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**Phylogeography and conservation genetics of endangered  
saproxylic beetles in Europe**

Ph.D. Thesis

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**Annotation**

This thesis introduces the use of molecular methods for the conservation of several species of endangered saproxylic beetles in Europe. It focuses on the questions related to the DNA preservation and microsatellites development, as well as the evolutionary history and conservation of threatened species. Using the combination of mitochondrial and nuclear markers, the genetic diversity and reintroduction history of *Cerambyx cerdo* was assessed and the phylogeography of *Rosalia alpina* from the whole range of its distribution was studied. This information is valuable for designing more efficient conservation strategies.

**Declaration [in Czech]**

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 10. 8. 2016

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Lukáš Drag

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## List of papers and author's contribution

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

- I. **Drag L.**, Zima J. Jr., Cizek L. (2013) Characterization of nine polymorphic microsatellite loci for a threatened saproxylic beetle *Rosalia alpina* (Coleoptera: Cerambycidae). *Conservation Genetics Resources* 5: 903–905 (IF = 1.136).  
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- II. **Drag L.**, Kosnar J., Cizek L. (2013) Development and characterization of ten polymorphic microsatellite loci for the Great Capricorn beetle (*Cerambyx cerdo*) (Coleoptera: Cerambycidae). *Conservation Genetics Resources* 5: 907–909 (IF = 1.136).  
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- III. Pokluda P., Čížek L., Stříbrná E., **Drag L.**, Lukeš J., Novotný V. (2014) A goodbye letter to alcohol: An alternative method for field preservation of arthropod specimens and DNA suitable for mass collecting methods. *European Journal of Entomology* 111: 175–179 (IF = 0.975).  
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- IV. **Drag L.** & Cizek L. (2014) Successful reintroduction of an endangered veteran tree specialist: Conservation and genetics of the Great Capricorn beetle (*Cerambyx cerdo*). *Conservation Genetics* 16: 267–276 (IF = 2.185).  
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- V. Basiita RK., Bruggemann JH., Cai N., Cáliz-Campal C., Chen C., Chen J., Cizek L.,... **Drag L.**, et al. (2015) Microsatellite records for volume 7, issue 4. Erratum to: *Conservation Genetics Resources* 7: 917–944 (IF = 1.172)  
*Lukáš Drag was responsible for all the laboratory work.*
- VI. **Drag L.**, Hauck D., Berces S., Michalcewicz J., Jelaska Šerić L., Aurenhammer S., Cizek L. (2015) Genetic differentiation of populations of the threatened saproxylic beetle *Rosalia longicorn*, *Rosalia alpina* (Coleoptera, Cerambycidae) in Central and Southeast Europe. *Biological Journal of the Linnean Society* 116: 911–925 (IF = 2.264).  
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- VII. **Drag L.**, Hauck D., Rican O., Cizek L. Phylogeography of the endangered saproxylic beetle *Rosalia longicorn*, *Rosalia alpina* (Coleoptera, Cerambycidae) corresponds with its main host, the European beech (*Fagus sylvatica*, Fagaceae) (manuscript)  
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**Co-author agreement**

Lukáš Čížek, the supervisor of this Ph.D. thesis and co-author of all presented papers, fully acknowledges the contribution of Lukáš Drag as the first and corresponding author of the papers I, II, IV, VI, VII and his substantial contributions in papers III and V as stated above.

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### Saproxylic beetles

According to Speight (1989), saproxylic invertebrates are defined as organisms „that are dependent, during some part of their life cycle, upon the dead or dying wood of moribund or dead trees (standing or fallen), or upon wood-inhabiting fungi, or upon the presence of other saproxylics“. Their role in the forest ecosystems is irreplaceable. The saproxylic fauna is mainly responsible for the natural decomposition of woody material (Cavalli and Mason, 2003). Most of the species feed on decaying trees, snags (standing dead trees) and logs (fallen trees) preparing them for colonization by other organisms. Also, they often serve as vector of other symbiotic organisms (mostly different species of fungi) that further humify wood (Gilbertson, 1984). Finally, saproxylic organisms are part of the complex food web systems and multiple types of animal-plant and animal-animal interactions (Quinto et al., 2012).

In Europe, however, saproxylic invertebrates (and especially beetles) are considered to be a highly threatened taxonomic group (Nieto and Alexander, 2010). According to IUCN Red List (Nieto and Alexander, 2010), nearly 11% (46 species) of evaluated saproxylic beetles are threatened, and almost 14% of species have declining populations. At the level of individual countries, most of the data comes from the Fennoscandia where saproxylic community is traditionally given high attention. For example, in Sweden 471 beetle species associated with forest habitats are included in the Swedish Red List (Gärdenfors, 2005). In Finland, almost 10% (333 species) of all assessed beetle species are considered as threatened (Rassi et al., 2010). As in Sweden, forest habitats are the most important; 42% (140 species) of the threatened beetles live primarily in forests. The situation in southern parts of Europe could be even more serious since the bulk of saproxylic diversity (species richness and the number of endemic species) occurs in these regions (Nieto and Alexander, 2010).

