time duration of the ice age cycles during the Late Quaternary was between 80,000 and 120,000 years, reoccurring in 100,000-year periods (Denton et al. 2010). According to the geological records, during the last 160,000 years, at least four major glaciations affected Eurasia (Svendsen et el. 2004). Last glacial maximum (LGM) was a last period, dated 24 – 18 thousand years ago, during which the ice cover reached its maximum (Hughes & Gibbard 2015). Many analyses based on pollen records, ice cores or marine sediments have been performed revealing important data about climate changes and vegetation during last glacial period (Fletcher et al. 2010).

In the Northern Hemisphere, large masses of the ice sheet covered north parts of the continents. For example, in North America, Laurentide Ice Mass, one of the broadest ice masses, covered area of Canada and reached to the North of America (Dyke et al. 2002). In Europe, the ice mass covered northern part of the continent, completely covered Scandinavia and Baltics and reached to the northern edge of the Poland and Germany (Svendsen et al. 2004).

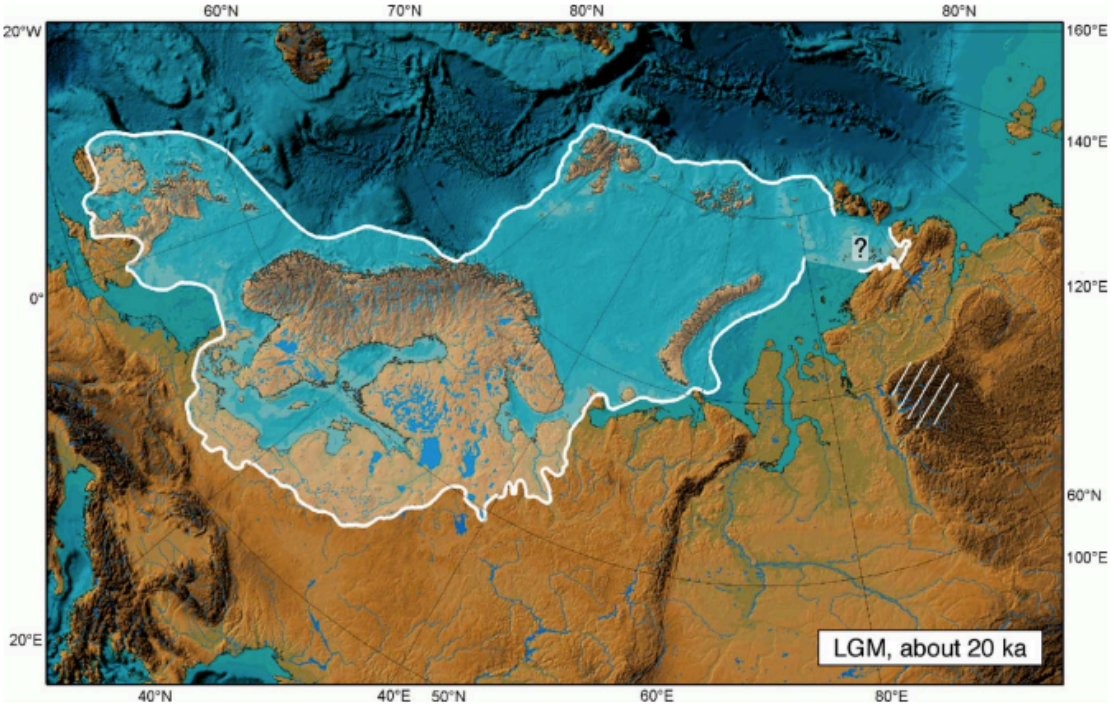

Fig. 1. Extent of the ice sheet in Europe during LGM (Svendsen et al. 2004).

However Hughes et al. (2013) state that timing and the extent of the glacials does not always correspond to the generally presented LGM timescale, based on many other works focusing on glaciation thorough the world. For example, Karents-Bara ice sheet, covering Russia had its peak at 90-80 kya, based on several analyses (Svedsen et al. 2004). Changes of the climate during the Quaternary influenced not only the temperate parts of the continents, but had an impact on the tropical areas as well. Decrease of the precipitation and thus, vegetation diversity, caused fragmentation of the tropical forests in Amazonia and reduction of the total area of the forest, which was even smaller than today. Decrease of humidity also led to the occurrence of more dry and open forests in Congo basin in Africa (Anhuf 2006). In Europe, the maximal glaciation during the LGM was around 21 kya, although the timescale information vary quite a lot and maximal extent slightly differed in different parts of the continent. The climate was at its coldest, the sea level was considerably lower, approximately 120m in comparison to todays level (Denton et al. 2010).

1.2. Refugia

Last 700,000 years were dominated mostly by ice-covered land in the glacial periods. Approximately 100,000 final year cycle was accompanied by relatively short periods of interglacials like today (Hewitt 1996). Climatic fluctuations during Quaternary influenced populations of many animal and plant species. These climate changes were a reason for many species to react to these changes, leading to extinction or subsequently to the shift of their range and distribution. The extension of the ice sheets caused many European species to seek refugia, which were found at the south of the continent (Hewitt 2011). Dynamics of range and distribution influenced, besides other factors, the genetic variability of the species (Santucci et al. 1998) and became a driver to the speciation and species diversity of the European biota (Hewitt 1999).

There are many data options according to which we can estimate the distribution of the species in the past, such as bones, skeletons, pollen grains or other remains in places like bottoms of the lakes, sea bed or other special sites (Hewitt 2011). For various animal or plant species, there are evidences of the range and distribution changes, mainly in pre-, during and postglacial periods, particularly in the last ice age (Sommer & Zachos 2009). Data records show that not all the species acted equally, and the responses to the temperature and climate oscillations were unique during the interglacials and current habitats of species are not necessarily exact in range or density as it was in the previous periods of time. Many various researches about processes connected to the LGM are important in describing and localization of the refugia through variable climatic conditions (Tzedakis 2003; Hewitt 2011).

Taberlet et al. (1998) identified three locations of the Mediterranean as the three main refugia of the European fauna and flora during the LGM (Fig. 2). These three areas, Iberian Peninsula, Italian Peninsula and Balkans respectively, are places, where many of the temperate species survived the cold conditions and also locations of the following south-to-north expansion (Santucci et al. 1998). However, later researches revealed that there were more additional places, where species were able to survive the unfavourable conditions during last glacial.

Sommer and Nadachowski (2006) state, that only fossil remains of the temperate species could be a reliable source for determining the glacial refugia during the maximal glaciation of LGM. They also mention the fact that these refugia are presented as models but without appropriate geographical data references. They also propose, based on the fossil records of several species from temperate Europe, existence of more refugia, where species could survive – areas of southern France and Carpathian region (Sommer & Zachos 2009). They present it on the fossils of the red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Studies of postglacial colonization of another species suggesting existence of more refugia than those located in Mediterranean exist, such as studies on bank voles and evidences of their presence in Central Europe, Ural or Carpathian regions (Deffontaine et al. 2005), several mustelids in Carpathians (Sommer & Benecke 2004) or bats from genus *Pipistrellus* in eastern and western Europe (Boston et al. 2014).

In case of plant species, there have been implications that cryptic refugia might be present further north of the classical southern refugia. Plant macrofossils, pollen records and genetic data of several tree species have been presented as evidences of existence of such refugia (Tzedakis et al. 2013). However, existence of various refugia during the LGM is complex subject and needs more research and detailed investigations.

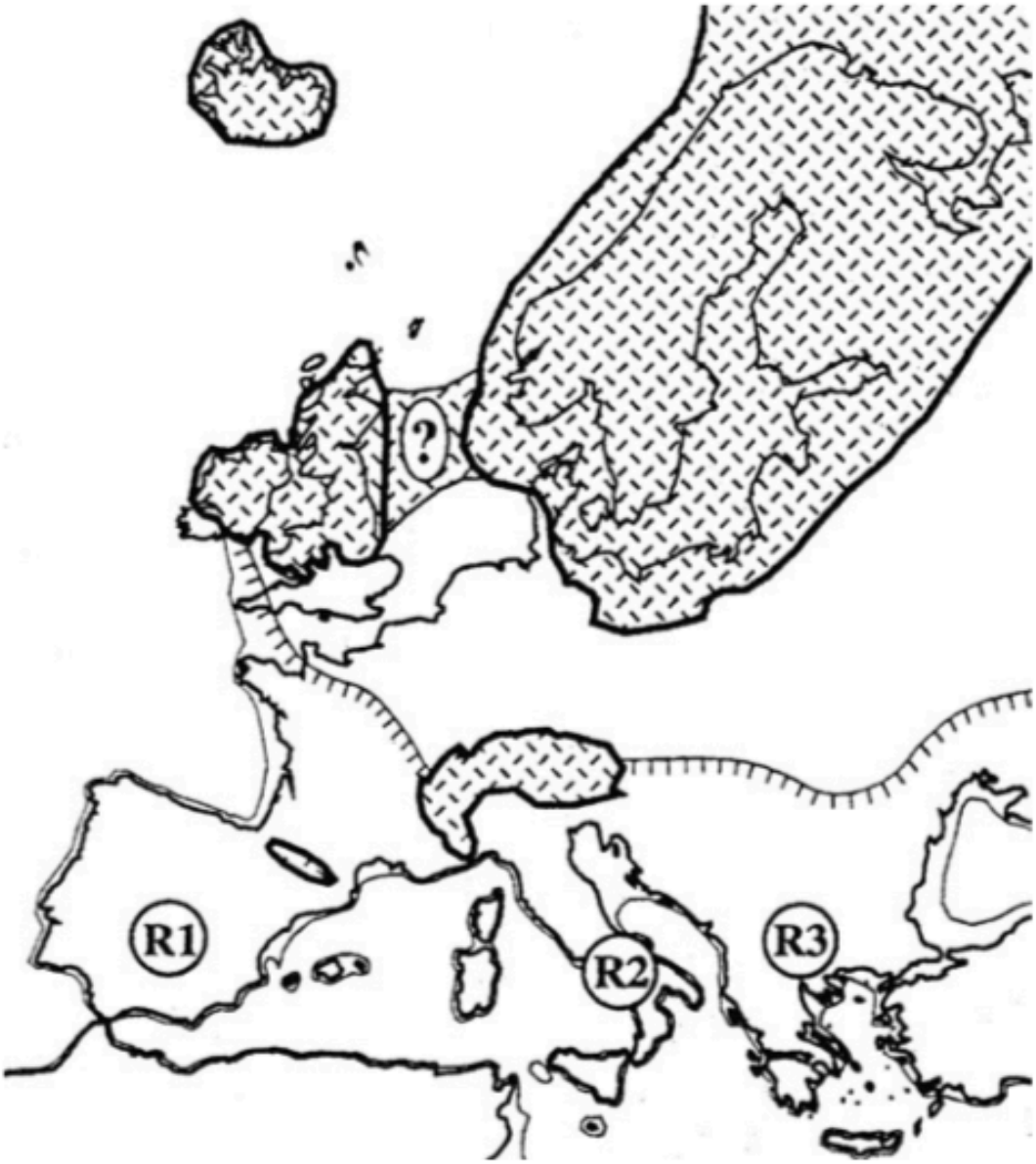

Fig. 2. Illustration of the dominant southern refugia (named R1-R3) of temperate European species (Taberlet et al. 1998).

1.2.1. Colonization routes from refugia

Colonization routes after the last glacial maximum and the following warming can tell the routes which the species underwent on their way to recolonize Europe, but also processes connected to it, as forming the secondary contacts and hybrid zones (Hewitt 2011). Sometimes the species, which recolonized the Europe, came from lineages from different refugia. It resulted in occurrence of fairly mixed biota (Hewitt 2004). Probable locations of refugia, along with the evidences of the specific genotypes found at those places, allow us to tell the supposed routes to current distribution from those refugia. Most of the evidences of it were found in Europe and North America but some examples of the colonization routes can be found in fact on every continent (Hewitt 2011). This thesis is focused on processes and their consequences solely in Europe.

With the ice sheets retreating after the end of the LGM, spreading of the species northwards began. Among species, which colonized Central Europe, were several forest plant species such as oak, pine, beech or alder (Hewitt 1999). Spreading of the forest plants allowed the expansion of other species connected to the specific environment, such as beetles and other animal species, which followed the expansion of the forest.

Great influence of the Quaternary climate on most of the European flora and fauna has already been discussed. Hewitt (1999) states three species paradigm patterns as examples for the colonization routes from refugia after the LGM, following the secondary contacts. He uses examples for grasshopper, hedgehog and bear. The colonization route of *Erinaceus* spp. is described in Chapter 1.4 below in more detail, with three main routes from Apennine, Italian and Balkan refugia.

Grasshoppers from *Chorthippus parallelus*, another species, often used as subject of the postglacial recolonization processes, on the other hand, with several subspecies, shows strong colonization route pattern originating in Balkans, with two well described contact zones – between French and Spanish subspecies and Italian/Austrian ones.

The third example pattern is demonstrated on brown bear (*Ursus arctos)*, whose distribution is largely influenced by human activities but DNA analyses discovered two distinct lineages, which colonized Europe – eastern, with its origin in Iberia and western, from Caucasus/Carpathian area. Both lineages formed a secondary contact zone in the Scandinavia (Sweden).

Interestingly, we can find these patterns in very similar way in other animal and plant species, for example oaks from the genus *Quercus* as parallel for *Erinaceus* spp. routes, *Alnus glutinosa* for grasshoppers or shrews from the genus *Sorex* for bears (Hewitt 1999).

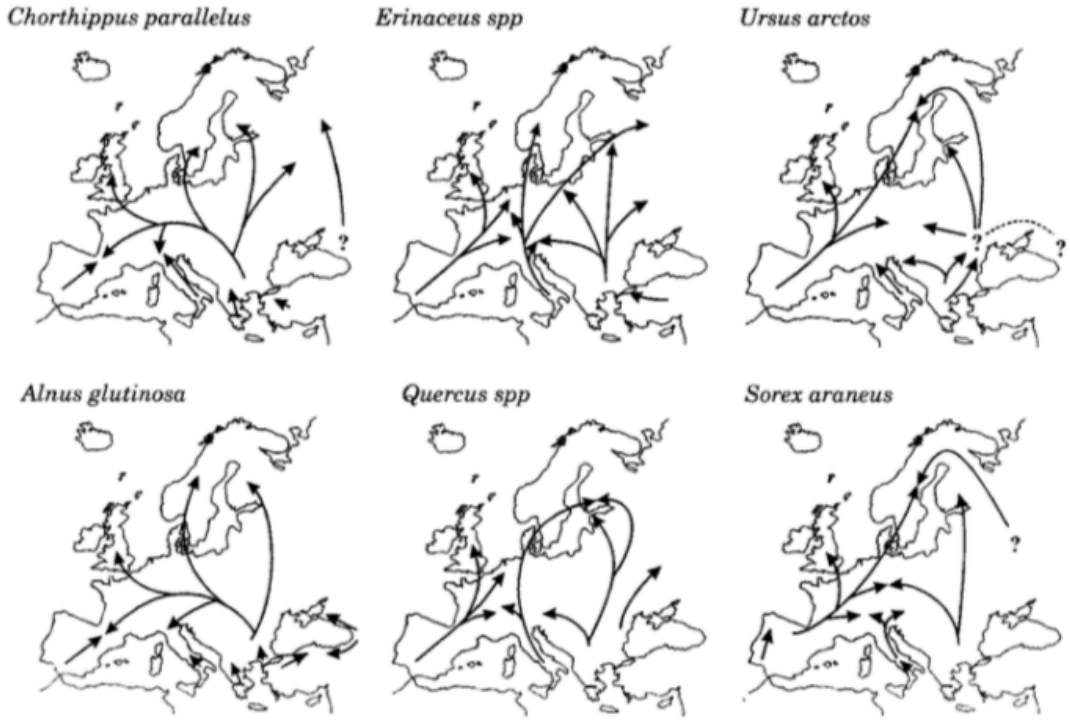

Fig. 3. Three patterns of postglacial expansion routes from southern refugia with three example species (above) and species which show similar pattern (below) (Hewitt 1999).

