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Faculty of Science

Phylogeography and conservation genetics of the European ground squirrel

Ph.D. Thesis

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Annotation

Phylogeography and conservation genetics of the European ground squirrel was studied in the whole range of its distribution revealing evolutionary conservation units which are important for future conservation management of the species.

Declaration [in Czech]

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Říčanová Š**, Koshev Y, Říčan O, Ćosič N, Ćirovič D, Sedláček F, Bryja J (in prep) Cryptic interglacial refugia of continental climate in Europe: Evidence from the multilocus phylogeography of the European ground squirrel (manuscript)
Štěpánka Říčanová participated in field work, laboratory work, statistical analysis and writing of the manuscript.

II. Cepáková E, **Hulová Š** (2002) Current distribution of the European souslik (*Spermophilus citellus*) in the Czech Republic. Lynx 22, 89-103
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III. Matějů J, Nová P, Uhlíková J, **Hulová Š**, Cepáková E (2008) Distribution of the European Ground Squirrel (*Spermophilus citellus*) in the Czech Republic. Lynx 39 (2), 277-294
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IV. **Říčanová Š**, Bryja J, Cosson JF, Gedeon Cs, Choleva L, Ambros M, Sedláček F (2011) Depleted genetic variation of the European ground squirrel in Central Europe in both microsatellites and the major histocompatibility complex: implications for conservation. Conservation Genetics 12, 1115- 1129 (IF=1.255)
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V. Matějů J, **Říčanová Š**, Ambros M, Kala B, Hapl E, Matějů K (2010) Reintroductions of the European Ground Squirrel (*Spermophilus citellus*) in Central Europe (Rodentia: Sciuridae). Lynx 41, 175-191
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Contents

1. Introduction

1.1. Conservation and landscape genetics

Humans have transformed much of the landscape of our planet into monoculture croplands and pastures to support 7 billion humans alive today. The replacement of wilderness by human exploited environments is causing a rapid loss of biodiversity at species and ecosystems level throughout the world (Mackay 2009). The continued growth of the human population imperils a large proportion of the wild species that now remains. Approximately 13% of birds, 20% of reptiles, 25% of mammals, 34% of fish, 41% of amphibians, and 9-34% of major plant taxa are threatened with extinction over the next few decades (IUCN 2011). In some regions as for example Europe, many species have to face to a human-altered environment and even some rodent species, usually considered to be very flexible and opportunistic, have been affected by increasing alternation of their natural environment (Smulders et al. 2003, Neumann et al. 2004, Biedrzycka and Radwan 2008).

Unlike species and ecosystem diversity, genetic diversity is still very little studied, although it is one of the three pillars of biodiversity conservation, as it was stated at the Earth Summit in Rio de Janeiro in 1992. In order to study and possibly remedy these grim facts the scientific field of conservation genetic became well established as it is today. It is an interdisciplinary science applying genetic methods to conservation and restoration of biodiversity. In fact, it represents a combination of other fields as population genetics, molecular ecology, biogeography, evolutionary biology and systematics (Freeland 2005). Genetic diversity is fundamental for conservation of biodiversity and withal it is important in conservation of species and ecosystem diversity. Further, conservation of genetic variability is important to long-term viability of populations, where decline in genetic variability leads to decrease of adaptive potential of population and reduction of fitness in changing environment.

Ultimately, loss and fragmentation of suitable habitats are the main factors that influence the genetic structure of wild populations (Garner et al. 2005, Kruckenhauser et al. 2009, Bužan et al. 2010). Landscape disconnectivity can inhibit gene flow between habitat patches, decrease the effective population size and lead to inbreeding depression (Lacy et al. 1993, Allendorf and Luikart 2007, Van Coillie et al. 2008). Inbreeding has a negative effect on fitness (Hedrick and Kalinowski 2000), as e.g. in the captive wolves affecting reduction in juvenile weight, reproduction and longevity (Laikre and Ryman

1991). Especially in small fragmented populations, the effect of genetic drift may reduce genetic variability and decrease adaptability to a changing environment (Frankham et al. 2002), and even lead to local extinction (Hedrick and Kalinowski 2000, Frankham 2005).

Extinction is a demographic process, in other words it is a failure of one generation to replace itself with a subsequent generation (Allendorf and Luikart 2007). Demography is of primary importance in managing populations for conservation (Lacy 1988, Lande 1988). There are two main types of threats causing extinction, i.e. deterministic and stochastic. Deterministic threats are habitat destruction, pollution, overexploitation, species translocation and global climate change. Stochastic threats are random changes in genetic, demographic, or environmental factors. Genetic stochasticity in form of random genetic change (genetic drift) and increased inbreeding (Shaffer 1981) leads to a loss of genetic variation (including beneficial alleles) and an increase in frequency and expression of harmful alleles. An example of demographic stochasticity is random variation in sex ratios, e.g. in the extreme the production of only male offspring. Environmental stochasticity is simply random environmental variation, such as the occasional occurrence of several harsh winters in a row.

The importance of gene flow as a means for the maintenance of genetic diversity and abolishing inbreeding depression is therefore evident and it is not surprising that one of the most common rescue-strategies adopted by conservation geneticists includes the increase of gene flow among populations (Guillaume and Perrin 2006, Hájková et al. 2007, Pertoldi et al. 2007, Hulová and Sedláček 2008, Luikart et al. 2011). In addition, the effect of population bottleneck is directly related to the increase of stochastic events associated with small population size, which leads in most cases to loss in genetic diversity (Hedrick 2005). Natural immigration and rescue effect (human-mediated) for bottlenecked populations could be a key to increase genetic variability and to reduce inbreeding. On the other hand, the negative genetic effects are not always present. For example, in the golden-mantled squirrel no evidence of increased inbreeding during or after the decline was recorded (McEachern et al. 2011) and the major factors of local population extinction risk in this species are more likely to be demographic (reproduction, immigration, predation) than genetic in origin. Despite these exceptions it is generally believed that sufficient levels of immigration and gene flow within the regional meta-population are critical for long-term viability of populations and highlight the importance of maintaining connectivity in natural populations (Garner et al. 2005, Luikart et al. 2011).