The decline of the saproxylic beetles is mostly connected with the changes in landscape caused by the human activities. One of the most serious problems is the removal and reduction in quality of dead and decaying wood within the forest (Davies et al., 2008; Müller et al., 2015). For example, 31% of all threatened beetle species in Finland are influenced by the decreasing amount of decaying wood (Rassi et al., 2010). A large number of saproxylic beetles are also dependent on ancient and veteran trees (Seibold et al., 2015). Therefore, a reduction of old-growth forests and the decreasing number of old large trees can also significantly influence the

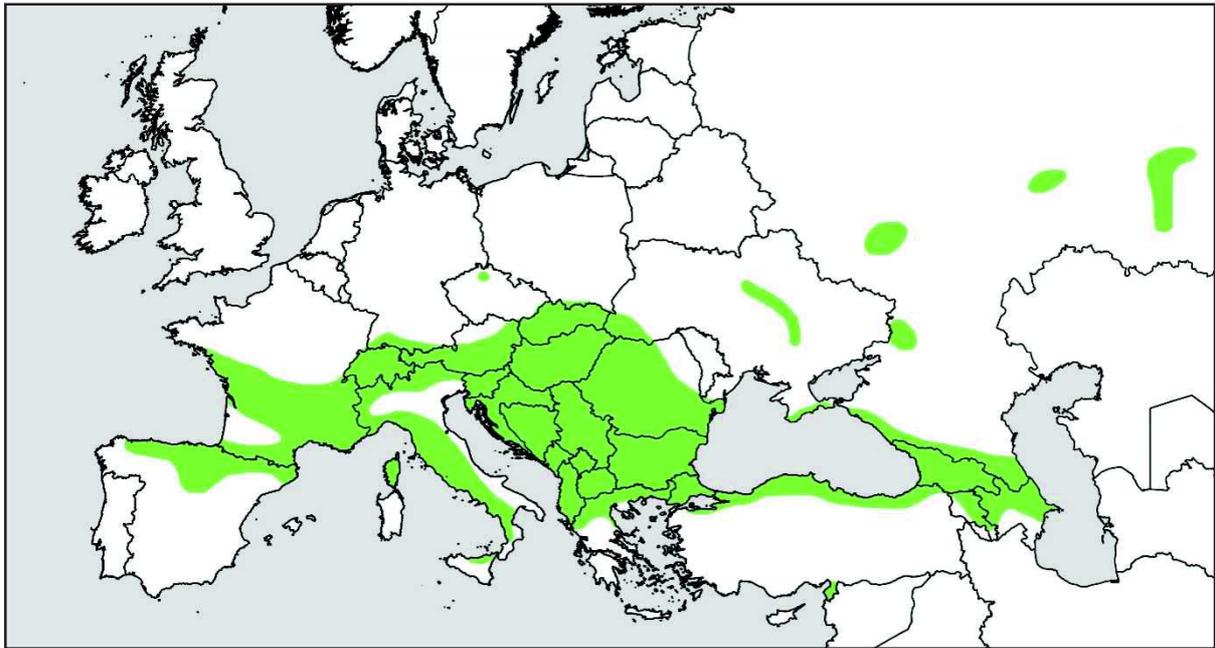
survival of many populations. Finally, it has been suggested that many saproxylic beetles prefer sun-exposed wood compared to shaded (Vodka et al., 2009). Overgrowing of the originally open habitats can thus constitute another threat for such organisms.

Saproxylic beetles are a diverse group with high ecological and economical importance (Grove, 2002). Although the major pest species, often causing damages to environment, forest and natural resources, have been the subject of various studies, less attention has been paid to the threatened species, some of them serving as important models in ecology, conservation biology, and/or as the umbrella species (Ranius, 2002; Buse et al., 2007). Large, conspicuous beetles are among the most attractive representatives of such guild. Even though the hermit beetle (*Osmoderma eremita* s.l.) (Scopoli, 1763) represents probably the most frequently studied species, its ecology and life history is very different from other endangered saproxylic beetles, especially the large cerambycids (Chiari et al., 2012; Chiari et al., 2013). Thus, focusing on them is highly desirable and it may bring new insight into the different life strategies.

### ***Rosalia alpina* as a model species**

*Rosalia longicorn*, *Rosalia alpina* (Linnaeus, 1758), belongs to the family *Cerambycidae*, tribe *Rosaliini* (Danilevsky, 2015). In Europe, there is only one species of this genus, although it has been suggested that the highly isolated population from southern Turkey (Hatay Province) might be considered as a subspecies *Rosalia alpina syriaca* (Pic, 1894) (for more details see the Chapter VII). Several other species within genus *Rosalia* have been described, but all of them inhabit Eastern Palaearctic and Oriental regions, mainly China and SE Asia (Löble and Smetana, 2010).

*R. alpina* currently inhabits a large part of Europe (Fig. 1) reaching as east as Caucasus and Ural Mountains (Sama, 2002). Its southern border is formed by northern Greece, Sicily and northern Spain. There are no indications that it occupies coasts of North Africa. In the northern parts of Europe, the beetle has experienced significant declines in the last decades. It has completely disappeared from Sweden (Lindhe et al., 2010) and Denmark (Horion, 1974), and from a large parts of Germany (Bense, 2002), Poland (Starzyk, 2004a) and the Czech Republic (Sláma, 1998).



**Figure 1** The distribution map of *Rosalia longicorn* (*Rosalia alpina*).

*R. alpina* is typically considered as a montane or sub-montane species associated with European beech (*Fagus sylvatica*) (Heyrovský, 1955; Sláma, 1998). In Western (Picard, 1929) and Southeastern (Serafim and Maincan, 2008) Europe, however, the beetle is known to display a broader host range and occupies more habitats including sea-shore and floodplain forests (Simandl, 2002). Recently, these lowland populations have also been repeatedly reported in Central Europe, mostly from the floodplains of the Danube and its tributaries in Slovakia, Austria, Hungary and Czech Republic (Jendek and Jendek, 2006; Cizek et al., 2009; Hovorka, 2011). While montane beech-associated populations (except those from the Alps and Carpathians) have almost disappeared from Central Europe, the lowland populations have started to spread and exploit other broadleaved tree species like maples, ashes and elms (Jendek and Jendek, 2006; Cizek et al., 2009; for detailed information see the Chapter VI). Furthermore, occasionally the development of the beetle has also been recorded in other broadleaved trees, including hornbeam and walnut (Merkl et al., 1996). Although the beech is generally preferred whenever present, beetles still have the ability to exploit a wide range of distantly related species of trees. Why such strange behavior exist, and which evolutionary mechanisms can maintain it is the subject of the Chapter VII.