1.3. Contact zones

Expansion towards north from different refugia could result in the formation of secondary contact zones where previously separated populations came into contact again (Hewitt 1999).

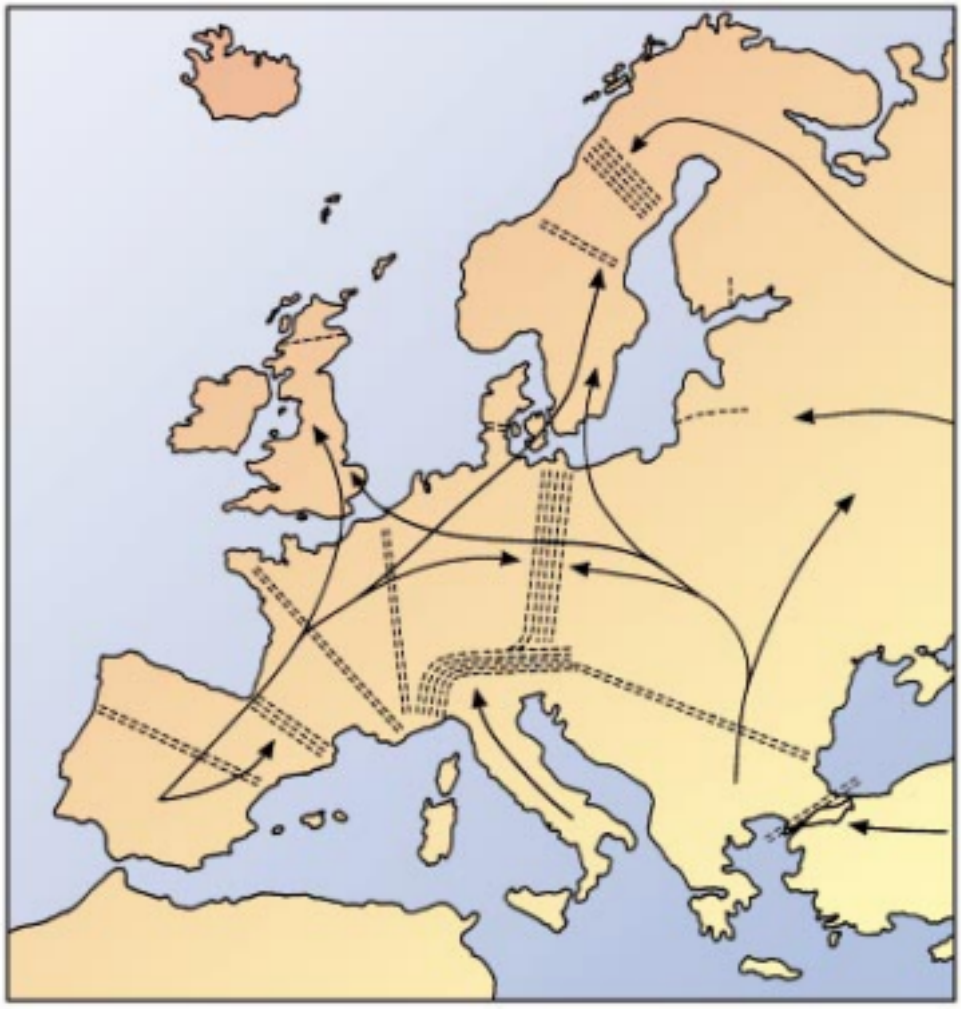

Fig. 4. Routes of recolonization of Europe from refugia (arrows) and several positions of known contact zones (double dashed lines) created by the secondary contacts (Hewitt 2000).

Depending on the form of speciation and the reproductive isolation mechanisms of the species, hybridization might or might not occur.

When the reproductive isolation is incomplete and two previously diverged species meet, hybridization might take place. Generally, mechanisms that prevent reproduction could be divided into two principal categories – prezygotic and postzygotic, which means if the barriers do act before or after fertilization (Santini et al. 2012). Prezygotic barriers involve mechanical or geographical barriers, which prevent interspecies crossing to even happen, postzygotic mechanisms are those, such as sterility of the hybrids or reduced viability.

Barriers preventing contact and therefore gene flow might be overcome through time and if the reproductive barriers are incomplete, it rises an opportunity for the gene flow to occur once more (Abbot 2013). In areas, where two parapatric species ranges overlap, contact zones are formed, where species meet, and also possibly mate and hybridize (Hewitt 1996).

Whereas in plants, hybridization is accepted as usually common instrument, in animals it is still often seen as unusual (Hewitt 2001; Mallet 2005) and for a long time, process of hybridization was considered to have no major influence on speciation (Capblanq et al. 2015). In the last years, many papers indicate that hybridization, mainly through introgression, can indeed promote the speciation (Mallet 2005) and be a contributor to the reproductive isolation (The Marie Curie SPECIATION Network 2012). Mack and Nachman (2017) suggest that comparison of the hybrids with mixed genetic background in laboratory conditions should be made with those occurring in the nature, when it comes to gene expression.

Most of the hybrid zones have been recorded in temperate parts of Europe (and North America). Studying the hybrid zones provides a better understanding of the history and evolution of the species lineages (Hewitt 2011). One of the most interesting areas for studying the zones of contact is Central Europe. It serves as a place where many secondary contacts are created as the result of the postglacial recolonization of the continent and for many animal and plant species, these zones are important in investigating processes connected to such previous events.

For example, Carrion crow (*Corvus corone*) and hooded crow (*Corvus cornix*), two closely related species, previously referred as subspecies, whose taxonomy is still unclear, created two contact zones. One contact zone is located on the British Isles and second goes through the middle of the Europe, from northern part of Germany to southern border between France and Italy where both species meet and occasionally hybridize (Poelstra et al. 2014). By genetic analyses it was found that despite clear phenotype distinction, the genetic differentiation is not very high (Wolf et al. 2010).

Another example can be found in the toad species *Bombina bombina* and *B. variegata*, which form complex variable secondary contact zone in Central Europe, based on ecological requirements of both species where they occasionally hybridize (Vörös et al. 2006). While *B. bombina* occupies lowlands, *B. variegata* is found at higher altitudes and they meet at the border areas of the two environments (Szymura 2000).

Two subspecies of mice from the genus *Mus* (*Mus musculus musculus* and *M. m. domesticus*) present in Europe form an intersection of their respective distributions across Europe, from the Jutland Peninsula, across Central Europe reaching to the Baltic Sea. Using mtDNA markers, presence of introgression has been found in several transects in Central Europe (Božíková et al. 2005).

All of abovementioned examples share similar pattern. Secondary contact zones have been formed, presumably as a result of the Pleistocene climatic fluctuations and recolonazation of Europe after the last glacial, and due to incomplete reproductive isolation, hybridization occurred in those, relatively narrow and stable zones. Also the taxonomy is often unclear and subject to many studies.

This thesis is focused on another model species of the postglacial processes – hedgehogs from the genus *Erinaceus*. Transect of the zone has been well studied in Czech Republic (Bolfíková & Hulva 2012); analyses of the rest of the contact zone should bring more information about the overlap.

1.4. Hedgehogs

1.4.1. Phylogeny

Tab. 1. Taxonomy of genus *Erinaceus* (Amori 2006).

Kingdom	Animalia
Phyllum	Chordata
Order	Mammalia
Class	Eulipotyphla
Family	Erinaceidae
Subfamily	Erinaceinae

Hedgehogs are small insectivorous mammals from Erinaceinae subfamily, which includes five genera – *Atelerix, Erinaceus, Hemiechinus, Mesechinus* and *Paraechinus* and 17 species within these genera (Amori 2006). Their natural range of occurrence is through Europe, Asia, parts of Africa and they have also been introduced to New Zealand (Kim et al. 2017). There are four species in the genus *Erinaceus* – *E. amurensis*, *E. concolor, E. europaeus* and *E. roumanicus*, all of these species present in Eurasia (Kim et al. 2017). Apart from the *E. amurensis*, the three remaining species are distributed in Western Palearctic (Djan et al. 2017).

Bannikova et al. (2014) performed research of the evolutionary history of Erinaceidae and brought important enlightening data. They performed multilocus analyses and determined divergence times of the particular branches. According to the results, the divergence of the hedgehog species was more recent then stated before (e.g. Santucci et al. 1998) and split of the *Erinaceus* spp. was dated to Pleistocene (1.0 – 2.2 Mya). The study acknowledges *E. amurensis* and *E. europaeus* as sister branches, as well as with *E. concolor* and *E. roumanicus*. Divergence between the *E. amurensis* and *E. europaeus* was estimated to 0.6-1.6 Mya and between the *E.concolor/roumanicus* to 0.4-1.4 Mya.

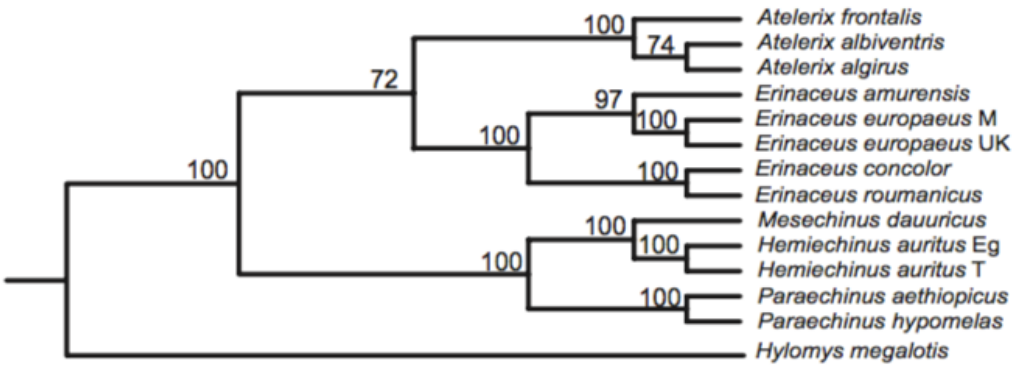

Fig. 5. Phylogenetic tree of the Erinaceidae family. Reconstruction based on multilocus analyses, using BEAST algorithm (Bannikova et al. 2014).

This thesis is focused mainly on the two of the *Erinaceus* species – *E. europaeus* and *E. roumanicus*, species with parapatric range that play an important role as model organisms for the phylogeography research and postglacial fluctuations in Europe (Bolfíková & Hulva 2012).

Erinaceus europaeus and *E. roumanicus* are parapatric species, considered on the macrogeographical scale (Bolfíková & Hulva 2012). Although hedgehogs are a classical subject to examining postglacial recolonization of Europe and processes connected to it (Černá Bolfíková et al. 2017), the history of their phylogenetic divergence and speciation history is not that sufficiently determined.

Though western hedgehog (*Erinaceus europaeus*) and his eastern counterparts are recognized as two separate species since 20th century (Kryštufek 2002), until recently, *Erinaceus roumanicus* was thought to be equivalent for *E. concolor* and was listed merely as morphotype (Sommer 2007) within the single species this way in scientific literature. According to several genetic (Santucci et al. 1998; Seddon et al. 2001) and morphological (Kryštufek 2002) test results and the morphological and genetical differences in those samples, it was demonstrated that *E. concolor* and *E. roumanicus* are two divergent sister species, occupying different habitats (Bogdanov et al. 2009). The distribution of the *Erinaceus* species itself and their genetic variability shows that several refugia and thus several colonization routes play part in nowadays hedgehog populations' diversity (Djan et al. 2017).

1.4.2. Distribution and phylogeography

Erinaceus europaeus is present in the Western Europe, part of Baltic Republics, western part of Russia and in south of Scandinavia. Area of distribution of *Erinaceus roumanicus* covers Central and Eastern Europe, Balkans, Baltic Republics and goes to Russia and Middle East.

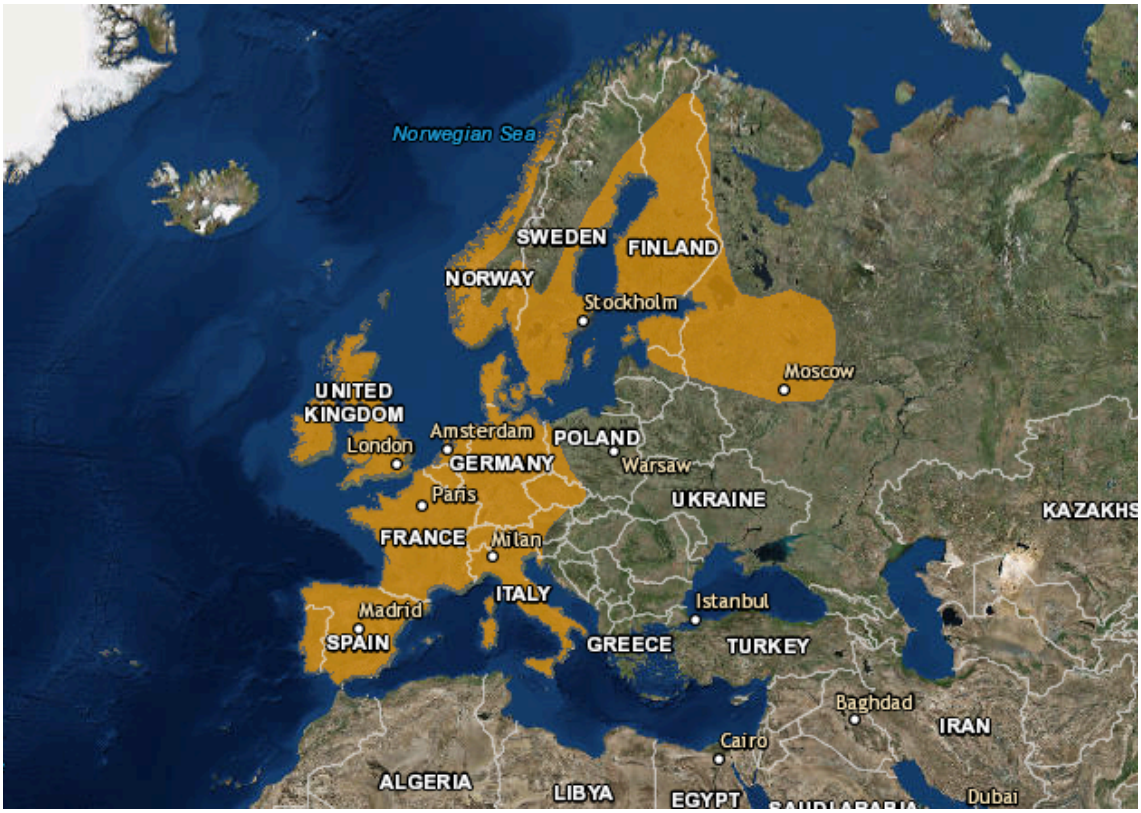

Fig. 6. Distribution of *Erinaceus europaeus* (The IUCN Red List of Threatened Species. Available from: www.iucnredlist.org/details/29650/0).