Recently, the improvements in molecular biology techniques, combined with existing or new statistical tools (e.g. geostatistics, maximum likelihood and Bayesian approaches) has given a birth to the field of landscape genetics, which is a fusion of molecular population genetics and landscape ecology (Turner et al. 2001). This discipline aims to provide information about the interaction between landscape features and microevolutionary processes, such as gene flow, genetic drift and selection. It helps to find cryptic boundaries limiting the gene flow across populations without any obvious reason, or the secondary contact zones among previously isolated and differentiated populations (Manel et al. 2003, Fontaine et al. 2007, Loxterman 2011). To understand the landscape features that limit gene flow, the spatial scales at which they act and the temporal dynamics of their effects on population substructure, it is essential to effectively use genetic data as a tool for evaluating population status and fragmentation. Using this knowledge to predict, localize and implement empirically based conservation corridors should greatly improve the successfulness of efforts to promote landscape connectivity of species at risk due to fragmentation (Segelbacher et al. 2010).

1.2. Adaptive genetic variability

Decreased fitness of individuals in fragmented populations is mainly caused by depletion in adaptive variation and increased homozygosity (Frankham 2002, Freeland 2005). There is growing evidence that maintenance of adaptive genetic diversity (i.e. adaptive potential) is particularly important for long-term survival of populations. One of the best studied examples of adaptive genetic variation are genes of the major histocompatibility complex (MHC). MHC are highly polymorphic genes in vertebrates coding molecules that bind to both self-peptide and non-self-peptide antigens, and present them to T-cells, thereby triggering a cascade of immune responses (Klein 1986, Edwards and Hedrick 1998, Meyer and Thomson 2001). MHC genes exist in a multigene family with two main subfamilies with immune function (class I and class II). Owing to differences in the pathways involved in antigen presentation, class I genes are associated primarily with intracellular pathogens and class II genes with extracellular pathogens (Jensen 2007).

Lower genetic diversity of MHC can reduce pathogen and parasite resistance or fertility (Ditchkoff et al. 2005, Lenz et al. 2009). The association of MHC genotypes or individual alleles and susceptibility to infection was recorded (Bonneaud et al. 2006, Deter et al. 2008, Schwensow et al. 2007) confirming that pressure from parasites is the primary source of selection on MHC, e.g. in sticklebacks the parasite diversity was positively associated with MHC diversity across populations (Wegner et al. 2003). To test the role of adaptive variation on fitness, MHC genes are particularly suitable (Sommer 2005, Knapp 2005, Piertney and Oliver 2006). While historical selection acting on MHC genes has been demonstrated in many wild vertebrate species (e.g. by analysis of non-synonymous and synonymous substitutions, Schad et al. 2004, Froeschke and Sommer 2005), it is more difficult to demonstrate selection acting on MHC genes in contemporary generations (Piertney and Oliver 2006). One promising approach is to compare population divergence levels identified on neutral (e.g. microsatellites) and MHC markers (Schierup et al. 2000, Bryja et al. 2007). In populations subject to heterozygote advantage or negative frequency-dependent selection (i.e. two most common forms of balancing selection), within-population MHC variability is higher compared with overall diversity, and population structure is lower at MHC than at neutral loci (Schierup et al. 2000). On the contrary, spatio-temporarily varying selection can cause stronger genetic differentiation in MHC than would be expected for neutral genes (Charlesworth et al. 1997), because some MHC alleles can be preferred in particular conditions (e.g. due to variable local compositions of pathogen communities) by local selection.

1.3. Phylogeography, evolutionary significant units and refugia

Major aim of phylogeography is to understand how historical events have shaped the recent geographical distribution of alleles, populations and species. The formulation of the field of phylogeography is dated to Avise in 1987 (Avise et al. 1987). This field can be defined as a discipline concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species (Avise 2000). Comparing the evolutionary relationships of genetic lineages with their geographical location, we can gain a better understanding of which factors have been most influential in the distribution of genetic variation. Phylogeography includes aspects of both time (evolutionary history and relationships) and space (geographical distributions). Only with the knowledge of a phylogeographical context covering the entire present species range is possible to correctly identify evolutionary significant units (ESUs), which are important for management decisions (Moritz 1994, Crandal et al. 2000, Moritz 2002, Grill et al. 2009). According to Moritz (1994) the criteria for recognition of these conservation units are that (1) ESUs should be reciprocally monophyletic for mitochondrial (mt) DNA alleles and show significant divergence of allele frequencies at nuclear loci, and 2) managements units (MU) should have a significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles (for useful critiques of the use of genetic data to define MUs, see also Taylor and Dizon 1999; Taylor et al. 2000). Further, Moritz (2002) mentioned that, in general, strategies for conservation planning should keep in mind: 1) To identify areas that maximize representation of species or historically isolated segments of widely distributed species (or both). 2) To add areas that represent specific evolutionary processes such as adaptive diversification or an admixture of historically isolated populations. 3) Within the areas identified in 1 and 2, to ensure that contiguous habitats of sufficient area are protected across major environmental gradients.