Despite this, *R. alpina* remains an endangered and strictly protected species in most of Europe. It is listed in the IUCN Red List of Threatened Species (IUCN, 2016) and the EU Habitats Directive as a priority species of community interest (Council of the European Communities, 1992). Because of the beetle's general publicity, its role as an umbrella and

flagship species providing protection for other beech-forest associated species has also been emphasized (Lachat et al., 2013).

While in lowlands the habitat requirements of *R. alpina* are still rather uncertain, in higher altitudes the beetle occupies mostly old and sun exposed trees in semi-open woodlands (Russo et al., 2011). Dead wood which is still mostly hard and dry is preferred in form of standing or fallen trunks, and large branches. Crevices and cracks on the surface probably facilitate female's oviposition. The development takes usually three years (Heyrovský, 1955) after that adults exit the wood through typical elliptic holes (Dominik and Starzyk, 1989).

The main threat for this species is the loss of suitable habitats, resulting in the fragmentation of populations with all the adverse consequences. Two contrary practices are usually involved. Intensive logging and massive dead-wood removal strongly affects the amount of breeding substrate available to *R. alpina*. On the other hand, a no management approach could lead to a closed canopy forest, which is also not suitable. Therefore, management at multiple scales is needed, including regular forest thinning (coppicing, pollarding) and dead wood retaining (Russo et al., 2011).

### ***Cerambyx cerdo* as a model species**

The Great Capricorn beetle, *Cerambyx cerdo* (Linnaeus, 1758), is a western Palearctic species distributed in most of Europe including the whole Mediterranean region and the Caucasus Mountains (Sláma, 1998; Sama, 2002). Although still relatively common in South France, Spain and Italy, the species is rapidly declining across northern parts of Europe (e.g. Sláma, 1998; Ehnström and Axelsson, 2002; Starzyk, 2004b; Jurc et al., 2008; Ellwanger, 2009). It has disappeared from large parts of Sweden, where the last population currently survives (Lindhe et al., 2010), Germany (Buse et al., 2007) and Poland (Starzyk, 2004b). In Great Britain, fossil evidences suggest that this species occurred until 3690 +/-100 BP and all the recent records have been described as importations (Alexander, 2002). In the southern parts of Europe, the species can be confused with other closely related species with the similar morphology, as for example *Cerambyx welensii* (Küster, 1846) or *Cerambyx miles* (Bonelli, 1812).

*Cerambyx cerdo* is one of the largest longhorn beetles living in Europe. Similar to *Rosalia alpina*, it is protected under the EU Habitats Directive (Council of the European Communities, 1992) and classified as globally vulnerable according to the IUCN Red List of Threatened Species (IUCN, 2016). Although the species prefers to develop in the wood of large and sun exposed oaks (Buse et al., 2007), it is at least occasionally able to accomplish its

development in oaks of a much smaller diameter (Albert et al., 2012), or even in a different tree species (Sláma, 1998). The vitality of trunk and canopy (the amount of dead or damaged branches) seems to be an important factor influencing the presence of this saproxylic beetle (Buse et al., 2007). Since larvae develop in the wood of weakened, but still living, trees, the adults prefer to attack older and injured trees. Also, the beetle requires open sides and sun-exposed trees; thus it is most commonly found in old open forests, orchards and landscape parks.

The reason that this species has declined in the last century is mainly due to changes in forest management practices resulting in fewer suitable oaks. Potential host trees have become too shaded by surrounding trees and shrubs with an unsuitable age structure. Thus, proper management should focus on creating a sufficient number of suitable trees that are the proper age, and on connecting such localities with the one already inhabited by this species.

*C. cerdo* is considered as an ecosystem engineer, probably due to its ability to change the characteristics of its host tree and thus provide more favorable conditions for other saproxylic insects (Buse et al., 2008a). Oaks colonized by this beetle had a higher number of other beetle fauna than uncolonized trees, and offered a habitat for numerous endangered beetle species (Buse et al., 2008b). For this reason, it has been suggested that reintroductions of *C. cerdo* where it has already disappeared from might be favorable for local fauna biodiversity (Buse et al., 2008a). Nevertheless, since the beetle is essentially a parasite of oaks, the possible negative effect of the reintroduction on the habitat should be considered (for more details see the Chapter IV).

### **Conservation priorities**

It has been generally suggested that, to prevent long-term loss and degradation of biodiversity, conservation activities needs to be prioritized (Brooks et al., 2006; Wilson et al., 2009). Such actions can help to effectively allocate usually limited amount of funds and resources. Setting conservation priorities usually includes the assessment of extinction risk of threatened taxa, as well as other ecological, phylogenetic, historical, economical, or cultural preferences for some taxa over others (e.g. Sarkar et al., 2006). The application of genetic methods is another important aspect that should be considered in any conservation decision (Marris, 2007; McMahon et al., 2014).

Different genetic approaches have been used in conservation biology for resolving wide ranges of questions related to the beetle's protection, e.g. estimating effective population size

(Kajtoch et al., 2014), detecting population bottlenecks (Gaublomme et al., 2013; Kajtoch et al., 2014), determining genetic diversity and population structure for recognition of conservation units (Kajtoch, 2011; Kajtoch et al., 2013; Ahrens et al., 2013; Cox et al., 2013; Sole and Scholtz 2013), detecting hybridization (Solano et al., 2016), resolving taxonomic uncertainties (Audisio et al., 2009; Solano et al., 2013), or assessing dispersal abilities and the gene-flow (Matern et al., 2009; Oleksa et al., 2013; Oleksa et al., 2015). An understanding of such patterns in populations of threatened species is vital for effective conservation strategies, and it may help to determine management actions according to the current needs (Avisé et al., 1987; Moritz, 1994).