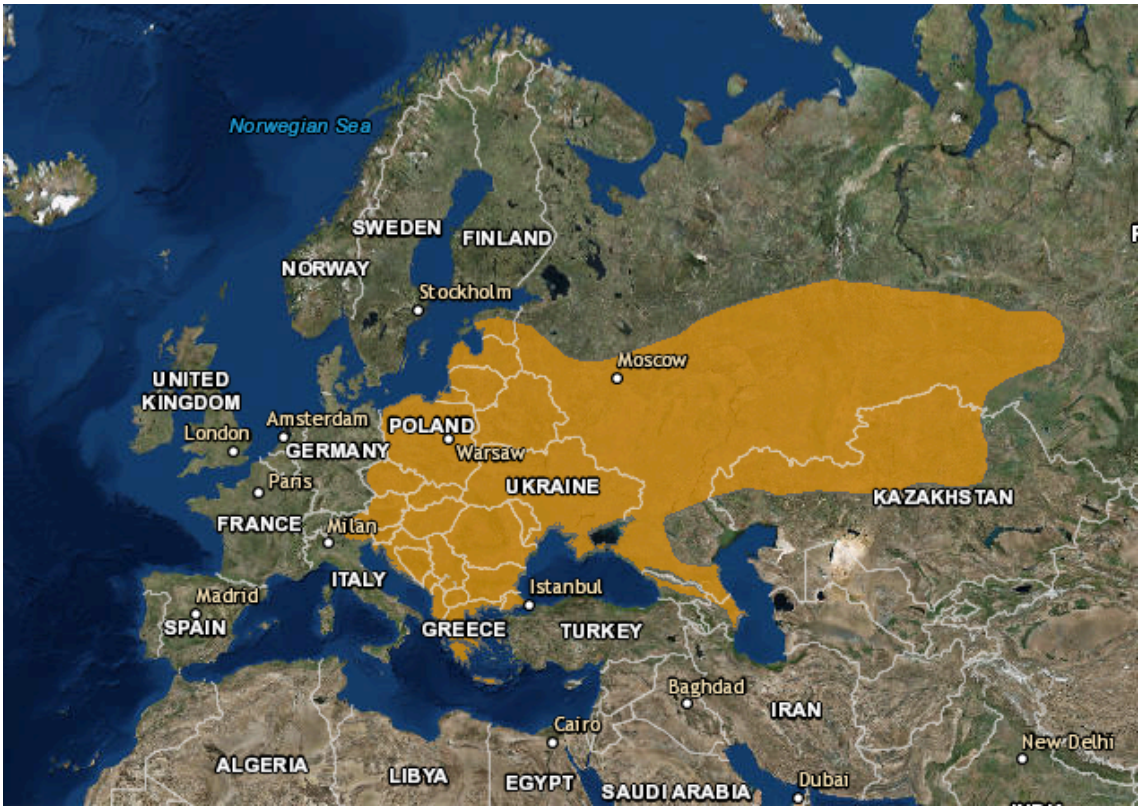

Fig. 7. Distribution of *Erinaceus roumanicus* (The IUCN Red List of Threatened Species. Available from: www.iucnredlist.org/details/136344/0).

The distributions of the two species have several zones of contact in Europe, where both of the species meet and occasionally live in sympatry. Two of these areas, contact zones, can be found within the distribution of *E. europaeus* and *E. roumanicus*. One of the zones is located in central Europe and covers the area of Czech Republic, Poland, Austria and Italy (Suchentrunk et al. 1998), main area for this works' research, the second contact zone is situated in the north-eastern part of Europe (Seddon et al. 2001).

While several studies exist about the contact zone in Central Europe, not much information is known about the northern contact zone. It spreads in the eastern and central regions of European part of Russia and western Estonia (Bogdanov et al. 2009) and was formed later, due to the longer distance from the southern refugia (Seddon et al. 2001).

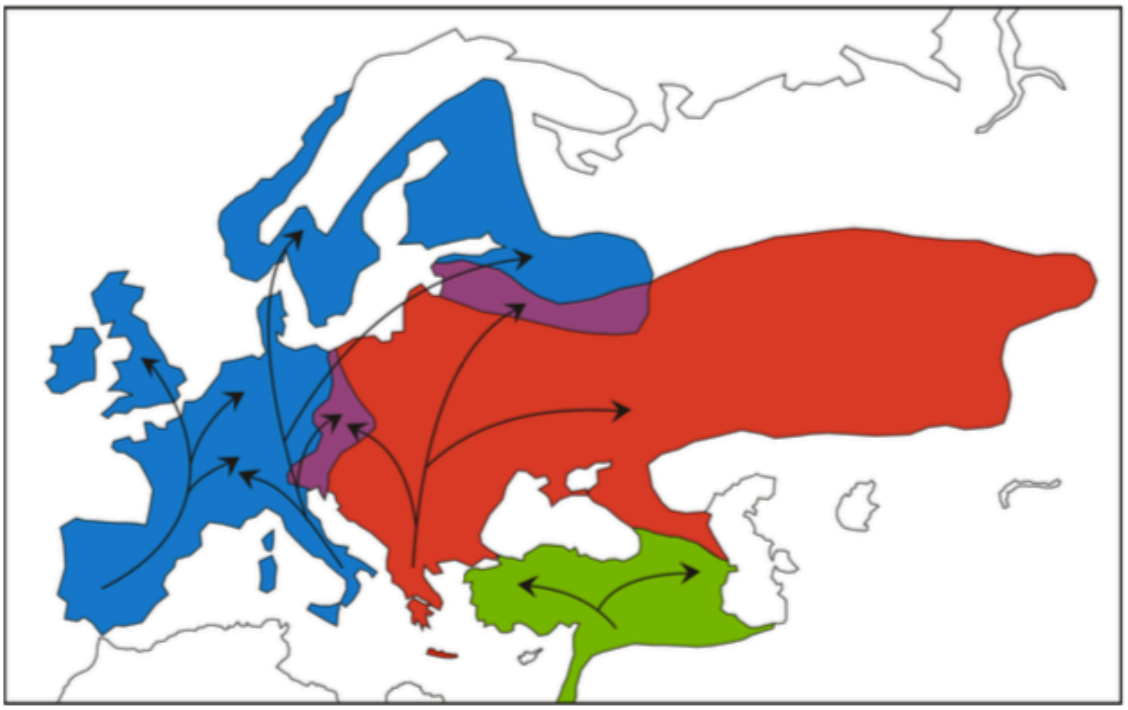

Fig. 8. Distribution of *E. europaeus* (blue), *E. roumanicus* (red) and *E. concolor* (green) and highlighted zones of sympatry (violet). Black arrows show routes of colonization from glacial refugia after LGM (Bolfíková & Hulva 2012).

In hedgehogs, morphological (Sommer 2007) and molecular (Seddon et al. 2001) analyses did show that different refugia gave rise to the particular hedgehog species. According to the Hewitt (1999), *Erinaceus europaeus* have its origin in the Iberian and Italian peninsula refugia, while *Erinaceus roumanicus* ancestors could be traced to the Balkans. *Erinaceus concolor* has formed in its own refugium, found at Caucasus and, with the Caucasus mountains and Bosphorus serving as natural borders, separating *E. conolor* and *E. roumanicus* and thus preventing the formation of the secondary contact zones and gene flow between these two closely related species (Seddon et al. 2001; Berggren et al. 2005).

Based on the fossil data, Sommer (2007) estimates that the contact between the two species in Central Europe occurred during Boreal and propose the occurrence of the *E. europaeus* as slightly earlier, which is suggested also by genetic data results (Bolfíková & Hulva 2012). Seddon et al. 2001 did perform analysis of the mitotypes, which showed clear diversification of the two species and also further division to monophyletic clades (E1-E3 in *E. europaeus* and C1-C2 in then *E. conocolor*, now *E. roumanicus* – C1 and *E. concolor* – C2) within the species.

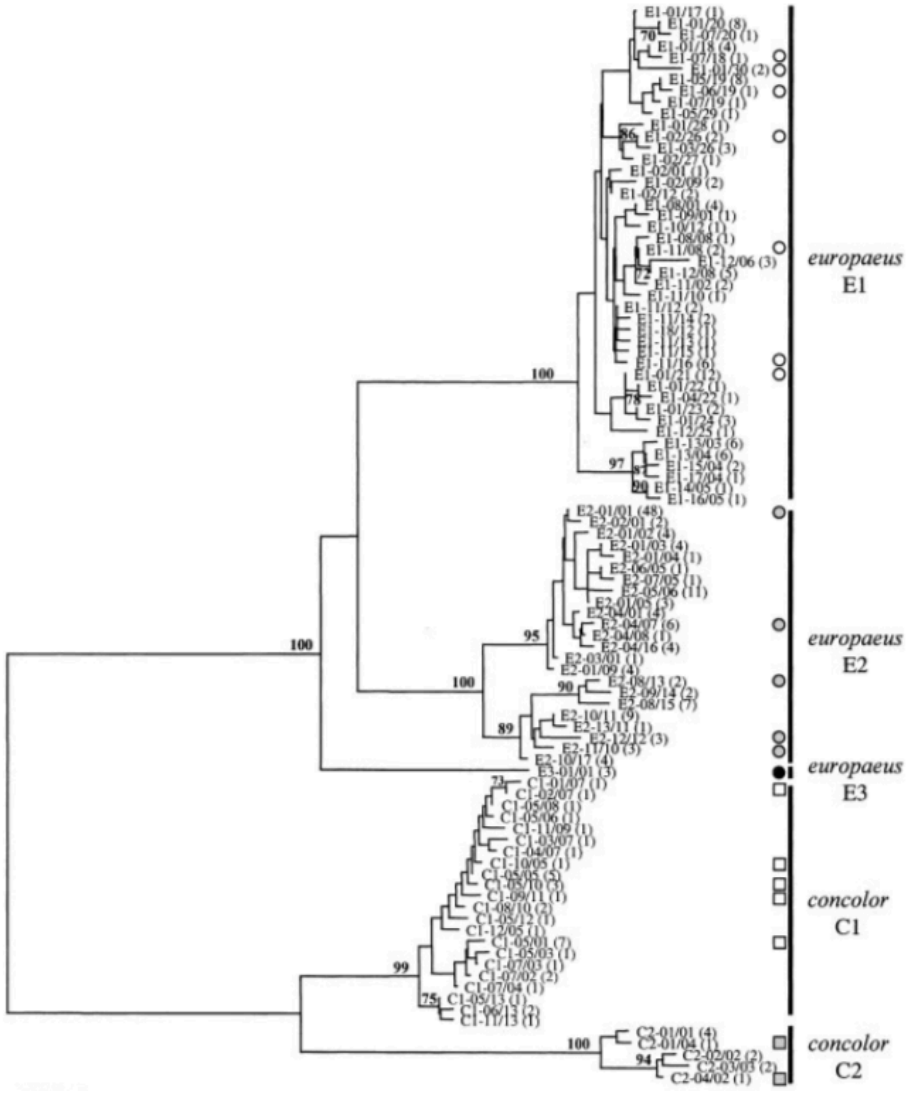

Fig. 9. Phylogenetic relations among mitotypes of *E. europaeus* and *E. concolor/roumanicus* (Seddon et al. 2001).

As stated before, although *E. europaeus* and *E. roumanicus* did evolve in separate refugia, and thereby, allopatrically, during postglacial recolonization of the Europe allowed these two species to form a secondary contact and evolve rather parapatrically, with their ranges overlapping at several areas (Černá Bolfíková et al. 2017).

Studies about the contact of the two species have been done recently, bringing important information about demographical, ecological and genetic differences between them. Suchentrunk et al. (1998) performed genetic analyses of 85 hedgehog specimens collected in central Europe including area of the contact zone in Austria represented by 15 samples. Although the number of the specimens was not high, the genetic variability within either species in the contact zone was detected as rather low.

In study by Bolfíková and Hulva (2012), research of the distribution and landscape patterns of both species based by genetic analyses was examined in the transect of the contact zone in the area of Czech Republic. This study brought important information about situation in this transect. The frequency ratio of samples from the transect was 1:3 in favour of *E. europaeus*. The size of the population of *E. europaeus* was indicated as almost constant, apart from the slightly increasing population of *E. roumanicus* and while distribution of *E. europaeus* was more widespread, *E. roumanicus* was located in rather lower altitudes (below 300 m a. s. l.). Landscape analyses of mtDNA data showed three mosaic subpopulations in *E. europaeus* while nuclear data analyses did not show differentiation in *E. europaeus;* in *E*. roumanicus, both analyses suggested two subpopulations. These outputs indicated less sex-biased distribution of *E. roumanicus* along with the higher level of gene flow.

Pfäffle et al. (2014) conducted a research regarding parasite prevalence of the *E. europaeus* and *E. roumanicus* in the transect of the contact zone in Czech Republic. 12 ecto- and endoparasite species were found at the specimens with distinguishable differences between the hedgehog species, especially concerning the intestine parasites, which could show ecological distinction between the species with possible differences in food strategy in *E. europaeus* and *E. roumanicus*.

Černá Bolfíková et al. (2017) aimed to determine consequences of the isolation in refugia and subsequent recolonization of Europe of *E. roumanicus*. Spatial and non-spatial analyses of genetic variability showed that population in contact zone in Central Europe along with the southern population within the range of the species are clearly differentiated. Genetic variability of the population in the contact zone was recognised as lower. This might be a consequence of the postglacial expansion, accompanied by such effects as bottlenecks. Demographic analyses also did not confirm significant increase of the population in the comparison with the study of Bolfíková and Hulva (2012).

In earlier works, focusing at contact zones of hedgehog species, no hybridization or introgression of two hedgehog species was detected in natural conditions and it was considered rather unlikely (Suchentrunk et al. 1998; Bolfíková & Hulva 2012). However, Bogdanov et al. (2009) found one hybrid individual during the research in northeastern contact zone, suggesting that possible hybridization in that zone might be higher. Bolfíková and Hulva (2012) later suggested that it might be due to the later formation of the eastern contact zone and therefore, due to the insufficient reproductive isolation mechanisms. They did not found any evidence of the hybridization or introgression in any of the specimens and assumed the reproductive isolation mechanisms to be formed in the sympatry zone. Afterwards, Černá Bolfíková et al. (2017) were first to report a hybrid in Central Europe, located in Slovakia, founding backcrossed individual of *E. europaeus* and *E. roumanicus*.

2. Aims of the Thesis

The aims of this thesis were to collect samples from the localities outside the Czech Republic and merge datasets from the two previously published papers with the new data, to reanalyze data from the published papers together with new data and evaluate the dynamics of the Central European contact zone. And finally, to compare the genetic structure of the two hedgehog species with special regard to previously published results.

3. Methods

Three sets of samples were used in analyses; a dataset of the samples processed by Bolfíková and Hulva (2012), containing specimens of *E. europaeus* (n=174*)*, dataset by Černá Bolfíková et al. (2017) with *E. roumanicus* individuals and the hybrid specimen (n=86) and new set of samples (n=51) processed by us, which included both *E. europaeus* and *E. roumanicus* species from Germany, Poland and Austria. Samples from the previous publications were chosen according to the landscape analysis of the nuclear data with individuals from Czech Republic, Slovakia and Hungary. Samples of *E. europaeus* came in majority from the dataset by Bolfíková and Hulva (2012) complemented by older specimens from Germany. These decisions were made with the aim to extend the base of the samples and obtain as many divergent samples and thus, information, as possible and for more objective assessment of the analyses outputs. List of the newly processed samples can be found in the attached table in Appendix 1. Maps of the distribution of the collected samples were created in ArcMap, ArcGIS software (www.esri.com).

The total number of analysed individuals was 311, from which 51 samples were new and went through the laboratory analyses. The rest of samples were obtained from the previous studies. The whole dataset included 203 individuals belonging to the species *Erinaceus europaeus*, 107 specimens of *E. roumanicus* and one hybrid individual (confirmed by Černá Bolfíková et al. 2017). The samples came from Czech Republic, Slovakia, Hungary, Poland, Germany and Austria.

The new samples were collected from the captured animals (Leon Barthel, Ekipa Ostoja), delivered from museum collections (Senckenberg Museum in Görlitz, Naturhistorisches Museum Wien) or obtained from road-killed animals. Samples contained tissue or hair follicles. The samples were stored in 96% ethanol at -20°C.

We possessed the outputs of laboratory analyses from the older datasets; all the newly obtained samples had to be processed as following.

3.1. Isolation of DNA

DNA of all the samples was isolated by DNeasy Blood & Tissue Kit (Qiagen) using the manufacturer protocol. The protocol was adjusted in the steps 1 and 8, by adding 15 µl of proteinase K and 100 µl of AE Buffer respectively; the step 9 was not performed.