In the reconstructions of species history an expansion and a contraction of populations have been often recognised as consequences of the abrupt climatic changes in the Quaternary (Hewitt 1996, Taberlet et al 1998). Glacial cycles have been affecting the biota significantly for at least the last 2,5 millions year (Agustí and Antón 2002). Within Europe the fossil and DNA evidence show that the peninsulas of Iberia, Italy and the Balkans were both major centres of endemism as well as major refugia which contributed variously but significantly to the postglacial recolonization of the more northern regions (Taberlet 1998, Hewitt 2004, Randi 2007). Southern glacial refugia and the northward postglacial recolonization of central and northern Europe from these areas has become an established biogeographical paradigm (Hewitt 2000). The typical scenario suggests reduced and fragmented populations surviving in southern refugia during the glacials. However, cold-adapted species like those living in the steppe or tundra habitats follow an opposite scenario and during interglacial period are restricted to their northern refugia (Stewart et al. 2010). Furthermore, cryptic refugia were described for many species as small islands with suitable conditions in the otherwise unfavourable surrounding areas (e.g. the southern European mountains for cold-adapted species; e.g. the rock ptarmigan (Holder et al. 1999) or the Arctic hare (Angus 1983); or the Carpathian basin for temperate species; e.g. the bank vole (Kotlík et al. 2006)). These classical and cryptic refugia are usually defined on the latitudinal direction because they are related mainly to temperature which has a latitudinal gradient.

Recently, Stewart et al. (2010) introduced a longitudinal perspective to the definition of refugia considering that the gradient of oceanic-continental climate was also significantly variable during Pleistocene glacial cycles. Some species may be adapted to the oceanic climate, which is more humid and less seasonally variable, while others require drier climate with pronounced seasonal variation. To my knowledge, the empirical testing of such hypothesis is still missing.

1.4. The European ground squirrel as a model species for conservation genetics and phylogeography

1.4.1. The European ground squirrel as Central European model for conservation genetics

The European ground squirrel (EGS), *Spermophilus citellus*, Linnaeus 1766 belongs to a monophyletic genus within the Marmotini tribe of the family Sciuridae based on mitochondrial DNA cytochrome *b* analyses (Harrison et al. 2003, Herron et al. 2004). According to a new revision (Helgen et al. 2009) also using mtDNA, *Spermophilus* sensu stricto is restricted only to Eurasia, while other genera are erected or resurrected for the remaining majority of species in North America. The EGS is distributed in Central and Southeast Europe and its distribution is divided into two geographic areas separated by the Carpathians. More specifically it ranges in the north from the west of the Czech Republic and Austria till the western part of Ukraine in the east and in the south from northern Greece to the European part of Turkey, and including all countries in between except those bordering the Adriatic sea (Mitchell-Jones et al. 1999, Wilson and Reeder 2005, see Fig. 1).

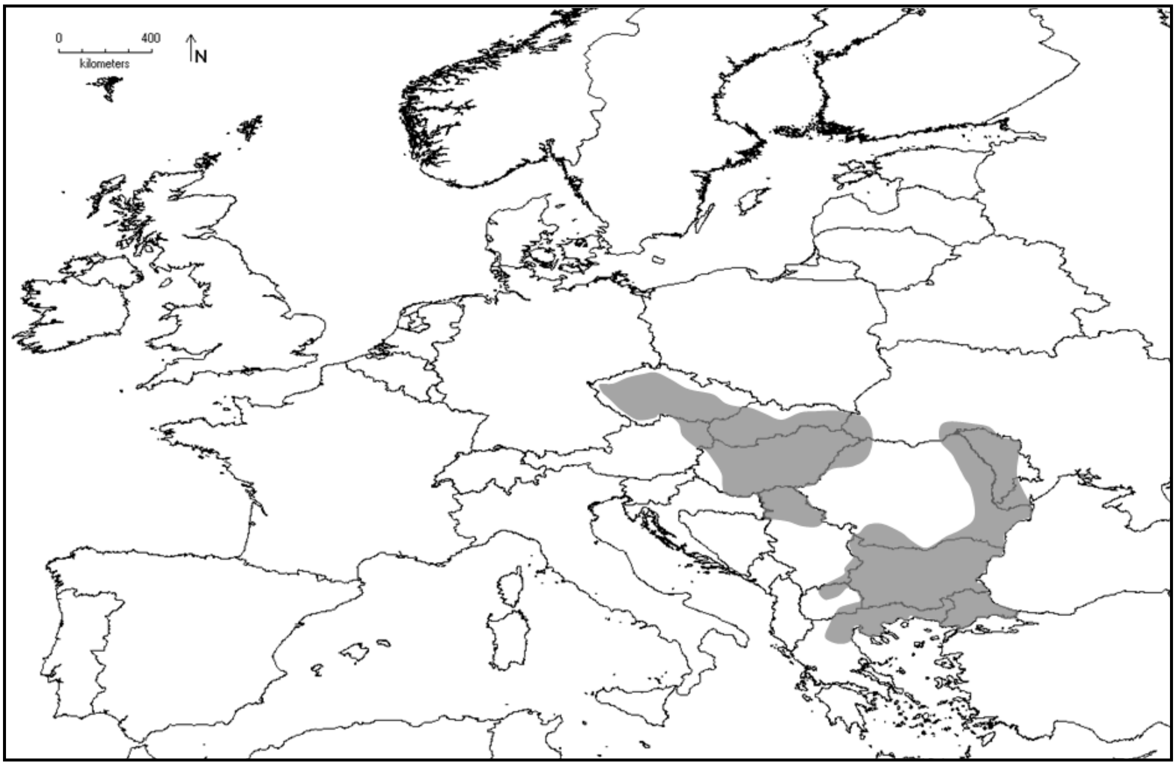

Fig. 1 Current distribution of the European ground squirrel (*Spermophilus citellus*) shown by dark colour, modified from Mitchel-Jones et al. (1999).