*Rosalia alpina* and *Cerambyx cerdo* are both considered as the two model species of saproxylic invertebrates for various ecological questions (Buse et al., 2007; Drag et al., 2011; Bosso et al., 2013), and at the same time they both serve as umbrella species in biodiversity conservation (Buse et al., 2008b; Lachat et al., 2013). Despite their significant importance, any population genetic studies are surprisingly missing.

## **Molecular markers**

Although current whole-genomic approaches constantly play a more important role in the study of genetic variation and can provide a great amount of new information for a reasonable price (McCormack et al., 2013), many questions in conservation genetics can still be satisfactorily answered using the conventional genetic markers as a mitochondrial DNA, microsatellites, or by the combination of these two approaches (McMahon et al., 2014).

Different mitochondrial gene sequences (mtDNA) have traditionally been used for studying genetic variations of insect populations, to reconstruct the phylogeny of recently diverged species, and to provide relevant information about the phylogeography of the study species. Because of its undeniable advantages (simple extraction from animal tissue, relatively high rate of polymorphism, universal primers across different taxa, and assumed no recombination), mtDNA has quickly become the marker of choice for many studies.

Despite its undeniable usefulness, employment of mtDNA is not always without complication. Questions about its suitability and reliability for resolving different evolutionary processes have been asked (e.g. Zhang and Hewitt, 1996). Firstly, it is important to expect the strict maternal inheritance of this marker in most cases. Consequently, it has been proposed that mtDNA does not reflect the history of the species as a whole but rather only of the female portion. Probably the major drawback of the marker is, however, the potential presence of the

mitochondrial copies in the nuclear genome (NUMTs or mitochondrial pseudogenes). Such occurrence can bias the inference of the phylogenetic relationships at the interspecific level (Ruiz et al., 2013), as well as being able to lead to the overestimation of the intraspecific genetic diversity and misinterpretation of the phylogeography (Haran et al., 2015). Another problem of the mtDNA arises with the maternally inherited endosymbionts (Hurst and Jiggins, 2005). These parasitic microorganisms promote the production and survival only of infected daughters (due to cytoplasmic incompatibility) thus altering the mtDNA diversity in the population. Two well-known examples of such microorganisms are *Wolbachia* and *Cardinium* (e.g. Gotoh et al., 2007; Arthofer et al., 2009). Finally, it has been suggested that mtDNA evolution is not completely neutral. Direct selection (positive or negative) can be responsible for discrepancies in the amount of genetic variation within a population compared to the mtDNA variation consistent with the neutral theory and can lead to an inappropriate estimation of the effective population sizes (Meiklejohn et al., 2007).

To avoid or at least reduce the potential error, the combination of mtDNA with other nuclear marker has been highly recommended (e.g. Hurst and Jiggins, 2005; Magri et al., 2006).

Microsatellites, also known as simple sequence repeats (SSRs), are tandemly repeating units of DNA one to six nucleotides in length that are widely distributed throughout the nuclear genomes of both eukaryotes and prokaryotes (Bhargava and Fuentes, 2010). Due to their high level of allele polymorphism (even in organisms displaying otherwise little genetic variation), relatively easy and straightforward identification, and easy scorability, microsatellites have become one of the most frequently used molecular markers in conservation and population genetics (Guichoux et al., 2011). They have been successfully used in a variety of population genetic studies, including insect ones (Behura, 2006).

However, SSRs also has some issues. The initial cost associated with microsatellites is usually high because of a lengthy and costly development phase (Wei et al., 2014). Another important problem associated with population genetic inferences using microsatellites is their mechanism of mutation (Bhargava and Fuentes, 2010). There are several different theoretical mutation models, but all of them have certain assumptions whose violation can lead to incorrect conclusions. Furthermore, often cited drawbacks of SSRs is the homoplasmy (Estoup et al., 2002) and the possible occurrence of null alleles (Dakin and Avise, 2004). Homoplasmy occurs when two individuals with different ancestries at a locus mutate to the same allele and become identical only in state, but not by descent. Such occurrences are even higher if microsatellites are genotyped by their length due to possible insertions or deletions within the flanking region. Null alleles usually arise during the PCR, when one of the alleles fails to be amplified and the

heterozygous individuals can be scored as homozygous. Both situations can complicate the correct interpretation of microsatellite allele frequencies leading to biased results. Nevertheless, if proper consideration and evaluation of the above mentioned limitations is involved, reliable inferences in population genetic studies can be expected. Microsatellites thus still serve as a relevant genetic marker useful in many situations.

The combination of mtDNA and microsatellites was commonly used in many recent phylogeographical studies dealing with vertebrates (e.g. Řičanová et al., 2013; Dufresnes et al., 2013; Gassert et al., 2013; Sztencel-Jablonka et al., 2015) as well as invertebrates (Kodandaramaia et al., 2012; Theissinger et al., 2013; Moreira et al., 2015). In beetles, such studies are mostly restricted to important pest species (Carter et al., 2010; Sánchez-Sánchez et al., 2012; Krascenitsová et al., 2013), for the rest of the group such an approach is still very rare (but see Kajtoch, 2011; Kajtoch et al., 2014). The employment of both markers can be, however, very beneficial not only because of the potential error of one of the used markers, but also because it brings a deeper insight into the genetic structure of the studied species. On the other hand, the direct comparison of the results based on these two markers may not be always appropriate, and discordances between mitochondrial and nuclear DNA have been found (reviewed in Toews and Brelsford, 2012).