3.1.1. Nanodrop

Amount of DNA in 1 µl of the isolate was measured by the NanoDrop™ 2000 Spectrophotometer and according to the measured values, the samples were chosen for the following processing. Samples with no signs of sufficient amount of the DNA (<1 ng/µl) in isolates and were not used in following analyses.

3.2. Mitochondrial genotyping

PCR reaction was performed using ProL-He and DLH-He primers to amplify D-loop of mtDNA.

Tab. 2. Control region sequences.

Primer	Sequence
ProL-He	5‘-ATACTCCTACCATCAACACCCAAAG-3‘
DLH-He	5‘-GTCCTGAAGAAAGAACCAGATGTC-3‘

A total amount of the PCR reaction volume for each sample was 25 µl, containing 12.5 µl of the Top-Bio PCR Master Mix, 8.5 µl of H_20, 1 µl of primer PRoL-He, 1 µl of primer DLH-He and 2 µl of DNA sample.

The mixtures were pipetted into 0.2 ml microtube strips and put into cyclers, where PCR was performed with following conditions:

Tab. 3. Setup of the cycler for DLOOP PCR.

Step	Temperature	Time [min]
1	94 °C	3:00
2	94 °C	1:00
3	56 °C	1:00
4	72 °C	1:00
5	72 °C	4:00
6	12 °C	∞

Steps 2 – 4 were 30x repeated.

3.2.1. Electrophoresis

For the visualization of the successful PCR product amplification, a gel electrophoresis was performed. One and a half per cent agarose gel with the 1.5 μl of ethidium bromide was prepared and GeneRuler 100 bp DNA ladder was used as a marker. Two microliters of each PCR product were pipetted into the holes and the electrophoresis was run at 120V for 30-35 min in TBE puffer-filled box and then visualized under UV light transluminator and photographed.

3.2.2. Purification

Successfully amplified samples were purified, using QIAquick PCR Purification Kit by Qiagen. The protocol was adjusted in the last step – 30 μl of EB Buffer were added.

3.2.3. Sequence analysis

Purified samples were processed in Sequencing Laboratory at the Faculty of Science of Charles University in Prague.

Reaction volume contained 8 µl of mixture – 0.5 µl of DLH-He primer, 6.5 µl H_20 and 1 µl of PCR product.

3.3. Microsatellite genotyping

3.4. PCR

All the samples were genotyped at 11 microsatellite loci. Two primer mixes were prepared - SB1 (containing six loci – EEU3, EEU5, EEU6, EEU54, EEU36) and SB2 (EEU1, EEU2, EEU4, EEU12, EEU37, EEU43). The primers were developed for *E. europaeus* by Becher and Griffiths (1997) and Henderson et al. (2000).

A reaction volume for every sample was 10 µl, containing 5 µl of Multiplex PCR MasterMix (Qiagen), 3 µl of H_20, 1 µl of primer SB1/SB2 and 1 µl of DNA, pipetted into 0.2 ml eppendorf strips and run in cyclers with following conditions:

Tab. 4. Setup of the cycler for SB1.

Step	Temperature	Time [min]
1	95 °C	5:00
2	95 °C	0:30
3	55-57 °C	1:00
4	72 °C	0:30
5	72 °C	5:00
6	12 °C	∞

Steps 2-4 were 32x repeated.

Tab. 5. Setup of the cycler for SB2.

Step	Temperature	Time [min]
1	95 °C	5:00
2	95 °C	0:30
3	58-62 °C	1:00
4	72 °C	1:00
5	72 °C	10:00
6	12 °C	∞

Steps 2-4 were 32x repeated.

3.4.1. Denaturation

A volume of 10 µl, containing 9 µl of formamide, 0.25 µl of LIZ 500 standard and 0.75 µl of PCR product was pipetted into 0.2 ml eppendorf strips, then run in the thermocycler. The mixture was heated to 95°C for 5 minutes and subsequently cooled down to 4°C.

Results were sent for the fragmentation analyses to the Sequence Laboratory at the Faculty of Science of Charles University in Prague.

3.5. Analyses of genetic variability

Sequences of the mitochondrial D-loop were edited in Geneious 10.2.2 (Kearse et al. 2012) and aligned using MAFFT (Katoh & Standley 2013).

MEGA7 (Kumar et al. 2016) software was used for the display of the phylogenetic tree. We constructed neighbour-joining tree with bootstrap set to 1000, with p-distance method and missing data were treated as partial deletion with 95% coverage.

DnaSP v6.0 (Rozas et al. 2017) software was used for obtaining descriptive statistical parameters for both species (*E. europaeus* and *E. roumanicus*). Numbers of haplotypes (N_h) and polymorphic sites (N_p), tests for nucleotide diversity (Π), haplotype diversity (H_d) and tests of neutrality were conducted. Significance of the three neutrality tests (Tajima's D, Fu's F_S and R^2) was tested.

Bayesian skyline plots were conducted for each species to visualize the dynamics of the population size using BEAST v1.8.4 program (Drummond et al. 2012). GTR substitution model with strict clock was set. Marcov Chain Monte Carlo (MCMC) runs were set to 20,000,000 iterations, logging the parameters every 1,000 iterations. For each species the BEAST program was run three times and the outputs were combined in LogCombiner. The Bayesian skyline plots were visualized in Tracer v1.5 (Rambaut et al. 2009).

Relationships among particular haplotypes were visualized in Network (http://www.fluxus-engineering.com/) by conduction of median-joining network for each species. Sequences with missing data (two *E. roumanicus* individuals from Poland – PL29, PL39; two *E. europaeus* individuals from Germany – R, V) were eliminated from median-joining network construction.

For analyses of the spatial genetic architecture, we used Geneland (Guillot 2009) running in the R program (http://cran.r-project.org/bin/windows/base/) using Bayesian clustering methods and TESS 2.3.1 (Chen et al. 2007; Durand et al. 2009). These two programs differ in the approach of the data assessment. TESS attempts to minimize the Wahlund effect by implementing of relationships in local scale. Geneland detects more straightforward linear genetic barriers, while TESS implements more complex individually-based admixture models.

In Geneland, we used both mitochondrial haplotype data and diploid microsatellite data to assess population memberships of each species and to determine the assumed number of clusters in HWE. Three independent runs were performed with number of clusters from K=1 to K=10, number of MCMC iterations was set to 500,000, storing every 100 steps for both data types. Only null allele model was set as true in case of nuclear data. The outputs were displayed graphically, using ArcGIS software. TESS works with microsatellite data only. The setup was as following: We chose BYM admixture model with maximal number of clusters set to K=5, number of sweeps to 50,000 with burn-in to 30,000.

Sizes of microsatellite loci were identified by GeneMarker V2.6.3 (www.softgenetics.com). Nine loci were used for further analyses. A total number of 273 samples were used for analyses of genetic population structure.

Presence and proportion of null alleles (false homozygotes) were estimated in Geneland (Guillot 2009).

Program GenAlEx 6.5 (Peakall & Smouse 2012) working in the Microsoft Excel environment was used for obtaining the descriptive parameters of microsatellite genetic diversity. We used functions for obtaining the number of alleles (N_a), expected (H_E) and observed heterozygosity (H_O) and coefficient of inbreeding (F_{IS}) for *E. roumanicus* and *E. europaeus*, respectively. Principal component analysis was also conducted by GenAlEx. This analysis is used to identify differences in samples of individual populations and their mutual genetic variance. Allelic richness (A_R) was calculated in FSTAT 2.9.3 (Goudet 2001) program. Values of allelic richness are used to estimate the number of alleles per locus in populations with equal sizes; individuals from the larger population are randomly chosen, so the number of individuals in both populations is identical. It allows comparison between the populations.

Deviations from Hardy–Weinberg equilibrium in both populations were tested in online version of Genepop 4.2 program (http://genepop.curtin.edu.au/) to assess the heterozygote deficiency or excess.

Structure V2.3.4 software (Pritchard et al. 2000) was used to assess the population structure. The program works by using Bayesian Clustering Analysis and suggests sorting of the population to the clusters (*K*) based on analysis of likelihood. Parameters of the final run were set as following: Number of the MCMC steps was 800,000 and the burn-in period was 200,000. Interval of the K was between 1 and 5; number of iterations was set to 3. Results of the final run were evaluated in Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) online program to estimate the maximum likelihood of number of clusters by Evanno method (Evanno et al. 2005).

4. Results

A total number of 311 individuals from six European countries were involved in analyses. In several samples, only microsatellite data are available and *vice versa* (see Appendix 1). A graphic representation of samples, their sampling sites and differentiation of the tree datasets can be found in Fig. 10.

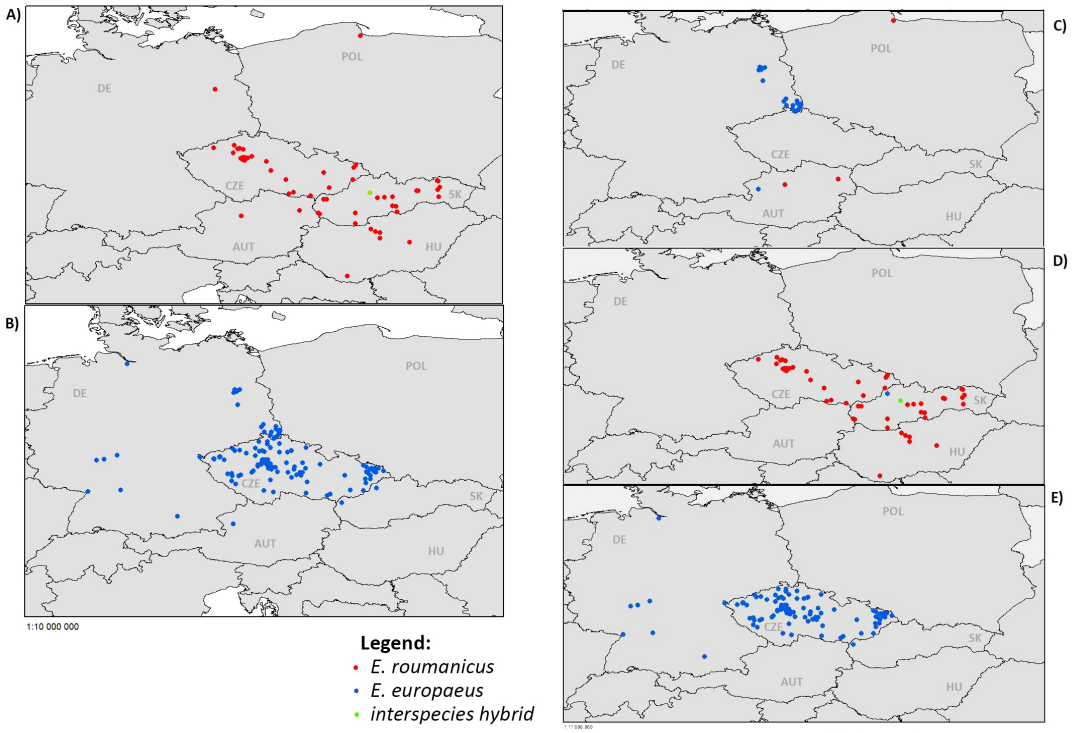

Fig. 10. Graphical display of sampling sites of all the individuals. A map with all the individuals of *E. roumanicus* (A) and *E. europaeus* (B) on the left and maps with the distinction of the new dataset (C), dataset with individuals of *E. roumanicus* by Černá Bolfíková et al. (2017) (D) and dataset by Bolfíková and Hulva (2012) with individuals of *E. europaeus* (E); individuals of *E. roumanicus* are displayed by red points, individuals of *E. europaeus* by blue points; Country codes: AUT, Austria; CZE, Czech republic; DE, Germany; HU, Hungary; POL, Poland; SK, Slovakia. The map was created by ArcMap.

4.1. Mitochondrial control region data

A total number of 270 sequences were used for the analyses, from which 37 sequences came from the new set of samples. The sequences contained 393 nucleotide sites.

Phylogenetic tree did show a shallow differentiation; one sample was clearly separated from the rest of the dataset. It was a sample obtained from the museum in Vienna, Austria and was identified as member of the *Atelerix* genus. This individual was omitted from further analyses. The remaining individuals were sorted to individual clusters according to the species affiliation without deeper differentiation. We tried to use Bayesian approach for further evaluation by using MrBayes (Ronquist et al. 2013) program but the tree did not show higher differentiation.

Genetic variability tests conducted in DnaSP were estimated for the *E. europaeus* and *E. roumanicus* populations respectively. The values can be seen in Tab. 6. Haplotype diversity, as well as nucleotide diversity, was higher in *E. europaeus*. The neutrality tests were not significant in *E. roumanicus*; Fu's F_S test in *E. europaeus* did show significance and shows negative values, which could indicate an expansion in the recent population structure.

Tab. 6. Descriptive parameters of genetic diversity of the two hedgehog species – *E. europaeus* (EE) and *E. roumanicus* (ER). Parameters describe the following: N, number of individuals; N_h, number of haplotypes; H_d, haplotype diversity; N_p, number of polymorphic sites; Π, nucleotide diversity; D, Tajima's D; Fu's F_S and Ramos-Onsins and Rozas' R^2. Significant value ($p<0.05$) is marked with an asterisk.

	N	N_h	H_d	N_p	Π	D	R^2	F_S
EE	178	25	0,8821	20	0,00687	-0,66875	0,0643	-6,915*
ER	92	10	0,516	10	0,00385	-0,7287	0,0686	-2,417

Number of haplotypes was determined as following: 10 haplotypes in *E. roumanicus* and 25 haplotypes in *E. europaeus*. Two median-joining networks (Fig. 11) by Network program were created, individually for each species.

In *E. roumanicus*, two major haplotypes were found thorough the population, forming over 85% of the total population. One haplotype included four individuals and mostly one or two individuals represented the remaining haplotypes. The dominant haplotype (present at 63 out of 92 individuals) was found across the major part of the mapped area.

In *E. europaeus*, the situation was more elaborate; two haplotypes were carried by less than 20% of population, five more were present at higher count of individuals and the rest of haplotypes could, again, be found at only several individuals. The more complex structure of net in the *E. europaeus* suggests more stable population.

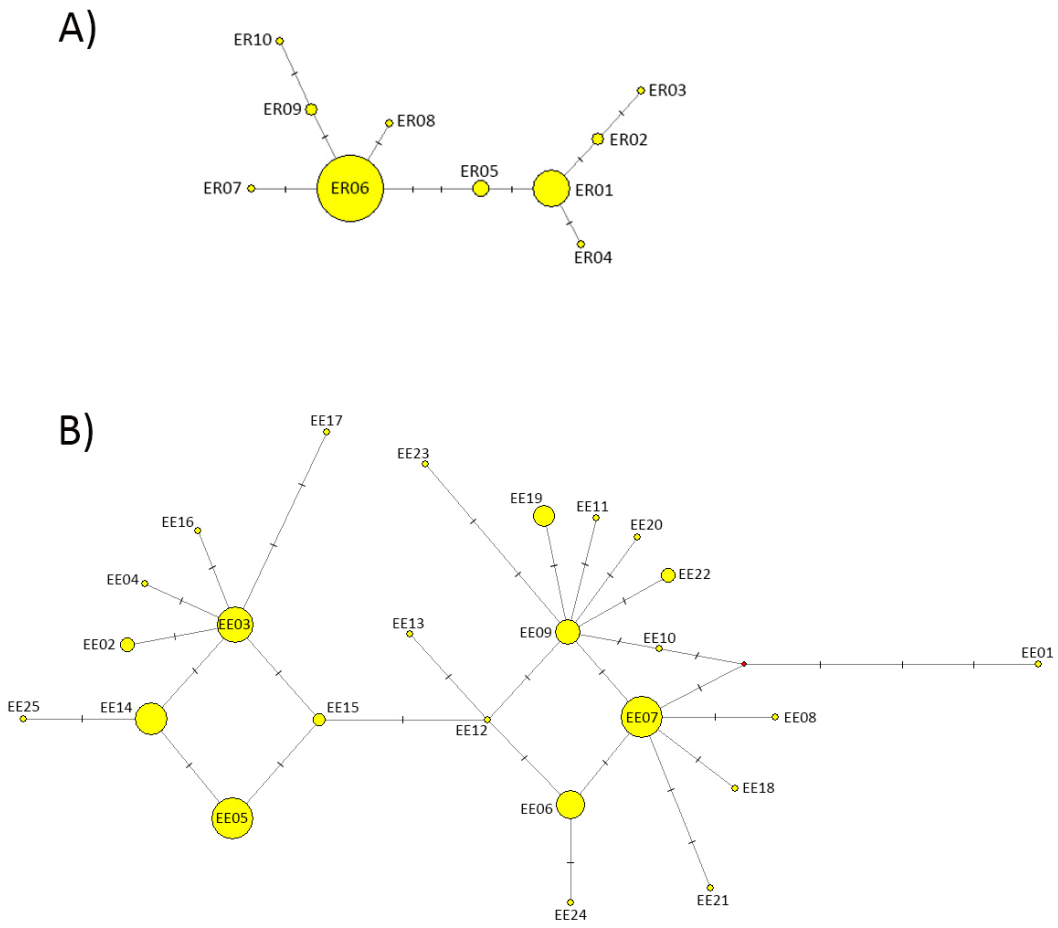

Fig. 11. Median-joining networks conducted by Network for *E. roumanicus* (A) and *E. europaeus* (B). The yellow circles represent haplotypes (EE01-EE25 in *E. europaeus*; ER01-ER10 in *E. roumanicus*), proportional to the haplotype frequencies; red circle represents hypothesized haplotype; dividing lines between nodes mark the number of mutated positions between the nodes.