A few decades ago the westernmost boundary of its distribution was in southeast Germany (Feiler 1988) and northernmost edge was in Poland (Męczyński 1985). Due to the extinction in these countries the Czech Republic became the westernmost and northernmost range of the natural EGS distribution. Recently it was reintroduced in Poland at two localities (Kamien Śląski and near the village Głębowice) (Matějů et al. 2010a).

The EGS is bound to steppe and steppe-like habitats, e.g. fields, meadows, pastures, balks, banks, tracks (Matějů et al. 2008). It hibernates during winter and survives only from fat reserves as it does not store corns or dry grass as for example the common hamster or the pika (Wilson and Reeder 2005). The diet consists mostly of grass (*Festuca, Poa, Agropyron, Stipa*, etc.) or different parts of plants (*Achillea, Cardus, Trifolium, Medicago*, etc.). During mating season and lactation females enrich their diet by insects (Coleoptera, Lepidoptera, Saltatoria) or even small vertebrates (Grulich 1960, Herzig-Straschil 1976).

Within Central Europe in the Czech Republic, the species lives in colonies with a few (20-50 animals) up to several hundreds of individuals, virtually all of which are in highly anthropomorphic habitats (Matějů et al. 2008). In other countries as Romania or Bulgaria it lives in colonies with a much higher abundance and in more natural steppe habitats (e.g.

Koshev 2008). The EGS used to be considered as a pest likewise the common hamster, but agrotechnical measures and direct persecution (deep ploughing, consolidation of fields, chasing and capture, targeted killing) had a fatal impact on this species (Grulich 1960) and caused that the EGS has become a rare and endangered species in the Czech Republic (Hulová and Sedláček 2008, Matějů et al. 2008). A decrease in abundance of the ground squirrel was also monitored in other European countries as in Slovakia (Ambros 2000, 2008) or even in Bulgaria (Koshev 2008). This species is endangered in many countries through its natural range and is protected under the Red List of Threatened Species IUCN (Coroiu et al. 2008) and by the European directive NATURA 2000.

Population declines connected with the reduction of genetic diversity was recorded in relative species of ground squirrels, such as *S. suslicus* (Biedrzycka and Konopiński 2008) and *S. brunneus* (Garner et al. 2005), or in the marmot *Marmota vancouverensis* (Aaltonen et al. 2009, Kruckenhauser et al. 2009). The main threat for the above mentioned species is usually habitat loss and very similar situation exists for EGS. Mainly in the Czech Republic large-scale habitat fragmentation, disturbance of natural migration corridors in the landscape and the loss of permanent grassy areas caused that suitable habitats for *S. citellus* became very scattered (Cepáková and Hulová 2002, Hulová and Sedláček 20008, Matějů et al. 2008). Consequently, the original population has become fragmented into small local populations, some of them too small to be able to persist in the long-term.

Habitat fragmentation could have affected neutral and adaptive variability in the EGS in Central Europe and to study such phenomenon two different markers were employed in this thesis. Firstly, microsatellite loci were used as neutral nuclear marker. They are known as simple sequence repeats (SSRs) or short tandem repeats (STRs), and consists of repetition units of 1-6 base pair of DNA. Microsatellites are widely used as molecular maker in studies of kinship, population structure, gene flow, and others (e.g. Gauffre et al. 2008, Mora et al. 2010, Patzenhauerová et al. 2010, Meshiry et al. 2011). Microsatellites developed for particular species can often be applied to closely related species, but the percentage of loci that successfully amplify may decrease with increasing genetic distance among species. For the EGS were used 12 loci designed originally for other sciurid rodents: *Marmota marmota* (Hanslik and Kruckenhauser 2000), *Spermophilus brunneus brunneus* (May et al. 1997) and *Spermophilus suslicus* (Gondek et al. 2006). Unfortunately, some of them have limitations known as null alleles due to primer failure in polymerase chain reaction (PCR), where the heterozygous individuals being scored as homozygous. Null alleles complicate the interpretation of microsatellite allele frequencies and thus make estimates of relatedness faulty. For more details and analysis of data please see attached papers I and IV.

Secondly, the exon 2 of the DRB gene (MHC class II) was used as a functionally important gene under selection. The exon 2 of the DRB gene is of special interest, because a part of this exon encodes the antigen binding sites (ABS) responsible for the recognition of antigens. It has been shown to have high levels of variation not only in the number of alleles but also in the extent of sequence variation between alleles (Hughes and Yeager 1998). In particular, the ABS sites display more non-synonymous than synonymous substitutions, which change the amino acid sequence of the peptide and thus allow binding of a diverse array of antigens (Brown et al. 1988, 1993). More details and information on data analysis are in attached paper IV.

1.4.2. The European ground squirrel as a model in phylogeography

So far, there is only one published study about the range-wide genetic structure of the EGS carried out by Kryštufek et al. (2009). This study is based on a limited number of populations and individuals and, more importantly, on a single genetic marker, i.e. mitochondrial DNA (cytochrome *b*) resulting in the fact that the phylogeography of the species is not sufficiently resolved. Kryštufek et al. (2009) revealed two major phylogenetic lineages, called northern and southern groups, with a distinct sublineage of the northern clade mentioned from Macedonia (Mountains of Jakupica). The divergence time between the two main lineages was estimated at 0.5 Mya based on 2% of mutation rate per million years and a possible contact of these lineages is reported from Romania (Gündüz et al. 2007, Kryštufek et al. 2009). The main result is pointed out as the refugium of this steppic taxa is evidenced in the southern Balkan, which is in general agreement of the traditional Mediterranean refugia (Hewitt 2000, Randi 2007).

In last decades there is a growing evidence of cryptic northern refugia in addition to the traditional southern refugia for a number of small mammal species including the field vole (Jaarola and Searle 2002), bank vole (Deffontaine et al. 2005, Kotlík et al. 2006) or pygmy shrew (Vega et al. 2010). Further, Stewart et al. (2010) recently introduced a longitudinal perspective to the definition of refugia and they argue that the gradient of oceanic-continental climate was also significantly variable during Pleistocene glacial cycles.