Given the above, the combination of the mtDNA and microsatellites has been decided as the most suitable to study the genetic structure of saproxylic beetle populations. A partial fragment (766 – 814 bp) of the mitochondrial gene for the cytochrome c oxidase subunit I (COI) has been amplified using the universal primers (C1-J-2183 and TL2-N-3014). This gene was proven to be sufficiently polymorphic and easy to amplify, even for insect populations, and thus it has become a standard genetic marker in many phylogeographical studies, as well as being used as a ‘barcode’ gene (Hebert et al., 2003). Unlike the mtDNA, microsatellites do not generally provide the possibility of the universal primers. Although loci can be relatively easily transferred between closely related species (cross-amplification), for most species being examined for the first time they need to be identified *de novo*. The employment of the next generation sequencing technologies has helped me to overcome some problems with SSR’s development previously described for butterflies (Zhang, 2004) or saproxylic beetles (Arthofer et al., 2007; Sallé et al., 2007). Finally, a set of polymorphic microsatellite loci was available for two endangered longhorn beetles, *Rosalia alpina* (Chapter I) and *Cerambyx cerdo* (Chapter II), as well as for one endangered scarab beetle, *Osmoderma barnabita* (Chapter V).

## Phylogeography

In the past 2.4 million years the biota on the Earth has been highly influenced by a series of climatic oscillations, called glacial and interglacial cycles. As the result, distribution range of many species in Europe has greatly changed (Hewitt, 2000). During the glacial periods, some populations, especially in northern parts of Europe, went extinct, while other had to retreat into southern refugia where they could survive. During interglacial periods, the warmer weather again created new habitats suitable for recolonization from the south (Hewitt, 1996). This must have occurred repeatedly. In Europe, the south-western orientation of the main mountain chains (Alps, Carpathians, and Pyrenees) had an important role in the species distribution, since they could have served as a significant barrier in the contraction and subsequent expansion of populations (Schmitt, 2009). Based on the fossil, pollen and later also DNA analyses it has been identified three major refugia in Europe (Taberlet et al., 1998; Hewitt, 1999; Hewitt, 2001). Populations inhabiting Iberian, Italian and Balkan Peninsulas were during the cold stage probably mostly isolated from each other with no migration or gene-flow between them resulting in development up to three distinct lineages that could have been involved in subsequent postglacial recolonization of the more northern regions in Europe (Schmitt, 2007).

With more detailed phylogeographical studies in the last years, it has become clear that the southern refugia did not constitute homogeneous regions during the ice ages, but for some taxa they represented structured mosaics of heterogeneous areas. This ‘refugia within refugia’ concept (Gómez and Lunt, 2007) assumes that individual southern regions comprise a multiple separate glacial refugia. Such a scenario has been described for all three main peninsulas, i.e. Iberian (Abellán and Svenning, 2014), Italian (Stefani et al., 2012) and Balkan (Pabijan et al., 2015).

The situation is even more complex if we consider that not all of the refugia were located in the southern Europe. Some species recolonized Europe or at least part of it from the east; typically boreo-temperate forest species (e.g. *Formica pratensis* Goropashnaya et al., 2004; *Myopus schisticolor* Fedorov et al., 2008) or species with the highest genetic diversity in Caucasus Mountains (e.g. *Lacerta agilis*; Kalyabina et al., 2001). Recently, it has also been suggested that for some thermophilic species the North Africa (Maghreb) could have served as an important differentiation center as well as another colonization source for Europe (Husemann et al., 2014).

Furthermore, Willis et al. (2000) indicated that for some temperate species in Europe, the higher latitudes could have served as an important cold-stage refugium during cold episodes.

This concept of cryptic refugia (Cruzan and Templeton, 2000) helped to explain some previous inconsistencies in paleontological and biogeographical studies (Clark et al., 1998; Stewart and Lister, 2001). Species that could not accommodate changes in climate (e.g. because of the limited dispersal ability) had a higher probability of extinction (Thomas et al., 2004). With the presence of cryptic refugia in northerly latitude, however, the species could have still survived and recolonized Europe faster than previously expected. Therefore, the dispersal capabilities of many organisms might have been overestimated (Provan and Bennett, 2008). Currently, there are many examples of temperate-climate organisms probably surviving the last glacial maximum outside the traditional refugia (reviewed in Schmitt and Varga, 2012).

Based on pollen, plant macrofossils, genetic records and distribution model evidences, it has been suggested that the concept of cryptic refugia can be also applied on numerous tree species (Willis et al., 2000; Willis and van Andel, 2004; Svenning et al., 2008). In Central and Eastern Europe, there is evidence that such areas could have served as a refugia for many boreal and/or montane species of conifers (*Picea*, *Larix*, *Pinus*, *Juniperus*, *Populus*) and some broadleaved trees (*Betula*, *Salix*, *Populus*, *Alnus*) (Svenning et al., 2008, Tzedakis et al., 2013). The temperate tree species (e.g. *Fagus*, *Quercus*, *Ulmus*, *Tilia*, *Fraxinus*) were probably confined to more southern areas (Tzedakis et al., 2013), although not necessarily to one of the three traditional peninsulas. For example, the area of south-eastern Alps and north-western Dinaric Alps has been suggested as the microrefugium for *Fagus sylvatica* playing an important role in the colonization of Central and Western Europe (Magri et al., 2006; Brus, 2010). The same region was also proposed as one of the possible refugium for *Fraxinus excelsior* (Heuertz et al., 2004).

Since all saproxylic beetles are closely connected at least in part of their life with their host tree species, it has been suggested that host's evolution history can also be an important factor influencing the genetic structure of the beetles (Avtzis et al., 2012). Although many studies have focused on such relationships, it still remains unclear how important the role is and to what extent the beetles are influenced.

Given the high ecological and economical importance, bark beetles probably represent the most frequently studied taxa within the saproxylic beetles. Thus, many studies have tried to discuss the phylogeographic patterns of bark beetles and compare it with their main hosts (Avtzis et al., 2012). Despite this, the results are still rather ambiguous. For some species (e.g. *Pityogenes chalcographus*) it has been suggested that the glacial refugia and the recolonization history correspond with its host (Avtzis et al., 2008; Bertheau et al., 2013), for other species (e.g. *Ips typographus*) the phylogeographic pattern just partly reflect the postglacial history of

the beetle's main host (Tollefsrud et al., 2008; Tollefsrud et al., 2009; Mayer et al., 2015). Although the relationship is not yet fully described, the close adaptation to a specific host can influence the beetles' life history and should be considered and discussed in all phylogeographic studies of saproxylic beetles (for more details see the Chapter VII).