The Bayesian skyline plots (Fig. 12) conducted for both species did confirm a stable population size for a longer period of time and recent growth for both of the species. Growth in *E. roumanicus* has been progressing more gradually, while in *E. europaeus*, the graph showed more abrupt increase in recent past of the species.

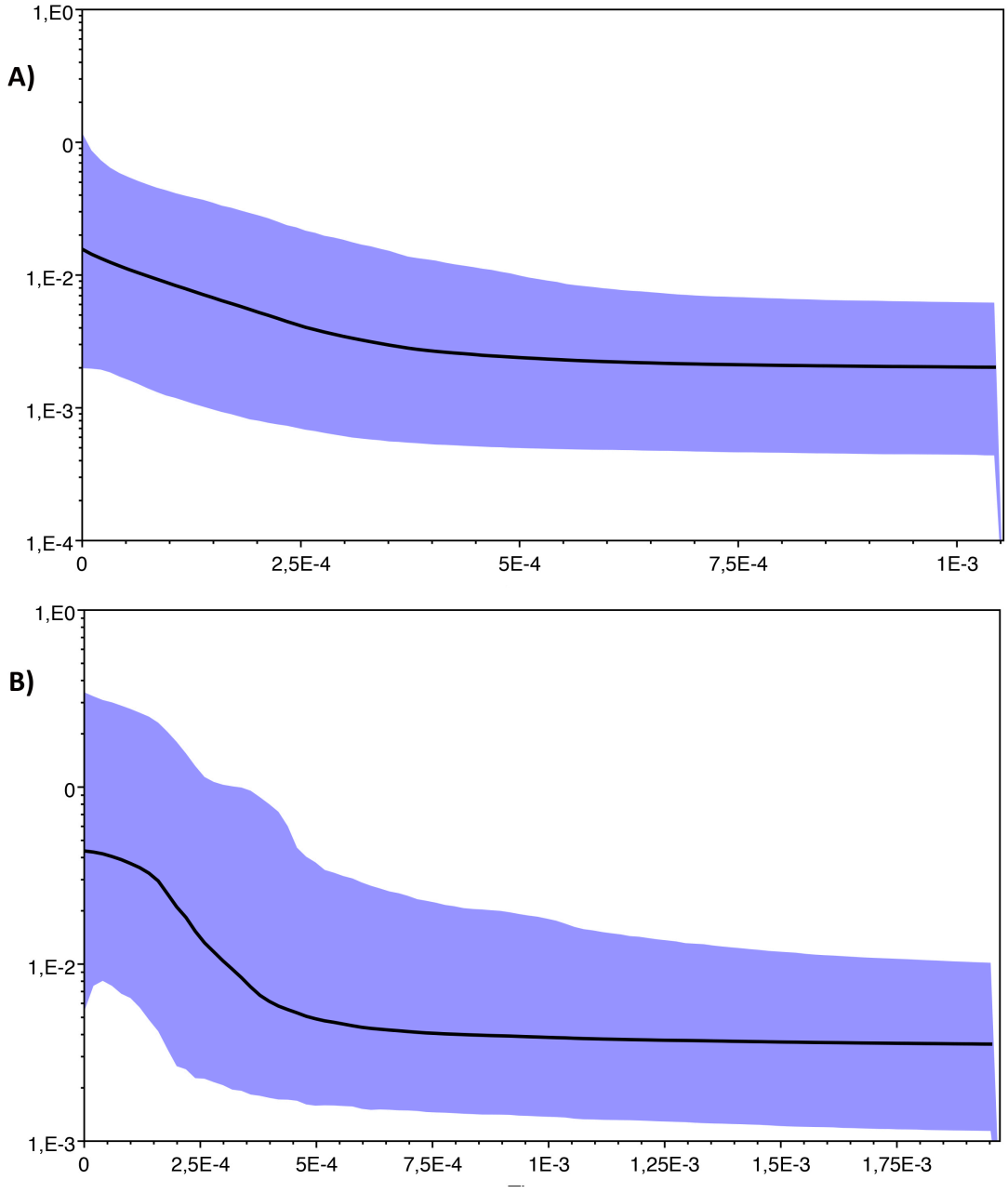

Fig. 12. Bayesian skyline plots for *E. roumanicus* (A) and *E. europaeus* (B). The x-axis represents timescale in mutation units and y-axis marks effective population sizes. The black line represents median of estimations and the blue area indicates 95% confident interval.

4.2. Microsatellite data

The data from *E. europaeus,* downloaded from the publication of Bolfíková and Hulva (2012) were analysed on different capillary sequencing machine and we needed standardization with our current dataset. Twenty samples were randomly chosen from the old datasets (ten specimens from each species) and the rest of the samples were binned according to the standard. This was highly demanding process and we admit that in case of *E. europaeus*, the data still show certain level of incompatibility.

The final dataset included 273 individuals genotyped at nine loci. From the 11 originally processed loci, one was monomorphic and one locus was insufficiently amplified. These loci were excluded from further analyses. Each of the used loci was polymorphic. Occurrence of the null allele presence was evaluated and none of the loci did show value over 15%, majority of the estimations were under 10%.

Parameters of genetic variability were calculated for each species and each respective locus (Tab. 7a; Tab. 7b). Each of the microsatellites contained between 2-21 and 4-23 alleles for *E. roumanicus* and *E. europaeus*, respectively. In both cases, expected heterozygosity was higher than the observed heterozygosity. Significant heterozygote deficiency was detected in the analysis by Genpop. Expected heterozygosity was higher in *E. europaeus* but observed heterozygosity was higher in *E. roumanicus*. Lower coefficient of inbreeding could be found in *E. roumanicus*. Principal component analysis divided the individuals to two populations; specimens of *E. roumanicus* form more composed structure in comparison with population of *E. europaeus*; the analysis did assign one sample of *E. europaeus* closer to the population of *E. roumanicus* and several others to the close proximity of the vertical axis (Fig. 13).

Tab. 7a. Descriptive parameters of genetic variability, given for each species and locus. Values are represented by following parameters: N_a, number of alleles; A_R, allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, coefficient of inbreeding.

Locus	N_a	A_R	H_E	H_O	F_{IS}
E. roumanicus					
Locus 1	14	12,807	0,737	0,538	0,270
Locus 2	14	8,998	0,845	0,670	0,208
Locus 3	20	15,884	0,891	0,702	0,213
Locus 4	12	9,97	0,669	0,520	0,223
Locus 5	2	2	0,028	0,028	-0,014
Locus 6	21	19,805	0,788	0,604	0,234
Locus 7	16	12,951	0,822	0,604	0,266
Locus 8	4	3,953	0,628	0,547	0,129
Locus 9	13	11	0,874	0,743	0,150
E. europaeus					
Locus 1	10	8,589	0,519	0,482	0,072
Locus 2	14	9,314	0,516	0,464	0,101
Locus 3	14	12,129	0,632	0,515	0,185
Locus 4	13	9,255	0,743	0,691	0,071
Locus 5	4	3,999	0,162	0,098	0,393
Locus 6	16	11,808	0,788	0,673	0,147
Locus 7	16	12,433	0,854	0,753	0,118
Locus 8	23	16,382	0,799	0,721	0,098
Locus 9	16	12,25	0,823	0,770	0,064

Tab. 7b. Mean values of descriptive parameters for each species.

	N_a	A_R	H_E	H_O	F_{IS}
E. roumanicus	*14*	*10,819*	*0,649*	*0,574*	*0,139*
E. europaeus	*12,889*	*10,684*	*0,698*	*0,551*	*0,186*

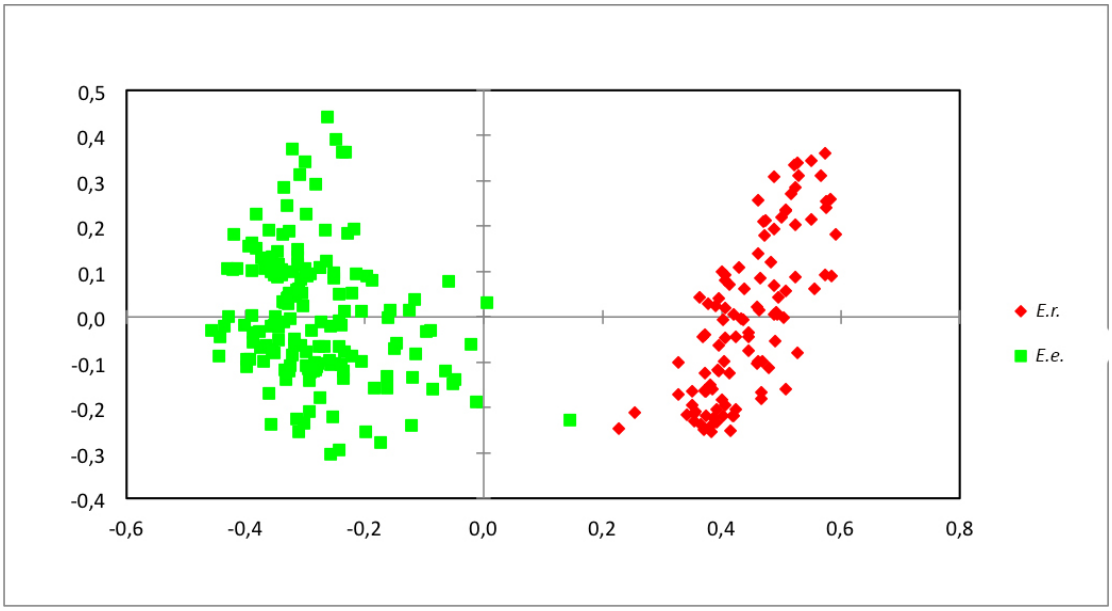

Fig. 13. Principal component analysis (PCA) generated in GenAlEx. It represents genetic variance among the individuals and sorts them into separate clusters. Individuals of *E. roumanicus* (n=106) shown in red, individuals of *E. europaeus* (n=166) in green.

Population assignment was assessed by Structure using Bayesian clustering approach. All the individuals were divided into three *a priori* populations according to the datasets. First population consisted of *E. roumanicus* individuals and one hybrid specimen from the dataset of Černá Bolfíková et al. (2017), second population did include *E. europaeus* from Bolfíková and Hulva (2012) and the last one was formed by new samples including both species. In K=2, apparent distribution of species into two separate populations according to their species affiliation was evident (Fig. 14). The evaluation in Structure Harvester did show a maximum likelihood for K=2, clearly dividing the dataset into two populations. From K=3 to K=5 the data did show noticeable distribution and intraspecific structure. It gives the impression, that discrepancies between older dataset and the new dataset of *E. europaeus* become apparent in higher number of K and that insufficient compatibility could be distinguishable from the outputs. The Structure was run for each species separately, where the variability among datasets was also noticeable (see Appendix 2).

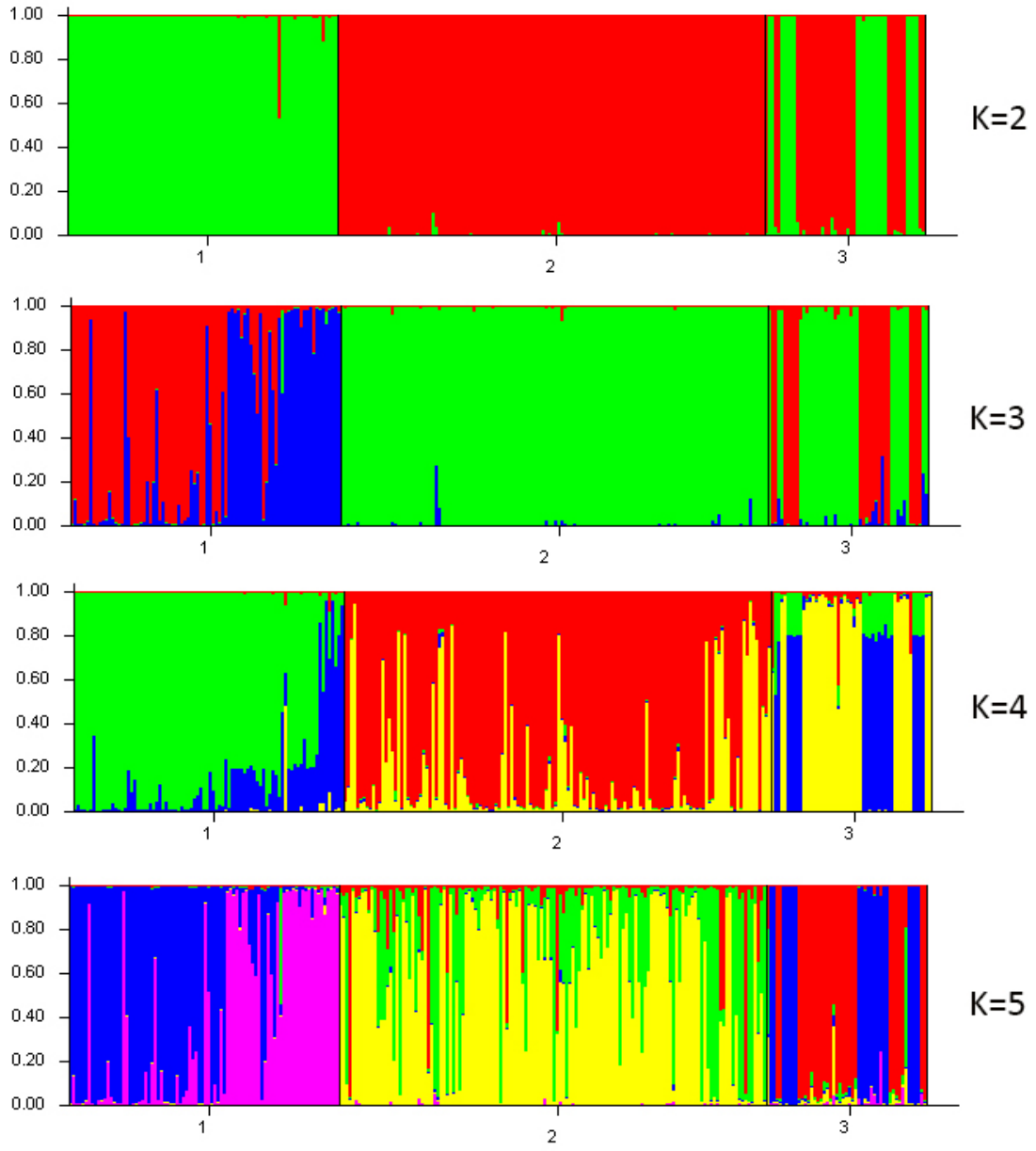

Fig. 14. Output of the Structure program, displaying distribution of populations, illustrated by different colours, when given the number of clusters from K=2 to K=5. Putative populations are marked as 1 (older dataset of *E. roumanicus*), 2 (older dataset of *E. europaeus*) and 3 (new dataset with both species).