Stewart et al. (2010) mentioned the ground squirrels as one of the examples of taxa (together with the pika and the saiga antelope) that have more restricted continental distribution in Palaearctic today than during some parts of the last glaciation. Interglacial refugia for continental-adapted taxa might be distributed mainly in steppes north of Caspian Sea, with cryptic western refugia in south-eastern Balkans or in Pannonia. The definition of a continental refugium is based only on comparison between the fossil record and modern distribution (Musil 1985), and no phylogeographic study testing this scenario is available to this date. The EGS seems to be a good candidate to search for the above mentioned continental refugium being a steppic species with continental distribution.

In order to reconstruct the species phylogeographic history it is optimal to use a broad variety of molecular data (including nuclear, cytoplasmatic, sex-linked, autosomal, conserved, neutral, high and low mutation rate). Many recent studies do provide such combined multiloci approach (e.g. mtDNA and microsatellite data) demonstrating the advantages of getting better insight into pre- and postglacial dynamics of the genetic structure in model species (Howes et al. 2006, Flanders et al. 2009, Bryja et al. 2010). Traditionally, the mtDNA gene of cytochrome *b* was used to study phylogeography of rodents. Cytochrome *b* is commonly used to determine phylogenetic relationships between organisms due to its sequence variability. It is considered to be most useful in determining relationships within families and genera. Comparative studies involving cytochrome *b* have resulted in new classification schemes and have been used to assign newly described species to a genus, as well as deepen the understanding of evolutionary relationships. Based on longevity theory divergence rate in cytochrome *b* varies across mammal species, third codon positions are renewed every 1-2 Myr, in average in the fastest evolving mammals, whereas it takes >100 Myr in slow-evolving lineages (Nabholz et al. 2008). Further, multivariable markers such as microsatellites are broadly used for gene flow estimation and investigation of current structure of populations, but for the purpose of phylogeography, they are less suitable because of their homoplasious mutations (Estoup et al. 2002, Hewitt 2004). On the other hand, Estoup et al. (2002) showed that homoplasy at microsatellites is not a significant problem for population studies because of the large amount of variability at microsatellite loci usually compensates their homoplasious evolution. More details on data analysis are in attached manuscript (Paper I).

1.5. Sampling method

To realize and to study all above mentioned aspects, the EGSs were needed to be captured and sampled for DNA analysis. The method as watering (Grulich 1960), I rejected as inhuman. I usually used live traps designed for rat or tubes (according to Koshev, pers. comm.), see Fig. 2 or snares. Individuals were captured during their active season, usually from April to September and a small piece of ear was cut off and stored in 96% ethanol for DNA extraction. Also I focused to sample the whole colony avoiding sampling of only one family group.

Fig. 2 Life traps used for catching of the European ground squirrel, usually baited with apples.

1.6. Aims of the dissertation project

The knowledge of genetic variation and structure of EGS is necessary for its successful conservation management. Therefore, the aims of this project were following:

1) By using molecular markers to reveal phylogeography of the EGS and to test the hypothesis of continental cryptic refugia for this steppic taxon in Europe.
2) To collect available information of the EGS distribution and changes in abundance of the populations in the Czech Republic.
3) To assess genetic diversity and level of population structure of EGS in Central Europe, where the vast changes in agriculture and landscape alternation have taken place and where the populations of EGS are strongly declining.
4) To review the reintroduction and translocation attempts within Central Europe in the past and to suggest a successful procedures for the future.

2. Summary of contributions of the presented work to the state of knowledge and conservation of the European ground squirrel in Europe

2.1. Historical biogeography and diversity of the EGS in its whole distribution area reveal cryptic continental refugium in Europe

Based on a unique sampling, which has demanded five field trips throughout the natural range of the EGS and contacts with many local researchers, it was accomplished that tissue samples from almost one thousand individuals covering the complete distribution area of the species are available for genetic analyses. This facilitated a first detailed phylogeographic study of the species using a combination of mtDNA (cytochrome b gene) and nuclear genetic markers (12 microsatellites).

Based on collected material the **paper I (Říčanová et al., in prep.)** could be realised. The classical and cryptic refugia are mainly related to temperature and defined within a latitudinal gradient, but also a longitudinal perspective of refugia is here considered. The gradient of oceanic-continental climate was significantly variable during glacial cycles affecting biota. Range-wide phylogeography of the EGS was used to interpret the evolutionary and paleogeographic history of the species in Europe and to shed light on its glacial-interglacial dynamic. The EGS is a steppe-inhabiting species and the westernmost member of the genus in the Palaearctic region.

The reconstructed phylogeography divides the species into two mitochondrial groups, the Southern and the Northern with deep structure within both groups. The ancestral area of the species with the highest genetic diversity is found in Bulgaria. The Southern group originated in the steppes of Bulgaria and European Turkey, while the Northern group in southern Serbia and northern Macedonia. The Northern group of the EGS, after reaching the Pannonian steppes by a single dispersal event across the Carpathian mountain chain survived in southern part of Pannonia throughout several glacial-interglacial cycles and probably repeatedly colonized areas further to the north and west during the glacial periods, while in the interglacial periods contracted back to this trans-Carpathian refugium. The EGS thus represents a species with a glacial expansion/interglacial contraction paleogeographic dynamics, and the Pannonian and south-eastern Balkanian steppes are supported as (usually overlooked) cryptic refugia of continental climate throughout Pleistocene interglacials.