### **Aims of the thesis**

The general aim of my theses is to introduce the molecular methods into the conservation of the threatened saproxylic beetles. My work includes testing of suitable DNA preservation liquids, a development of the new genetic markers (microsatellites), and their application on the specific ecological and conservation problems. My results provide new insight into the genetic diversity and population structure of several species of endangered saproxylic beetles in Europe. I believe that my findings will contribute to better understanding of their ecology and the evolution history as well as they will be of great importance for designing more efficient conservation strategies. The specific ecological and conservation questions include:

- a) to discover the reintroduction history of the Czech population of *Cerambyx cerdo* in Hluboka nad Vltavou
- b) to assess the population genetic structure of *Rosalia alpina* species and to test the hypothesis of differentiation between lowland and upland populations
- c) to reveal the phylogeography patterns of *Rosalia alpina* implementing the samples from the entire range of its distribution
- d) to compare the phylogeography of *Rosalia alpina* with its main host tree (*Fagus sylvatica*) to test the hypothesis about the ecological specialization of the beetle



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*The following passage (pages 14–18) is a published paper (DOI 10.1007/s12686-013-9929-1) and it was removed from the online version of the thesis.*

### Characterization of nine polymorphic microsatellite loci for a threatened saproxylic beetle *Rosalia alpina* (Coleoptera: Cerambycidae).

Drag L., Zima J. Jr., Cizek L.

#### Conservation Genetics Resources

##### **Abstract**

Nine polymorphic microsatellite loci were developed and characterized for the *Rosalia longicorn*, an endangered icon of European saproxylic biodiversity. The detected number of alleles per locus ranged from 2 to 4, and the observed and expected heterozygosities varied from 0.044 to 0.622, and from 0.086 to 0.613, respectively. All loci were in Hardy–Weinberg equilibrium as well, as no evidence of linkage disequilibrium was found. Despite the low level of polymorphism, all loci described in this study will provide a useful tool in future genetic studies of the *Rosalia alpina* species.

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*The following passage (pages 20–24) is a published paper (DOI 10.1007/s12686-013-9930-8) and it was removed from the online version of the thesis.*

### Development and characterization of ten polymorphic microsatellite loci for the Great Capricorn beetle (*Cerambyx cerdo*) (Coleoptera: Cerambycidae).

**Drag L., Kosnar J., Cizek L.**

#### Conservation Genetics Resources

##### **Abstract**

Ten polymorphic microsatellite loci, developed using next-generation sequencing technology, are described for the Great Capricorn beetle, *Cerambyx cerdo*; an endangered and internationally protected European longicorn. Based on 30 individuals from South-East Czech Republic, the number of alleles per locus ranged from 4 to 8, and the observed and expected heterozygosity was 0.36–0.79 (mean = 0.57) and 0.5–0.77 (mean = 0.63), respectively. In one locus tests indicated a deviation from the Hardy–Weinberg equilibrium, as well as the presence of null alleles. Future studies using markers described here may provide additional information for the efficient protection of this threatened species.

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*The following passage (pages 26–32) is a published paper (DOI 10.14411/eje.2014.024) and it was removed from the online version of the thesis.*

### A goodbye letter to alcohol: An alternative method for field preservation of arthropod specimens and DNA suitable for mass collecting methods.

Pokluda P., Čížek L., Stríbrná E., **Drag L.**, Lukeš J., Novotný V.

European Journal of Entomology

#### **Abstract**

Despite its limitations, ethanol remains the most commonly used liquid for the preservation of arthropod specimens and their DNA in the field. Arthropod ecology and taxonomy have witnessed a substantial increase in the use of various trapping and molecular methods in the past two decades. However, the methods of collecting and the preservation liquids most widely used in arthropod traps do not properly preserve DNA. Trap-collected specimens are typically of limited utility for molecular studies due to the poor preservation of DNA. A stable and cheap substance that can be used to trap arthropods in and preserve their DNA is therefore needed. Here we test whether (i) 2% SDS, 100mM EDTA, (ii) 1% SDS, 50mM EDTA and (iii) 0.66% SDS, 33mM EDTA can preserve DNA of small and medium-sized beetles for one, four and eight weeks. Preservation of DNA was tested using PCR amplification of parts of the mitochondrial cytochrome c oxidase I (Cox1) and nuclear 28S rRN A genes. All the solutions tested preserved DNA for at least up to eight weeks and we recommend 2% SDS and 100mM EDTA as a cheap, stable and easily transportable alternative to ethanol for preserving specimens and their DNA collected in the field. This solution is also suitable for using as the collection and preservation liquid in arthropod traps.

*The following passage (pages 34–44) is a published paper (DOI 10.1007/s10592-014-0656-2) and it was removed from the online version of the thesis.*

# Successful reintroduction of an endangered veteran tree specialist: Conservation and genetics of the Great Capricorn beetle (*Cerambyx cerdo*).

Drag L. & Cizek L.

## Conservation Genetics

### Abstract

Habitat fragmentation is one of the main threats to biodiversity. Reintroductions or translocations may mitigate its effects by allowing species with limited dispersal ability to exploit otherwise inaccessible habitat patches. Despite the fact that reintroductions are among the most effective conservation measures, they are rarely used for invertebrates. In this study we investigate the potential of reintroductions as a conservation measure for beetles, and present the first genetic results for an endangered veteran tree specialist. After translocation of 10 adults in 1987, a population of the Great Capricorn beetle reappeared in Hluboká nad Vltavou (Czech Republic) in 1990s. Using population genetic analyses of 79 individuals based on nine microsatellite loci and 82 individuals based on the mitochondrial COI gene we assessed the origin of this population, and compared its genetic variation, population structure and demography to the alleged source population (southern Moravia) and to the closest autochthonous population (Třeboňsko). Although the reintroduced and the closest autochthonous populations are geographically close (24 km), their mutual genetic distance was much higher than that between each of them and the geographically distant ([150 km) potential source population in southern Moravia. The genetic diversity of the reintroduced population was the lowest from the three studied populations and represented a subset of the alleged source population suggesting its establishment due to a translocation from southern Moravia. Despite the lower genetic variation at the reintroduced site, our results suggest that reintroductions could serve as a highly effective measure in biodiversity conservation and in some cases it may be the only chance to prevent extirpation of many endangered populations.