4.3. Spatial analyses of genetic variability

Spatial genetic analyses of mitochondrial data made by Geneland did classify the individuals of *E. roumanicus* to two clusters based on georeferenced haplotype data. In *E. europaeus* we detected three subpopulations within the population. Visualizations of the spatial distribution of the clusters are shown in Fig. 15. The distribution of the subpopulations in *E. roumanicus* shows relatively clear transition, dividing a subpopulation with individuals from Germany, Poland, Czech Republic, western Slovakia and western Hungary (green area) and subpopulation from eastern Slovakia and majority of Hungary (white area), while in *E. europaeus* the distribution appears to have a more complex mosaic structure. A posterior probability of the individuals, belonging to the particular clusters was estimated and displayed graphically. The areas with the lightest shadow of yellow mark the values of the highest likelihood of membership to a subpopulation. Black lines (isoclines) indicate the genetic landscape extension and inclusion probability. More abrupt transitions between the subpopulations indicate greater mutual distances. For comparison of the maps of cluster memberships and posterior probabilities of both species for mtDNA and nuclear data see Appendix 3.

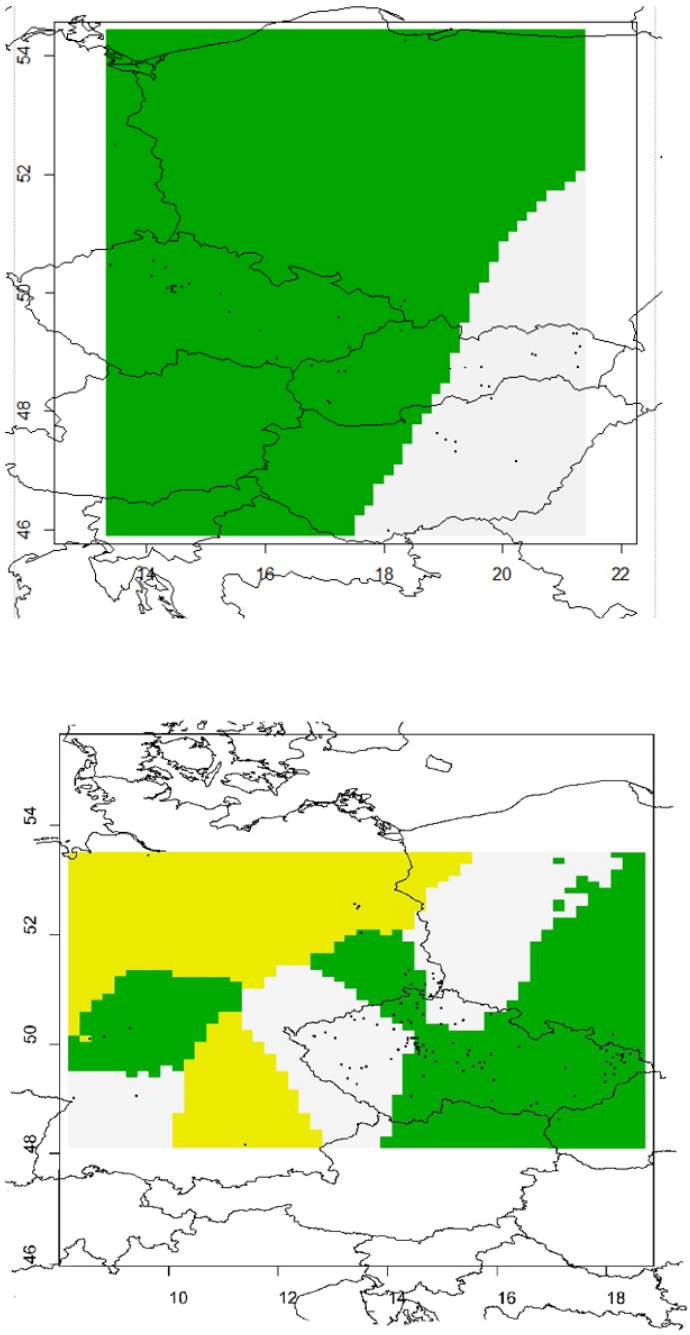

Fig. 15. Cluster memberships for *E. roumanicus* (above) and *E. europaeus* (below) using mitochondrial data, made by Geneland, georeferenced in ArcMap; x-axis represents longitude, y-axis represents latitude; points show localities of sampling sites; colours represent subpopulations.

Landscape analysis of nuclear data by Geneland detected two subpopulations in *E. europaeus*, while four subpopulations in *E. roumanicus*, which is in direct contradiction with the spatial analyses of the mitochondrial data. The hybrid individual formed one of the subpopulations so the run was repeated once more without the hybrid specimen. Subsequently, three populations were found by the analysis. The largest subpopulation of *E. roumanicus* contained individuals form Czech Republic and Slovakia (white). Individuals from Hungary are present in the second one (green) and the third population includes new individuals from Poland and Austria (yellow).

Another spatial analysis using Bayesian clustering was conducted by TESS. It provides similar outputs but the algorithm is based on different approach, what gives us a possibility to assess the data from broader perspective. In *E. europaeus,* the results showed division to two clusters as well. Four subpopulations were detected in connection with *E. roumanicus* data (Fig. 16). The samples from Czech Republic and Austria did cluster together (violet) and samples from Slovakia (yellow), Hungary (blue) and Poland (green) each formed a separate subpopulation.

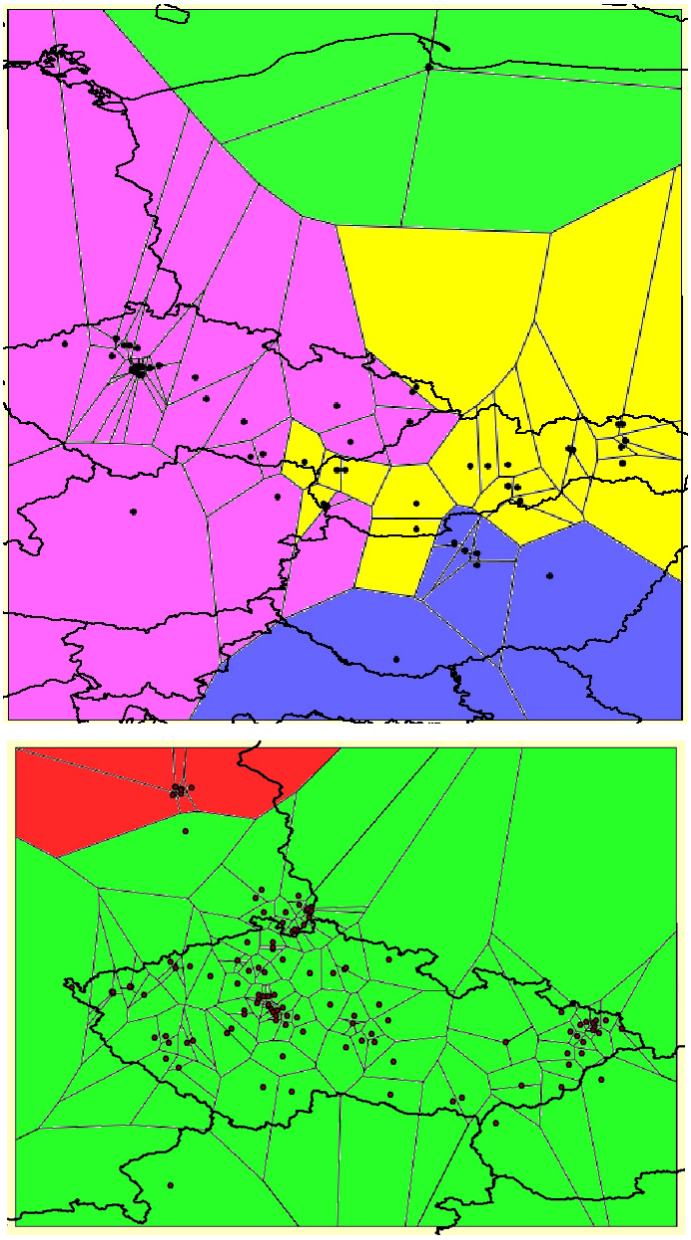

Fig. 16. Tessellation maps by TESS for *E. roumanicus* (above) and *E. europaeus* (below) showing the likely distribution of samples to subpopulations.

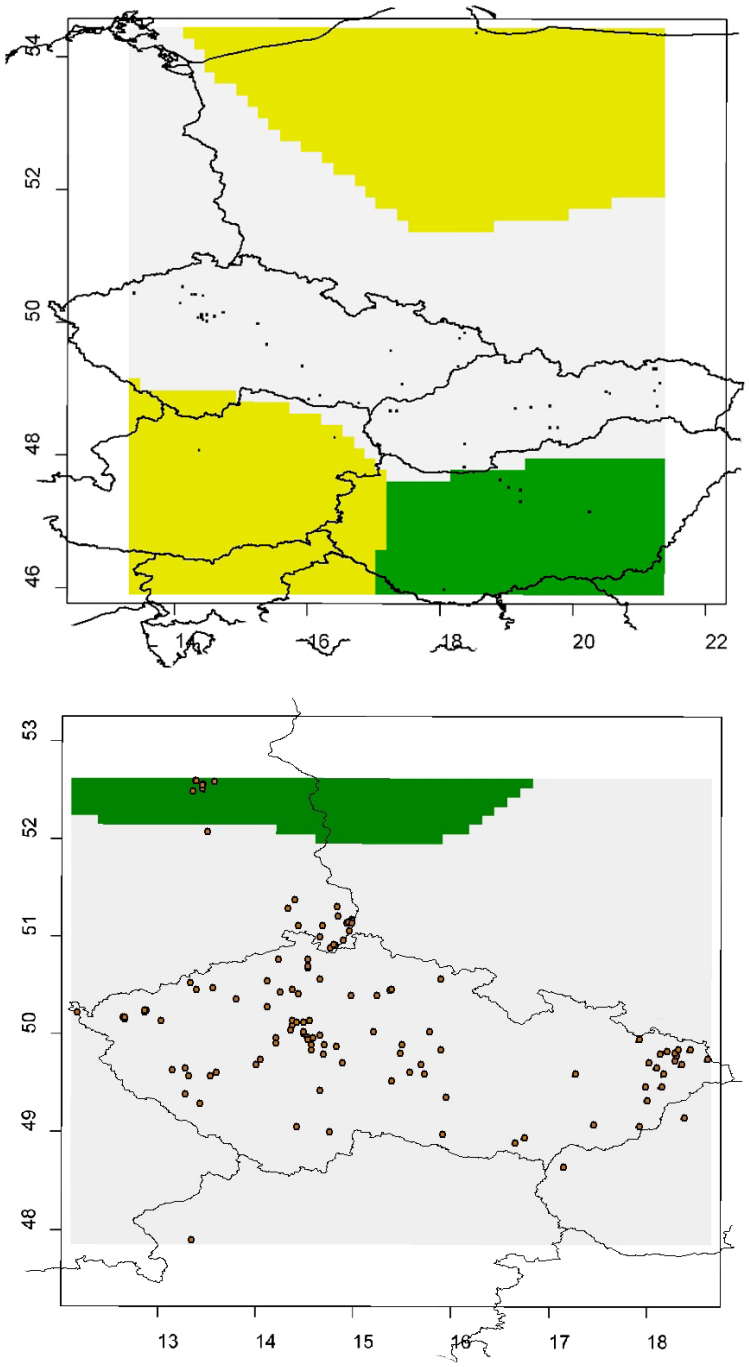

Fig. 17. Cluster membership for *E. roumanicus* (above) and *E. europaeus* (below) using nuclear data; x-axis represents longitude, y-axis represents latitude.

5. Discussion

A research of the comparative population genetics of *E. europaeus* and *E. roumanicus* was carried out by Bolfíková and Hulva (2012) in transect of the contact zone located at the area of Czech Republic. The same working group added more information about *E. roumanicus* in later work (Černá Bolfíková et al. 2017). This thesis follows up the research with aim to broaden the knowledge about the Central European contact zone by mapping the rest of the area with newly collected and processed samples.

The contact zone of hedgehogs from *Erinaceus* genus in Central Europe is well known and described in many works handling this topic. The sympatry zone in Czech Republic has been taken into account since the half of the last century. Kratochvíl (1975) performed a demographic research showing the distribution of both species in the area with overlap (map in Appendix 4). Following research did confirm the presence of the contact zone and proved the expansion of both species in the area and progressive extension of the zone (Bolfíková & Hulva 2012; Černá Bolfíková et al. 2017). We aimed to evaluate the dynamic processes of the zone with using the older dataset and simultaneously implementing the new samples to the analyses and assess the outputs. The low number of newly obtained samples did not allow considerable assessment of the dynamics but did bring a new insight to the internal structure of the populations.

One sample obtained in Berlin, Germany was classified as *E. roumanicus* according to the analyses of mitochondrial sequences but in the analyses of microsatellite data, the sample did show status of the *E. europaeus* species. This might have a cause in making of a mistake during the laboratory procedures. The D-loop was twice repeated with the same result (*E. roumanicus*). The microsatellite genotyping should also be repeated in the future for reassurance. Unfortunately, the isolation cannot be carried out again because we obtained the DNA from the hair follicles in this case and no remaining material is available. However, the presence of the *E. roumanicus* in this area is possible; we assume that this area is within the border of area of distribution of *E. roumanicus*. Lower densities of individuals of the same species in these areas could cause hybridizations (Liao et al. 2015; Černá Bolfíková et al. 2017).

Another individual from the same locality did show closer inclination to the population of *E. roumanicus* then to *E. europaeus* in the principal component analysis, although in all mitochondrial and the rest of the nuclear data analyses, it did not show such signs. More sampling in this locality is recommended for better understanding of the structure in this area. The cytonuclear incompatibility might be a sign of the interspecies interaction and propose hybridization (Barnard-Kubow et al. 2016).

Although both of the species share compatible karyotypes, not many cases of hybridization between these two species have been reported. Poduschka and Poduschka (1983) managed to breed hybrid individuals in captivity even with subsequent backcrossing with maternal species (only with *E. roumanicus*). First evidence of hybrid occurrence in nature was recorded in the Moscow region by Bogdanov et al. (2009) in the area of contact zone located in the Northeastern part of Europe. Afterwards, Černá Bolfíková et al. (2017) were first to report a hybrid individual in the Central Europe at the borders of the contact zone. We used this specimen in analyses of microsatellite data for wider data assessment. We did not detect any evidences of the hybridization among the new samples but more sampling and additional data information is necessary for obtaining clearer image of the real situation but detection of one individual in the area suggests that the reproductive isolation mechanisms are not necessarily complete and that hybridization could possibly occur in more areas, especially around the edges of the individual species' ranges.

Demographic analyses of mitochondrial data showed relatively high interspecies similarity; in case of neutrality tests, conducted for both species, none of the tests were statistically significant for *E. roumanicus*. It corresponds to findings of Černá Bolfíková et al. (2017) for the population of *E. roumanicus* located in transect of the contact zone and also to findings of Bolfíková and Hulva (2012). As for *E. europaeus*, the only significant value ($p<0.05$) was shown in case of Fu's F_S neutrality test, which is in contrast with the latter work. Also both values of this parameter were negative, opposed from Bolfíková and Hulva (2012) where only *E. roumanicus* did show a negative value, though not statistically significant. This could indicate recent expansion in each population or a presence of genetic hitchhiking. Genetic variability of both species was higher in this study compared to results of Bolfíková and Hulva (2012) and Černá Bolfíková et al. (2017).