2.2. *Situation of EGS in the Czech Republic, the most fragmented area*

Detailed distribution of the EGS within the Czech Republic was summarised in two papers (**Cepáková and Hulová 2002**, **Matějů et al. 2008)** giving source data for preparation of the Action plan for the species (**Matějů et al. 2010b**, see part 3). In **paper II (Cepáková and Hulová 2002)** occurrence of the EGS in the Czech Republic was summarized and recorded at or reported from 83 sites between 1990-2001. The species inhabits mostly regularly managed grassland areas and only several sites with natural or semi-natural steppe-like habitats. Although two sites were situated in the altitude of 640 m a. s. l., the majority of the records (64%) were between 200 and 340 m a. s. l., i.e. in lowlands. Of the total, only 26 sites were verified to host EGS colonies in the years 2000-2001. The size of most colonies is very small, exceeding the estimated abundance of 100 individuals in seven sites only. Considering the fact that 30 sites were abandoned in the period 1990-2001, it can be concluded that the decline of the EGS and fragmentation of its range in the Czech Republic has been continuing. As evident from the list of sites, the species inhabits mostly grassland areas regularly managed by man such as airfields, camping sites, a golf course, etc. Farmland habitats, typical of the species in the past (Grulich 1960), are not used any more. The reason is the absence of regularly mowed or pastured plots (balks, meadows, orchards, etc.) caused by intensification of agricultural management in the last decades. Natural or semi-natural steppe-like habitats are occupied only in several localities.

In **paper III (Matějů et al. 2008),** the occurrence of the EGS was recorded from 45 sites in the Czech Republic between years 2002–2008. However, only 35 sites were verified to host ground squirrel colonies in 2008. Again as in previous paper III, most of the colonies (91%) were found in some kind of artificial habitat with high anthropogenic influence. Only 9% of the EGS colonies occurred in natural steppe habitats. Although three sites (9%) were recorded at altitudes above 550 m, the majority of the sites (60%) were situated below 300 m a. s. l. In more than half of the colonies (54%), the estimated abundance was 50 individuals or less. Only one third of the colonies hosted 100 individuals or more. Considering the fact that seven sites were abandoned in the period 2002-2008, it can be alarmingly concluded that the decline of the EGS in the Czech Republic continues. The higher number of known populations was not caused by migration and founding of new colonies, but by higher research activity on the species and finding of new relict colonies mainly in southern Moravia.

2.3. The role of fragmentation on genetic architecture of EGS populations in Central Europe

Ground squirrels collected in the highly anthropogenic landscape of Central Europe, namely the Czech Republic, Slovakia and Hungary, were studied genetically in order to assess the role of fragmentation level on the genetic structure and variation of populations. This study is presented in **paper IV (Říčanová et al. 2011).** Specifically, two types of molecular markers were used to test how fragmentation affects the genetic architecture of presumably selectively neutral (12 microsatellites) and non-neutral (the MHC class II DRB gene) loci. In total 382 individuals were analysed from 16 populations in this part of Central Europe, covering almost half of its natural range. Spatial genetic analyses defined two groups of populations, “western” (the Czech Republic and extreme west of Slovakia) and “eastern” (Slovakia and Hungary), with a significantly higher level of habitat fragmentation in the former group. The highly fragmented western populations had significantly lower genetic diversity in both types of markers. Only one allele of the DRB gene predominated in the populations of the western group, while four alleles were evenly distributed across the eastern populations. Higher values of coefficient of inbreeding were observed in the western than in the eastern populations. Inter-population differentiation was very high, but similar in both groups. Comparison of genetic variability and structure on microsatellites and the DRB gene does not provide any evidence for contemporary selection on MHC genes. Probably genetic drift in small bottlenecked and fragmented populations may overact the role of balancing selection.

2.4. Reintroductions of the European ground squirrel in Central Europe

Knowledge of disappearing of the EGS from landscape has motivated a few introduction or translocation attempts in the past (Jansová 1992, Budayová 1995, Hapl et al. 2006). In **paper V (Matějů et al. 2010a)** we collected primary data on 13 European ground squirrel reintroduction projects carried out in the Czech Republic, Slovakia and Poland since 1989. During these projects more than 3,200 ground squirrels were reintroduced at 15 sites or used for reinforcement of five populations. Reintroductions can be considered successful at seven sites where settlement and reproduction of the released individuals were observed. At other seven sites reintroductions failed and the result of reintroduction is still unknown at one site. Proportion of long existing reintroduced colonies is even much lower. Results of reinforcements are unclear at all five sites. The main problems of reintroductions were the low number of released individuals, unsuitable methodology of releasing and inappropriate site management. For future reintroduction attempts we recommend to release a sufficient number of individuals and to use artificial burrows as well as temporary fencing of the site of release. Long-term management of the site and regular monitoring of the newly established population are also necessary.

3. Conclusions and future prospect

During the whole project dealing with the EGS in the last decade (including the work on this thesis) very interesting and important aspects have appeared. A first detailed view on genetic variability and structure in the whole natural range is presented here including completely new information about history of the EGS and a novel view on refugia suggesting the presence of refugium of continental climate in Europe. The EGS has a deep history not only in Balkan representing an ancestral area, but also in Central Europe based on fossil records but also on mitochondrial lineages which are there still present. The EGS is a taxon responding to expansion of steppe habitat and being in refugium during interglacial period.

The optimal strategy for conservation management of the EGS would be a cooperation of Nature Protection Agencies on international level. The main topics of recent threats are probably a lack of grazing animals as sheep, goat or cattle or regular moving at EGS localities. Villages and countryside are more abandoned and the meadows are exposed to a rapid succession, which is not a problem only in Central Europe but also in Balkan. For sure, a special attention should be paid to status of populations in Balkan area as ancestral area conserving the most genetic variability, but also the situation in Central Europe. The area of Balkan would be worth to continue with a study focusing on detailed landscape genetic and gene flow.