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*The following passage (pages 46–54) is a published paper (DOI 10.1007/s12686-015-0493-8) and it was removed from the online version of the thesis.*

### Microsatellite records for volume 7, issue 4. Erratum to: Conservation Genetics Resources 7: 917–944.

(first four pages of the original paper + erratum + supplementary material)

Basiita R.K., Bruggemann J.H., Cai N., Cáliz-Campal C., Chen C.,  
Chen J., Cizek L.,... **Drag L.**, et al.

#### Conservation Genetics Resources

##### **Abstract**

Fourteen microsatellite loci are described for the eastern European hermit beetles, *Osmoderma barnabita*, a vulnerable and internationally protected species associated with mature hollow trees. Based on 45 individuals from Poland, 13 of 14 loci were polymorphic. The number of alleles per polymorphic locus ranged from 2 to 13, and the observed and expected heterozygosity was 0 – 0.889 (mean = 0.231) and 0.033 – 0.868 (mean = 0.253), respectively. Three loci showed deviation from Hardy–Weinberg equilibrium. The probability of null alleles was negligible for all but one locus. Seven loci cross-amplified in the closely related *Osmoderma eremita*. The markers reported here can be valuable tool for detecting genetic structure and gene flow in *O. barnabita*.

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\* ) Explanation of the complicated title: After being submitted in Conservation Genetics Resources as the “Primer note”, the MS was accepted in the form of “Microsatellite records” publishing all primer sequences from different authors together with the editor as the only author. Later, erratum to this issue was released giving the correct list of all contributed authors in alphabetical order. The original Primer note can be downloaded in the form of Supplementary material 2.

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*The following passage (pages 56–72) is a published paper (DOI 10.1111/bij.12624) and it was removed from the online version of the thesis.*

### Genetic differentiation of populations of the threatened saproxylic beetle *Rosalia longicorn*, *Rosalia alpina* (Coleoptera, Cerambycidae) in Central and Southeast Europe.

Drag L., Hauck D., Berces S., Michalcewicz J., Jelaska Šerić L., Aurenhammer S., Cizek L.

Biological Journal of the Linnean Society

#### Abstract

Knowledge of patterns of genetic diversity in populations of threatened species is vital for their effective conservation. *Rosalia longicorn* (*Rosalia alpina*) is an endangered and strictly protected beetle. Despite a marked decline in part of its range, the beetle has recently expanded to the lowlands of Central Europe. To facilitate a better understanding of the species' biology, recent expansion and more effective conservation measures, we investigated patterns of genetic structure among 32 populations across Central and South-east Europe. Eight microsatellite loci and a partial mitochondrial gene (cytochrome c oxidase subunit I) were used as markers. Both markers showed a significant decline in genetic diversity with latitude, suggesting a glacial refugium in northwestern Greece. The cluster analysis of the nuclear marker indicated the existence of two genetically distinct lineages meeting near the border between the Western and Eastern Carpathians. By contrast, one widespread mtDNA haplotype was dominant in most populations, leading to the assumption that a rapid expansion of a single lineage occurred across the study area. The genetic differentiation among populations from the northwestern part of the study area was, however, surprisingly low. They lacked any substructure and isolation-by-distance on a scale of up to 600 km. This result suggests a strong dispersal capacity of the species, as well as a lack of migration barriers throughout the study area. That the lowland populations are closely related to those from the nearby mountains indicates repeated colonization of the lowlands. Our results further suggest that *R. alpina* mostly lives in large, open populations. Large-scale conservation measures need to be applied to allow for its continued existence.

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*The following passage (pages 74–102) is a manuscript in preparation and it was removed from the online version of the thesis.*

### Phylogeography of the endangered saproxylic beetle *Rosalia longicorn*, *Rosalia alpina* (Coleoptera, Cerambycidae) corresponds with its main host, the European beech (*Fagus sylvatica*, Fagaceae)

**Drag L., Hauck D., Rican O., Cizek L.**

#### **Abstract**

Highly host-specific organisms, such as phytophages, are often forced to react to the environmental changes within the frame set by their hosts. Thus, the population genetic structure of the specialists should at least partly mirror that of their host. *Rosalia alpina* is an endangered xylophagous beetle that is mainly associated with the European beech (*Fagus sylvatica*), although it is also able to exploit a wide range of other distantly related hosts. To investigate such surprising association we studied the phylogeographical pattern of *R. alpina* over the entire range of species distribution using both mitochondrial and nuclear markers. Furthermore, we compared these results with the known patterns of genetic structure and demographic history of its frequent host species. We found five distinct clades in *R. alpina* populations; three of them were endemic to Italy, one to Turkey and the remaining clade probably originated in SW Balkan Peninsula and colonized the rest of the species range from Iberian Peninsula to the Caucasus and the Ural Mountains. The phylogeographic pattern of *R. alpina* was most congruent with that of the beetle's main host, the European beech, and both species thus probably share the common history. Furthermore, we probably witness broadening of the host range of the beetle. This may happen periodically during interglacials, since the populations not exploiting beech are likely to go extinct during the next glacial periods. Such mechanism offers the answer to the question of why the beetle exhibits surprisingly close and constraining association with only one of the numerous hosts.