In case of *E. europaeus* it was not very apparent with the results varying only very little; in case of *E. roumanicus* the diversity was slightly higher in comparison with the Černá Bolfíková et al. (2017) and even more with the findings of Bolfíková and Hulva (2012). Seddon et al. (2001) presented the value of the genetic diversity higher than in our results but dataset of their research included also individuals from southern areas as Greece and Balkans, where the diversity could be higher in some areas (Černá Bolfíková et al. 2017). Differences between the two species were less obvious in nuclear data, regarding the genetic variability. It might be influenced by the ploidy of data and the subsequent effective population size, which is four times higher at diploid nuclear data than in haploid mtDNA and thus, more sensitive to bottlenecks and other phenomena connected with genetic variability decrease.

Median-joining networks did show occurrence of several haplotypes in both populations in the area of the contact zone. While *E. roumanicus* showed more consistent population with most individuals belonging to two haplotype groups, the population of *E. europaeus* indicated more complex distribution with higher amount of differentiation between haplotypes. In comparison with Bolfíková and Hulva (2012), we detected higher number of haplotypes for both *E. europaeus* and *E. roumanicus*. In both cases, we had also more individuals from broader geographic area. The most common haplotype found in *E. roumanicus* was present in the majority of the sampling sites, as well as two most common haplotypes of *E. europaeus*. The sampling site localities in Germany did show high variability of haplotypes (17 haplotypes out of 25 detected were present in one or more samples from Germany), supporting the suggestion of the local variability in this area. This might be a consequence of population expansion and placement of the locality on the margin of population range, accompanied by various phenomena as allele surfing.

Both of the skyline plots also suggest recent expansion of the populations, which is in correlation with the descriptive parameters of mitochondrial data; both of these conclusions are in contradiction with the previous study by Bolfíková and Hulva (2012) when it comes to outcomes for *E. europaeus*. This fact might be a consequence of the presence of the new samples from Germany, included in analyses.

Tessellation maps (Fig. 15; Fig. 17) show a separate subpopulation containing sites located in Germany (yellow; green); according to the proposed routes of recolonization by Hewitt (2000) (see Fig. 4), the area of Germany (especially the north-eastern part, where the new sampling sites are located) could be colonized not only from the refugium located in Apennine peninsula but also from the Iberian refugium and thus, it could imply the increase of the values in case of *E. europaeus*. The high diversity in Germany population was also observed by Seddon et al. (2001). They speculate that it might be a result of the movements of refugial areas in their research of geographical distribution of mitotypes in European hedgehogs analysed by nested clade analysis. Contrary to this implication, previous studies of relationships between haplotypes did show closer proximity of the haplotypes found in Germany to the haplotypes from mainland Italy than to those found in Spain, France or UK (Santucci et al. 1998). Seddon et al. (2001) also presents division into three mitotypes of *E. europaeus* in Europe and confirms the findings of German and Italian specimens as the members of one clade; nevertheless, found samples of the individuals belonging to the western clade were also detected in the south-western Germany, indicating that the expansion from the Spain could go more easterly than expected.

Landscape analyses of mitochondrial data revealed more compound population structure in *E. europaeus* species. The most complex structure for the population of *E. europaeus* was found in the area of Germany, which could be a consequence of the already discussed origin of the samples. Not only the Italian lineage but also the lineage from Iberia is assumed to pass across the area (see Fig. 8 in Chapter 1.4.2), so the mosaic structure of the subpopulation for this species could be influenced by the fact that more lineages could form present population structure. Also the other factors of the patchy distribution might play the role; several studies conducted in UK discussed the food availability or presence of predators (Micol et al. 1994) or subsequent higher density in urban areas than in the adjacent countryside areas to possibly play a role in the distribution (Hubert et al. 2011). Nuclear data showed less differentiation in case of *E. europaeus*, dividing the individuals to only two subpopulations.

The similar output was observed in research made by Bolfíková and Hulva (2012) where microsatellite data did show only one consistent population in the examined area. They determined only one cluster covering the whole dataset in the area of Czech Republic and Slovakia. This is in agreement with our output; we detected one population including the individuals of the Czech Republic, Slovakia and the part of samples from Germany, geographically closer to the rest of the samples from the cluster; and the second subpopulation in the area of Berlin, Germany. The findings in *E. europaeus* might suggest lower sensitivity of the microsatellite markers to the recent changes in population structure, what has been proposed already by Bolfíková and Hulva (2012). The results might be biased by the dataset incompatibilities but in this case, the samples form the new dataset were present in both clusters. The more complex structure of the population of *E. europaeus* in the mtDNA data could as well be a consequence of the earlier occurrence of the species in the Central Europe than its eastern counterpart (Sommer 2007). The arrival of the hedgehogs to Central Europe is estimated to the Boreal, with the findings of fossils located also in Germany in the early Boreal, thus is could suggest relatively rapid expansion from refugia.

The situation in *E. roumanicus* was clearer, given the mtDNA data; two subpopulations were estimated for the species, dividing the Slovakia in half from north to south and continuing down to Hungary and going roughly alongside the Danube River. It could be the river, what served as a natural barrier in the area of Hungary and the presence of the mountain complex in the centre of Slovakia, what divided the subpopulations or the fact that *E. roumanicus* prefers open habitats and lowlands as indicated by Bolfíková and Hulva (2012) and areas with higher altitude present limiting barriers in this case. More detailed sampling of the open areas, mainly in regions of Poland, is recommended to find out more answers about the structure of the subpopulations in the Central Europe. The situation concerning nuclear data was particularly different. The dataset was divided into several subpopulations. The difference can be found in the assessment of samples from Hungary and Slovakia. According to the mitochondrial data, Hungarian samples cluster together with samples from eastern Slovakia, while in nuclear data outputs, the Hungarian population is restricted and the samples from Slovakia cluster with samples from Czech Republic or they are separated as well.

Nevertheless, nuclear data inconsistency between the datasets is clearly shown here and it should be taken into account especially in this case of the assessment of spatial data and the outcomes of these analyses.

The outputs of analyses did bring interesting comparison between results of older research and the latest one while implemented the new samples. The number of *E. europaeus* individuals was twice as high as in the *E. roumanicus*, so the higher frequency of *E. europaeus* agrees with the other works but in this case it could be biased by chosen sampling site localities with great mutual distances. While in previous studies the population expansion was described at *E. roumanicus* and the found populations of *E. europaeus* were more or less constant, we detected a signal for expansion in both populations, supported by significant value of Fu's F_S neutrality test in *E. europaeus* and also by Bayesian skyline plot outputs. Landscape analyses did show similar results for *E. europaeus* in assessing the outputs of both mitochondrial and microsatellite data analyses as in the original research by Bolfíková and Hulva (2012) but the differences of the outputs for *E. roumanicus* were in contradiction with the previous studies, showing more compound intra-species structure. These discrepancies are in contradiction with findings and conclusions made by Bolfíková and Hulva (2012) about less sex-biased data of *E. roumanicus*. Our data did show greater variability in diploid nuclear data over matrilineal haploid mitochondrial data in this species, suggesting the more provoked matrilineal gene flow.

The differences between results of mitochondrial and microsatellite data analyses could be influenced by several facts. We did not possess equal count of the mitochondrial sequences and microsatellites; in some samples, only one type of the data was available. Full available data was present at 234 out of 311 samples, that it slightly over 75%. Another issue was already mentioned above, concerning the incompatibility of certain older and new data. Further research will be carried out, which should handle the findings presented in this thesis, deal with the inconveniences and try to provide another, wider view, enriched by the initial results by us. The differences in the outputs of the analyses working with both types of data could be due to the mean of inheritance. While mtDNA is haploid and solely matrilineal, it does not undergo recombination and accumulates a high number of mutations. The nuclear DNA is repetitive and highly variable, diploid and is inherited from both parents. The problem in microsatellite data and fragmentation analyses is the difficulty in comparing the more datasets due to variability in processing the data, for example the determination of the sizes of loci, used devices et cetera, as presented in our research. The microsatellite data were edited several times and therefore any errors generated during the processing of the data could have an impact to the results. Use of approaches using more progressive methods of assessment is recommended in future research, such as Single Nucleotide Polymorphisms (SNPs). The main issue was the insufficiency of new samples from localities of the overlap of the two species, what should be resolved for further analyses and is being actively sought for and also the incompatibility of the datasets, where more methods should be tested to get more satisfying results and less unbiased outputs of analyses.

6. Conclusions

This thesis proposes a wider view on the contact zone in Central Europe with the sampling sites located outside of the described transect covering the area of Czech Republic, Slovakia and close surroundings. We assessed the mtDNA and microsatellite data and described demographic processes, differences between the populations and used landscape analyses to compare the distribution patterns in both species. Presented results were compared with other studies, in particular with the original research, which we based our thesis on.

We detected recent population growth according to the mtDNA analyses in both species. Higher intra-species diversity was found in the area of Germany with one species with ambiguous species affiliation but we did not detect any hybrid individual in newly evaluated dataset. We discussed differences in interpretation of results from mitochondrial and nuclear data analyses.

With newly obtained data, we were able to get the broader perspective on the area of the contact zone and evaluate it with original data. Further research should continue in our work and attempt to perform the analyses for larger amount of data from various sampling sites and handle the possibility of implementing new approaches to current methods of data assessment.

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Appendices

List of the Appendices:

Appendix 1: Table with information about all newly processed samples.

Appendix 2: Outputs from Structure given for each species separately from K=2 to K=5.

Appendix 3: Comparison of tessellation maps with maps of posterior probability of cluster membership.

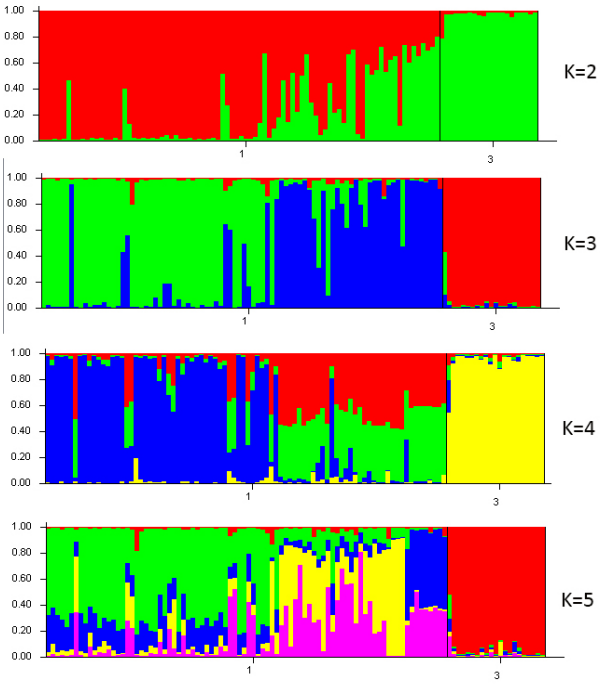
Appendix 4: Map of the contact zone in Czech Republic by Kratochvíl (1975).

Appendix 1: Table with information about all newly processed samples.

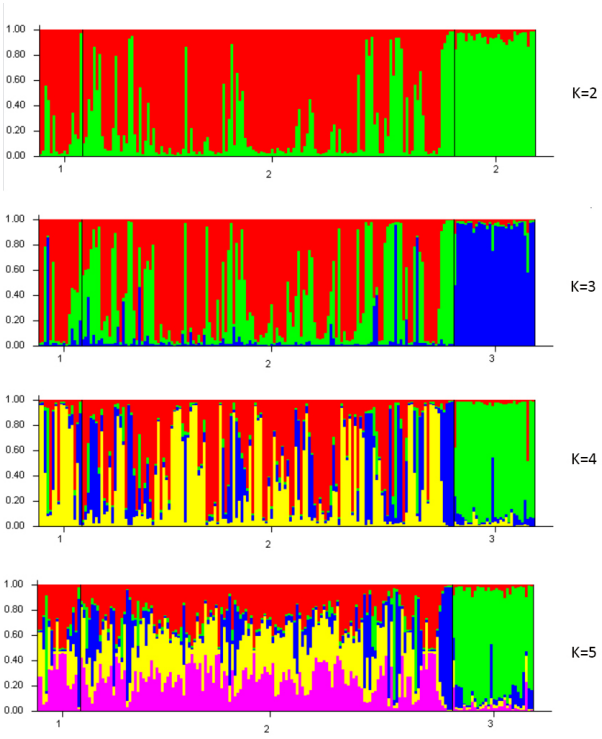
sample	species	latitude (N)	longitude (E)	haplotype	mtDNA	nuclear DNA
NMW24947	*E. europaeus*	47,90	13,34		✔	✗
NMW29796	*E. roumanicus*	48,28	16,41		✔	✗
NMW42576	*E. roumanicus*	48,08	14,37		✔	✗
B	*E. europaeus*	52,49	13,46	EE01	✔	✔
D		52,49	13,46	ER06	✔	✔
PL11	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL21	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL24	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL27	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL28	*E. roumanicus*	54,36	18,54	ER09	✔	✔
ZC	*E. europaeus*	52,54	13,47		✔	✗
M11658	*E. europaeus*	51,19	14,84	EE08	✔	✔
M11659	*E. europaeus*	51,16	14,98	EE06	✔	✔
M11660	*E. europaeus*	51,29	14,83	EE07	✔	✔
M11661	*E. europaeus*	51,04	14,95	EE07	✔	✔
M11662	*E. europaeus*	51,1	14,68	EE04	✔	✔
M11663	*E. europaeus*	50,87	14,76	EE07	✔	✔
M13478	*E. europaeus*	51,36	14,4	EE10	✔	✔
M13479	*E. europaeus*	51,12	14,92	EE06	✔	✔
M13480	*E. europaeus*	51,16	14,98	EE07	✔	✔
M13481	*E. europaeus*	51,15	14,98	EE09	✔	✔
M13482	*E. europaeus*	51,13	14,94	EE06	✔	✔
M3879	*E. europaeus*	51,12	14,98	EE07	✔	✔
M5676	*E. europaeus*	50,89	14,81	EE05	✔	✔
M5677	*E. europaeus*	52,05	13,51	EE06	✔	✔
M5678	*E. europaeus*	51,27	14,33	EE05	✔	✔
M5679	*E. europaeus*	50,95	14,89	EE06	✔	✔
M5680	*E. europaeus*	50,9	14,79	EE06	✔	✔
M9632	*E. europaeus*	50,98	14,65	EE03	✔	✔
PL5	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL19	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL20	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL22	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL13	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL23	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL29	*E. roumanicus*	54,36	18,54		✔	✗
PL47	*E. roumanicus*	54,36	18,54	ER06	✔	✔
PL52	*E. roumanicus*	54,36	18,54		✔	✗
PL54	*E. roumanicus*	54,36	18,54		✔	✗
H	*E. europaeus*	52,57	13,39	EE02	✔	✔
ZB	*E. europaeus*	52,54	13,47	EE11	✔	✔
A	*E. europaeus*	52,49	13,46		✔	✗
G	*E. europaeus*	52,57	13,4		✔	✗
J	*E. europaeus*	52,53	13,46		✔	✗
M3210	*E. europaeus*	51,1	14,43	EE06	✔	✔
PL25	*E. roumanicus*	54,36	18,54	ER09	✔	✔
PL26	*E. roumanicus*	54,36	18,54	ER10	✔	✔
PL35	*E. roumanicus*	54,36	18,54		✔	✗
PL39	*E. roumanicus*	54,36	18,54		✔	✗
R	*E. europaeus*	52,47	13,36		✔	✗
V	*E. europaeus*	52,56	13,58		✔	✗

Appendix 2: Outputs from Structure given for each species separately from K=2 to K=5.