Within Central Europe, it is highly recommended to avoid mistakes from the past translocation or reintroduction attempts summarized in **Matějů et al. (2010a)**. It is necessary to release a sufficient number of individuals and to use artificial burrows as well as temporary fencing of the site of release (a soft version of releasing method). Long-term management of the site and regular monitoring of the newly established population are also necessary. Very important would be to succeed in reproduction of the EGS in captivity in cooperation with ZOOs and not to use animals from recent colonies.

Based on results of monitoring carried by Agency of Nature Protection and the summary of distribution of the EGS in the Czech Republic in the two last decades (**Cepáková and Hulová 2002**, **Matějů et al. 2008**) and the first genetic study of the EGS (**Hulová and Sedláček 2008**), the outline for an Action plan was laid out and eventually the Action plan for the EGS in the Czech Republic was created (**Matějů et al. 2010b**). The populations of the EGS have been monitored from the year 2002 systematically.

Within the action plan both long-term and medium-term aims were established.

The long-term aims are:

1) Ensure the existence of the EGS in the highest possible number of existing localities, while focusing on sites of community importance and more numerous populations with a positive development perspective and with the possibility of enlarging the area of the colony.

2) Create a total of five metapopulation systems of the EGS occurrence in the Czech Republic, with a total number of at least 2,500 individuals in each of the five metapopulations for at least 10 years. Considering local conditions, these metapopulations will be established in two ways:

a) Allowing for the natural spread of the EGS (i.e. by the settlement of young individuals to the surroundings), supported by creating suitable conditions in areas near more numerous colonies.

b) Establishing new colonies by distributing individuals bred in semi-natural breeding programmes in the vicinity of existing colonies, and at the same time, by preparing areas that will allow for an exchange of individuals between existent and newly created colonies.

The medium-terms aims have been defined for the first 10 years upon adopting the Action Plan:

1) Create conditions to maintain EGS numbers in the Czech Republic.

2) Gather information on known and possibly not yet known localities of the species.

3) Predict suitable localities for the natural spread and re-introduction of the EGS.

4) Establish at least one prosperous, semi-natural breeding programme of the EGS.

5) Complete information of the EGS biology and ecology in the Czech Republic (detailed research of genetic variability and structure, parasitofauna and demographics).

6) Ensure education and awareness at the local and regional levels.

This Action Plan has been currently in progress and it is considered as a basic tool to sustain EGS population in the Czech Republic. To accomplish the aims, the results from **Říčanová et al. (2011)** should be taken seriously and should be “brought to life”. Within Central Europe, namely the Czech Republic, Slovakia and Hungary, two groups have been considered. Surely, these two groups were created relatively recently due to genetic drift, they do not represent two ESUs (based on phylogeography). Based on the resulting risk of inbreeding depression in the western populations (Czech populations), population management by crossbreeding between the western and eastern (Slovakian and Hungarian) populations was suggested. Since creating a net of corridors is highly unrealistic in the current Central European anthropogenic landscape, it would be necessary to substitute natural migration with introductions and/or translocations of individuals between western and eastern populations. This could recover genetic variability and increase the adaptive variation of EGS colonies, and thus prevent the extinction of the species in Central Europe.

Here, the EGS represents a perfect model species where we can track changes in viability of colonies and populations in time. We know the situation from certain period and when any translocation or introduction of individuals would be realized, we can monitor the development.

Further, together with tissue samples, I have collected faeces which were investigated for parasites as Coccidia, Nematoda and Cestoda. So far, we have just preliminary data (J. Kvičerová, preliminary results), but we suppose to look on their distribution throughout the whole EGS range which has been sampled. Another lack of information is in field of demography of recent colonies and correlation of demographic development and dynamics with genetic variability.

During the project I had an opportunity to visit and see all types of habitats where the EGS occurs, i.e. lowlands along the Danube river or high elevated populations (over 2000 metres a.s.l.) in Macedonia and Bulgaria, or very anthropogenic sites in Central Europe. The area of Moldova has turned to be a very interesting place, where two species of ground squirrels meet, i.e. *S. citellus* and *S. suslicus*. Our observations and preliminary results (mtDNA) indicate that these two species could hybridize (Š. Říčanová and Y. Koshev, unpubl. data). With cooperation with Bulgarian colleague we noticed distinguishable differences in vocalization between *S. citellus*, *S. suslicus* and their possible hybrids. The question of interspecific hybridization surely needs to be studied further.

Finally, I am positive that the unique sampling (one thousand individuals) of the EGS represents the greatest collection covering its whole natural range. The tissue samples (in 96% ethanol) and also stored extracted DNA constitute a valuable material and a broad base for future studies using newly emerging molecular techniques (like next-generation sequencing).

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5. Attached papers

Paper I

Říčanová Š, Koshev Y, Říčan O, Ćosić N, Ćirović D, Sedláček F, Bryja J (in prep) Cryptic interglacial refugia of continental climate in Europe: Evidence from the multilocus phylogeography of the European ground squirrel (manuscript)

Cryptic interglacial refugia of continental climate in Europe: Evidence from the multilocus phylogeography of the European ground squirrel