## Summary and conclusions

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Genetic diversity is one of the three essential levels of biodiversity which has a direct relevance to conservation efforts. In the last decade, various genetic approaches have become more accessible and been applied to address wide range of ecological questions. Genetic diversity, however, was surprisingly rarely considered in studies focused on threatened saproxylic insects. Hence, in this thesis I focused on the investigation of some rather technical questions related to the DNA preservation and microsatellites development, as well as on crucial questions of ecology, evolutionary history and conservation of several threatened saproxylic beetle species.

Numerous genetic approaches are currently available to answer different ecological questions. Some of the methods are relatively easy to handle and can be applied to a wide range of relevant questions. Nevertheless, if more detailed information about the population structure is required, more effort needs to be made. Microsatellites are of particular interest to ecologists because they are one of the few molecular markers that allow researchers insight into fine-scale ecological questions. Although the process of isolating new microsatellite markers has become faster and less expensive, it may still represent an obstacle, especially when working with non-model organisms. The **Chapters I, II, and V** consists of three technical notes describing the *de novo* development of microsatellites for three endangered saproxylic beetles (*Rosalia alpina*, *Cerambyx cerdo* and *Osmoderma barnabita*). All newly described microsatellite loci thus represent a valuable tool for future population and conservation genetic studies of these threatened species.

The collection and preservation of insect samples in the field which are suitable for genetic analyses might be a problem, particularly when using mass collecting methods. Since widely used ethanol suffers with several limitations, in the **Chapter III** we tested the mixture of SDS (chelator sodium dodecyl sulfate) and EDTA (ethylenediaminetetraacetic acid) in different concentrations to examine the effectiveness of such solutions in preserving insect DNA. Our results suggest that the mixture is suitable as a cheap, stable and easily transportable alternative to ethanol, although its use should be limited to small or medium-sized insects.

*Cerambyx cerdo* is considered an ecosystem engineer due to its ability to provide more favorable conditions for other saproxylic insects. Therefore, its reintroduction at localities where the species no longer exists, might be favorable for local fauna biodiversity. The **Chapter IV** of my thesis is devoted to the origin of the one reintroduced population of this species in Czech Republic. It also presents the first genetic evidence supporting the success of an endangered saproxylic beetle reintroduction, as well as the first genetic study of this species across the three populations. The most important part of this chapter is where I consider the findings that as long as suitable conditions are present, just a few individuals are enough to found a viable population, thus minimising the potentially negative effect of removing individuals from the source population. On the other hand, the genetic consequences are detectable (substantial loss of the mitochondrial and the partial loss of the nuclear diversity), and it remains unclear if it can negatively influence the long-term survival of the reintroduced population.

**Chapters VI and VII** were dedicated to *Rosalia alpina*. Here, I present the first study of the genetic variability and structure covering the whole range of the species distribution. Due to this the unique sample collection and the combination of the nuclear (microsatellites) and mitochondrial (cytochrom c oxidase subunit I) markers, new information about the beetle's biology and history could be inferred.

Surprisingly, the genetic differentiation of populations from the large part of the species distribution range (up to 620 km) was very low. Such high admixture might be a result of the strong dispersal capacity of the species and/or the lack of migration barriers. Also, it seems possible that the effective sizes of *R. alpina*'s populations are in general rather large maintaining the low genetic differentiation due to the low effect of the genetic drift. This would indicate that the beetle might be unable to exist in small, isolated populations, possibly as a result of some intrinsic reasons related to the species' biology or behavior. Such hypothesis may then explain why a mobile species with the ability to exploit a broad range of habitats has disappeared from substantial part of its range in Europe.

Also, no genetic differences between lowland and upland populations of this species were recorded. As a consequence, we assume that the recently inhabited lowland areas in Central Europe were probably colonized from the nearby upland populations, rather than that the beetle would spread from other lowland populations thus forming a distinct lowland lineage.

Range-wide phylogeography of *R. alpina* also revealed some interesting patterns. While the Hatay population (south Turkey) and the populations inhabiting Sicily and the Apennine

Peninsula represented just three endemic populations that did not contribute to the rest of Europe, a large part of Europe including the western Asia was colonized from the single refugium located in the mountains of SW Balkan peninsula. Such a pattern would suggest that neither the Pyrenees nor the Caucasus Mountains served as a glacial refugia for this species, and that both were colonized from the same source. Also, the populations inhabiting SW Balkan peninsula have clearly the highest genetic diversity (documented by both markers), thus representing the most valuable parts of the species distribution range.

Such an unusual pattern of genetic structure was also observed for *R. alpina*'s main host, European beech (*Fagus sylvatica*) (Magri et al., 2006). Unlike other potential hosts of *R. alpina* (*Tilia* spp., *Fraxinus* spp., *Ulmus* spp., and *Acer* spp.), *F. sylvatica*'s populations survived the last glaciation in Italy, but did not cross the Alps. Furthermore, SW Balkan served as an important refugium displaying the highest genetic diversity, and the large part of Europe was dominated by the single haplotype. The phylogeographies of *R. alpina* and the beech thus seem to be tightly matched, and both species probably shared a common history. This is rather surprising since the beetle is able to sustain its populations on a phylogenetically wide range of trees. It is thus possible that although the beetle appears to be polyphagous, it is historically connected with only a single host. Its broadening of host range thus might be a recent event which periodically happens during interglacial periods, meaning that the populations currently not exploiting beech are likely to go extinct during next glaciation.



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*The following passage (pages 118–120) is a part of the published paper (DOI 10.1007/s10592-014-0656-2) and it was removed from the online version of the thesis.*

## **Appendix I**

### Supporting information for Chapter IV

*The following passage (pages 122–126) is a part of the published paper (DOI 10.1111/bij.12624) and it was removed from the online version of the thesis.*

## **Appendix II**

### Supporting information for Chapter VI

*The following passage (pages 128–135) is a part of the manuscript in preparation and it was removed from the online version of the thesis.*

## **Appendix III**

### Supporting information for Chapter VII

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