A) *E. roumanicus*

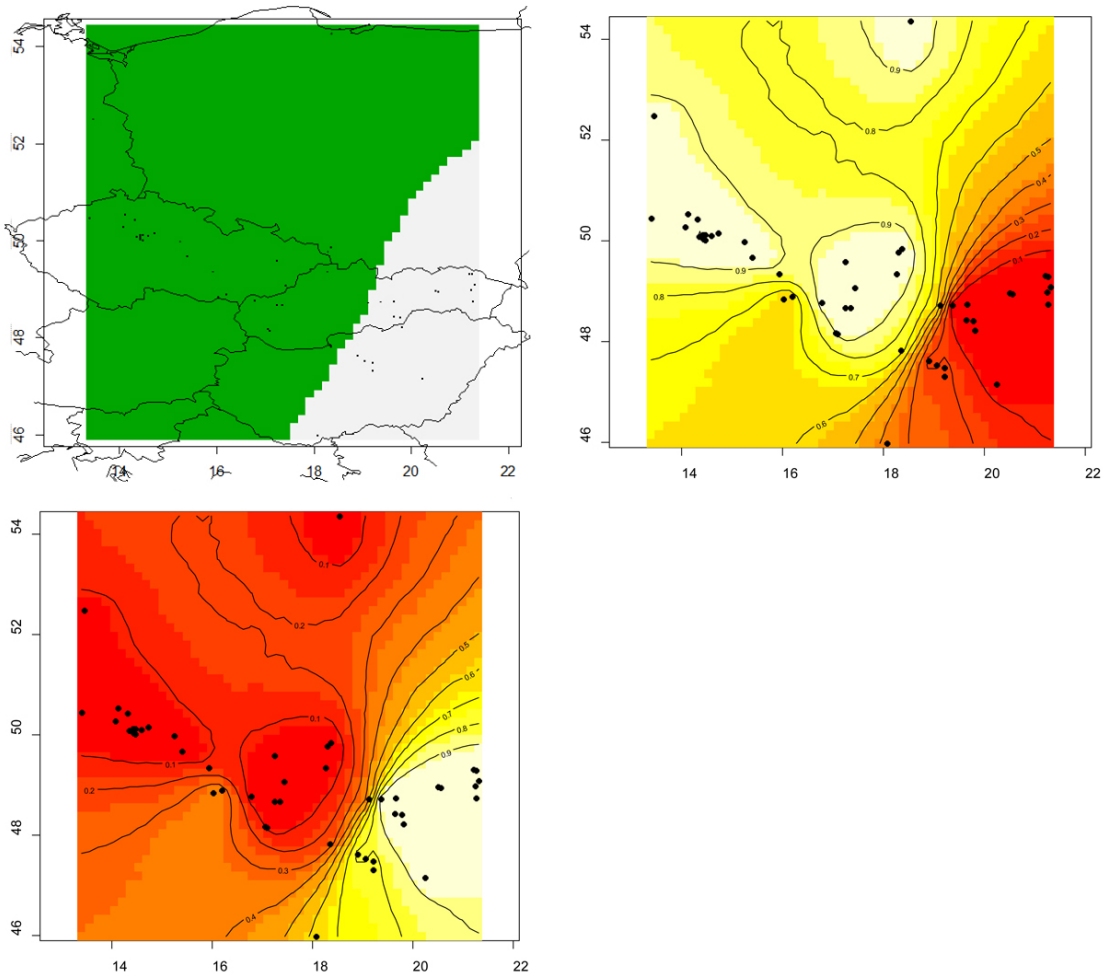


B) *E. europaeus*

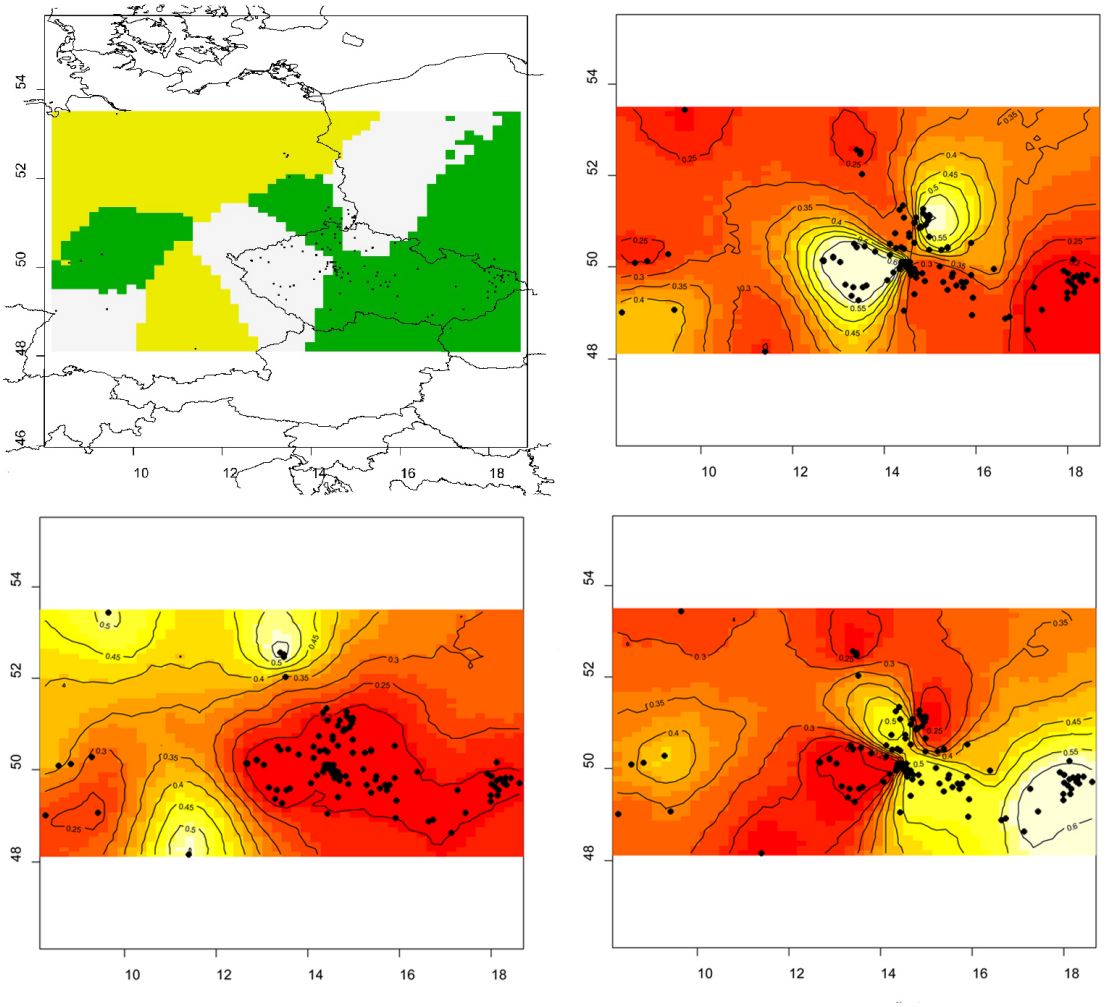


Appendix 3: Comparison of tessellation maps with maps of posterior probability of cluster membership in Geneland, using both mtDNA and microsatellite data.

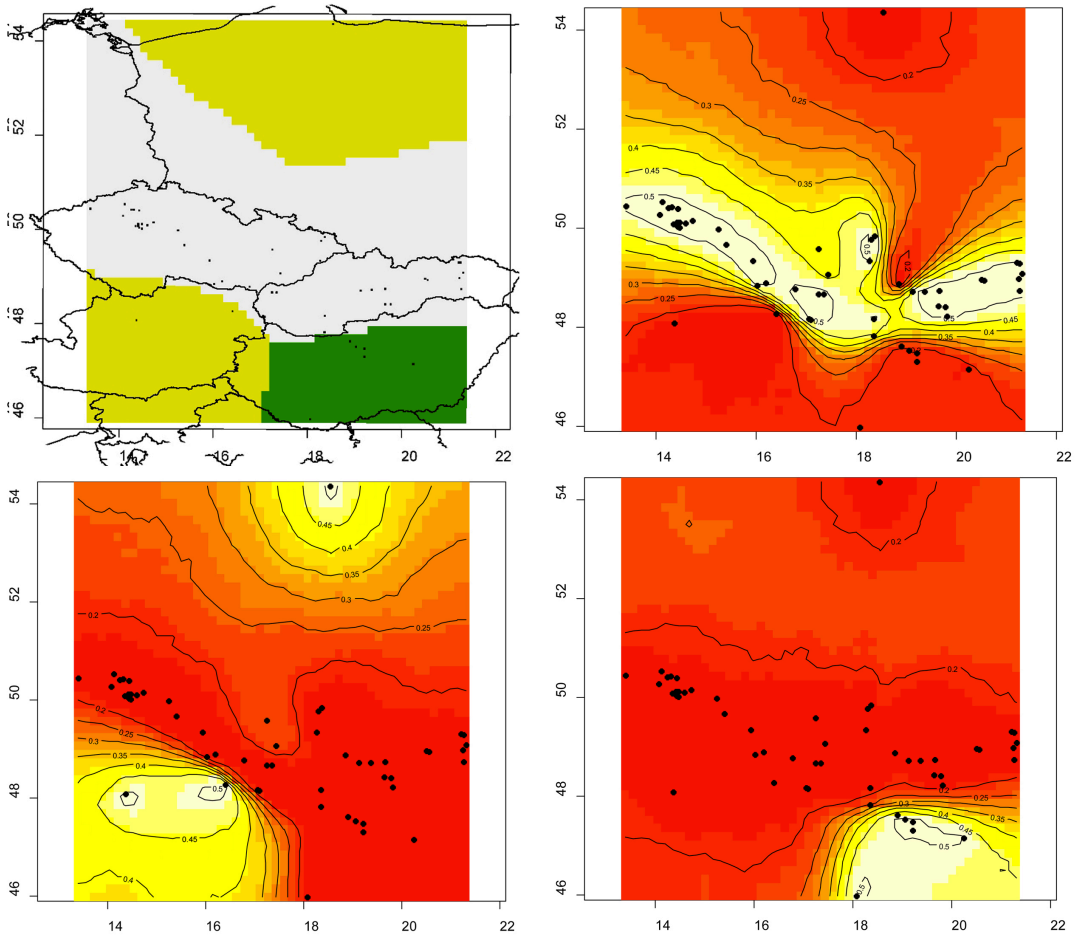
A) mtDNA, *E. roumanicus*



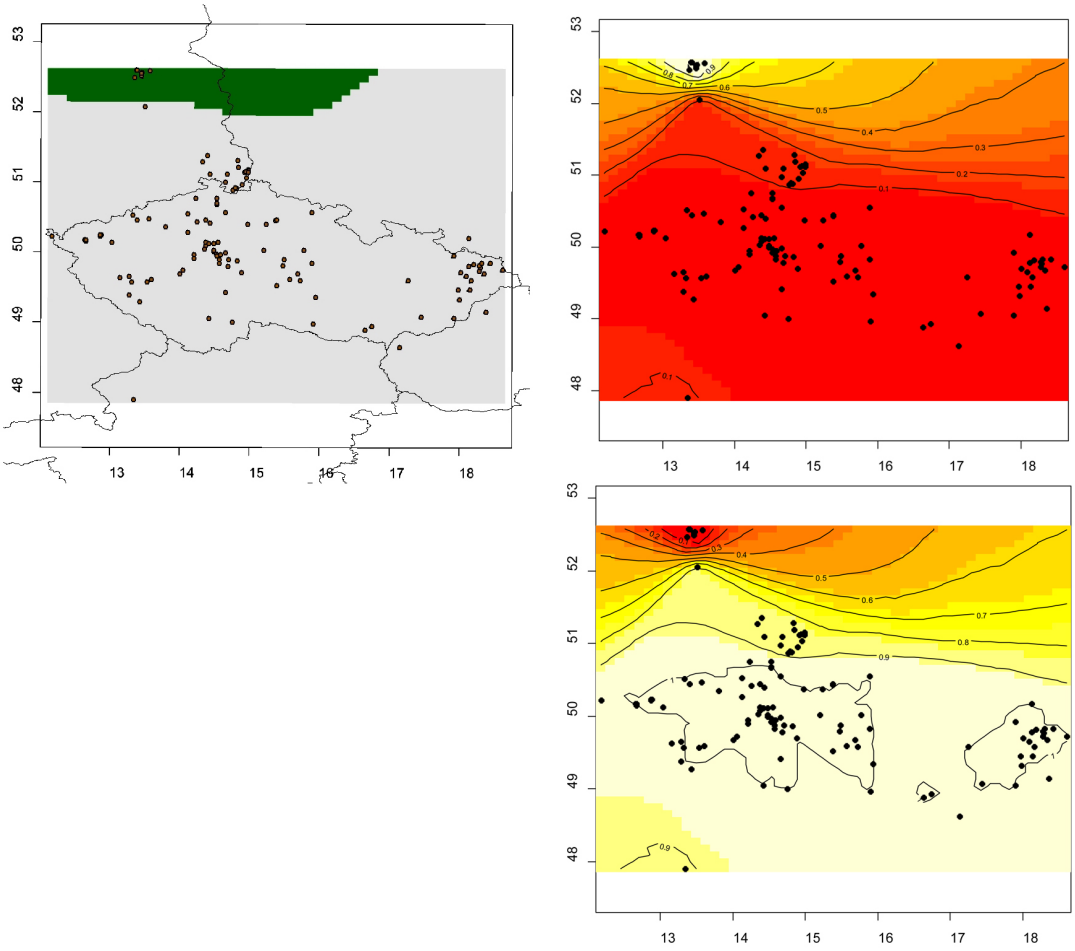
B) mtDNA, *E. europaeus*



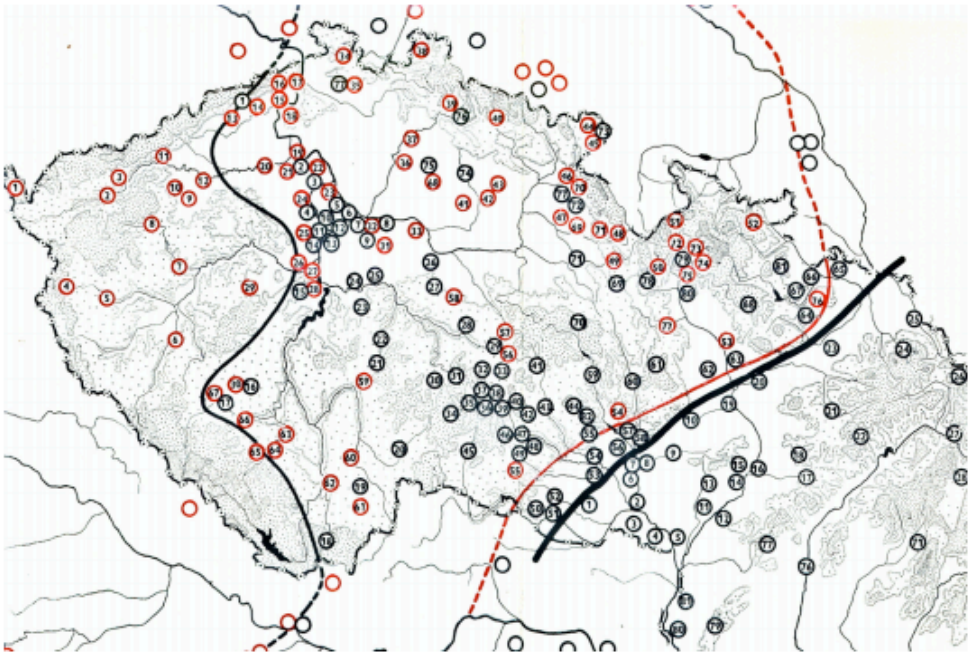
C) Microsatellites, *E. roumanicus*



D) Microsatellites*, E. europaeus*



Appendix 4: Map of the contact zone in Czech Republic by Kratochvíl (1975).



Distribution of *E. roumanicus* (black dots) and *E. europaeus* (red dots) in the area of Czech Republic in 1966.