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Abstract

The classical and cryptic refugia are mainly related to temperature and defined within a latitudinal gradient, but also a longitudinal perspective of refugia is here considered. The gradient of oceanic-continental climate was significantly variable during glacial cycles affecting biota. Range-wide phylogeography of the European ground squirrel (EGS; *Spermophilus citellus*) was used to interpret the evolutionary and paleogeographic history of the species in Europe and to shed light on its glacial-interglacial dynamic. The EGS is a steppe-inhabiting species and the westernmost member of the genus in the Palaearctic region. We analyzed almost one thousand specimens of the species throughout its present natural range by employing mitochondrial DNA sequences of the cytochrome *b* gene and 12 nuclear microsatellite markers. The reconstructed phylogeography divides the species into two mitochondrial groups, the Southern and the Northern with deep structure within both groups. The ancestral area of the species with the highest genetic diversity is found in Bulgaria. The Southern group originated in the steppes of Bulgaria and European Turkey, while the Northern group in southern Serbia and northern Macedonia. The Northern group of the EGS, after reaching the Pannonian steppes by a single dispersal event across the Carpathian mountain chain survived in southern part of Pannonia throughout several glacial-interglacial cycles and probably repeatedly colonized areas further to the north and west during the glacial periods, while in the interglacial periods contracted back to this trans-Carpathian refugium. The EGS thus represents a species with a glacial expansion/interglacial contraction paleogeographic dynamics, and the Pannonian and south-eastern Balkanian steppes are supported as (usually overlooked) cryptic refugia of continental climate throughout Pleistocene interglacials.

This manuscript will be submitted next month in Molecular Ecology.

Paper II

Cepáková E, **Hulová Š** (2002) Current distribution of the European souslik (*Spermophilus citellus*) in the Czech Republic. Lynx 22, 89-103

Abstract

Occurrence of the European souslik was recorded at or reported from 83 sites in the Czech Republic in 1990.2001. The species inhabits mostly regularly managed grassland areas and only several sites with natural or semi-natural steppe-like habitats. Although two sites were situated in the altitude of 640 m a. s. l., maximum of the records (64%) were made between 200 and 340 m a. s. l., i. e. in lowlands. Of the total, only 26 sites were verified to host souslik colonies in the years 2000.2001. The size of most colonies is very small, exceeding the estimated abundance of 100 individuals in 7 sites only. Considering the fact that 30 sites were abandoned in the period 1990.2001, it can be concluded that the decline of the European souslik and fragmentation of its range in the Czech Republic has been continuing.

Paper III

Matějů J, Nová P, Uhlíková J, **Hulová Š**, Cepáková E (2008) Distribution of the European Ground Squirrel (*Spermophilus citellus*) in the Czech Republic. Lynx 39 (2), 277-294

Abstract

In 2002–2008, occurrence of the European ground squirrel was recorded at or reported from 45 sites in the Czech Republic. However, only 35 sites were verified to host ground squirrel colonies in 2008. Most of the colonies (91%) were found in some kind of artificial habitat with high anthropogenic influence. Only (9%) of the European ground squirrel colonies occurred in natural steppe habitats. Although three sites (9%) were recorded at altitudes above 550 m, majority of the sites (60%) were situated below 300 m a. s. l. In most (54%) of the colonies, the estimated abundance was 50 individuals or less. Only one third of the colonies hosted 100 individuals or more. Considering the fact that 7 sites were abandoned in the period 2002–2008, it can be concluded that the decline of the European ground squirrel in the Czech Republic continues.

Paper IV

With kind permission of Springer Science and Business Media:

Říčanová Š, Bryja J, Cosson JF, Gedeon Cs, Choleva L, Ambros M, Sedláček F (2011) Depleted genetic variation of the European ground squirrel in Central Europe in both microsatellites and the major histocompatibility complex: implications for conservation. Conservation Genetics 12, 1115- 1129

Abstract

Habitat fragmentation may influence the genetic make-up and adaptability of endangered populations. To facilitate genetic monitoring of the endangered European ground squirrel (EGS), we analyzed 382 individuals from 16 populations in Central Europe, covering almost half of its natural range. We tested how fragmentation affects the genetic architecture of presumably selectively neutral (12 microsatellites) and non-neutral (the major histocompatibility class II DRB gene) loci. Spatial genetic analyses defined two groups of populations, ‘‘western’’ and ‘‘eastern’’, with a significantly higher level of habitat fragmentation in the former group. The highly fragmented western populations had significantly lower genetic diversity in both types of markers. Only one allele of the DRB gene predominated in populations of the western group, while four alleles were evenly distributed across the eastern populations. Coefficient of inbreeding values (FIS) calculated from microsatellites were significantly higher in the western (0.27–0.79) than in eastern populations (-0.060–0.119). Inter-population differentiation was very high, but similar in both groups (western FST = 0.23, eastern FST = 0.25). The test of isolation by distance was significant for the whole dataset, as well as for the two groups analyzed separately. Comparison of genetic variability and structure on microsatellites and the DRB gene does not provide any evidence for contemporary selection on MHC genes. We suggest that genetic drift in small bottlenecked and fragmented populations may overact the role of balancing selection. Based on the resulting risk of inbreeding depression in the western populations, we support population management by crossbreeding between the western and eastern populations.

Paper V

Matějů J, **Říčanová Š**, Ambros M, Kala B, Hapl E, Matějů K (2010) Reintroductions of the European Ground Squirrel (*Spermophilus citellus*) in Central Europe (Rodentia: Sciuridae). Lynx 41, 175-191

Abstract

We collected primary data on 13 European ground squirrel reintroduction projects carried out in the Czech Republic, Slovakia and Poland since 1989. During these projects more than 3,200 ground squirrels were reintroduced at 15 sites or used for reinforcement of 5 populations. Reintroductions can be considered successful at 7 sites where settlement and reproduction of the released individuals were observed. At other 7 sites reintroductions failed and the result of reintroduction is still unknown at one site. Proportion of long existing reintroduced colonies is even much lower. Results of reinforcements are unclear at all 5 sites. The main problems of reintroductions were the low number of released individuals, unsuitable methodology of releasing and inappropriate site management. For future reintroduction attempts we recommend to release a sufficient number of individuals and to use artificial burrows as well as temporary fencing of the site of release. Long-term management of the site and regular monitoring of the newly established population are also necessary.