# Linking Genetics and Ecology: Reconstructing the History of Relict Populations of an Endangered Semi-Aquatic Beetle

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**Abstract** Population ecology and genetic studies are complementary approaches to address central questions of conservation biology and can provide information for the protection of biodiversity and the improvement of conservation measures which may otherwise be unattainable. This contribution highlights the benefit of combining diverse approaches for obtaining knowledge on a relict species and for implementing suitable conservation measures. We use the example of *Carabus variolosus*, a flightless European beetle that is listed as a Species of Community Interest in the EU Habitats and Species Directive. Ongoing investigations established the species' habitat choice, population ecology and population genetics, and yield first results from mitochondrial DNA analysis (COI). In small isolated habitat patches we found small sized populations that are genetically strongly differentiated even if adjacent. Evidence for a number of glacial refugia was found. However, all *C. v. nodulosus* populations studied North of the Alps share the same haplotype. We will discuss our findings in the light of the history of the species, of its taxonomic classification and of their implications for conservation.

## 1 Introduction

The flightless ground beetle *Carabus variolosus* Fabricius, 1787 (Coleoptera: Carabidae) is a rare and threatened habitat specialist of headwater areas and swamps in deciduous woodlands. Both larvae and adults live close to the water edge where

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they forage (Sturani 1963). Populations persist in localised areas under pristine habitat conditions of which *C. variolosus* is considered to be an indicator (Turin et al. 2003). Remaining populations are spread in a disjunct range throughout Central and South Eastern Europe. While already confined to a restricted range, the distribution of *C. variolosus* is further contracting presumably due to habitat destruction and pollution (Pavicevic and Mesaros 1997; Turin et al. 2003; Matern and Assmann 2004), resulting in its status as a "relict species" (sensu Fryxell 1962).

Taxonomically, *C. variolosus* has been subdivided into two allopatric subspecies, which can be distinguished reliably only by the different tip shape of the male genitalia (Breuning 1926). The Western *C. v. nodulosus* Creutzer, 1799 extends to Germany and France and along the Dinaric mountains Southwards to Macedonia (Breuning 1926; Turin et al. 2003). The Eastern *C. variolosus* s. str. ranges from the Czech Republic and South Poland along the Carpathian mountains to the Western edge of the Balkan mountains in Bulgaria (Breuning 1926; Turin et al. 2003). Over the past 20 years some authors considered both taxa as sister species (e.g. Casale et al. 1982; Deuve 1994), triggering a debate about their taxonomic rank. In 2004, *C. variolosus* was listed as a "species of Community interest" under the EU Habitat and Species Directive, which requires the European member states, to secure its long-term survival. This may be achieved by strict measures for protection, including the designation of reserves, the application of suitable management plans, and the establishment and maintenance of a "favorable conservation status" for the species (The Council of the European Communities 2004). However, expanding basic knowledge about this rarely studied beetle is necessary to assess and monitor its conservation status and devise effective management measures.

Population ecology and population genetics are complementary approaches to address questions about species conservation management. Combined, they offer valuable insights into viable population size and structure, population dynamics and isolation, dispersal and colonization ability, while addressing different spatial and temporal scales (e.g. Ranius 2006; Schmeller and Merilä 2007; Schwartz et al. 2007). This is of great importance for the protection of biodiversity and the improvement of conservation measures, providing information on, for example, the habitat requirements of species (e.g. Gröning et al. 2007), the levels of genetic variability in populations (e.g. Böhme et al. 2007), and the recognition of distinct gene pools and evolutionary lineages (e.g. Palsbøll et al. 2007).

Here, we review our ongoing investigations on the population ecology and population genetics of *C. variolosus*, including recent results from mtDNA data. We discuss the findings with a specific focus on the (1) postglacial history of the Western subspecies *C. v. nodulosus* and (2) the implications of the results for conservation issues showing the benefit of combining field-based and genetic approaches.

## 2 Habitat Choice and Demography

Demography and habitat choice of *C. variolosus* were investigated in a 2 year mark-recapture study at two representative relict populations of *C. v. nodulosus* in North-Western Germany. Parameters determining the habitat preferences of adult beetles

were estimated from habitat suitability models (e.g. Matern et al. 2007). These studies have highlighted and further refined which specific requirements determine habitat associations (Matern et al. 2007). Key habitat variables governing the occurrence of the adult beetles were found to be: short distance to water, high soil moisture, open woodland vegetation cover, a near-neutral pH of the soil and the lack of ground cover. Accordingly, the extent of suitable habitat was rather restricted (Fig. 1). Continuous observations of individual beetles confirmed their confinement to regions of wet soil and close to, or covered by, water (Drees et al. 2008).

Mark-recapture data showed *C. variolosus* to be a spring breeder in the sense of Larsson (1939), i.e. reproduction took place after the emergence of overwintering adults between April and June. Estimates of population sizes at both sites were very low (Matern et al. 2008). During the spring activity period in 2004 the total number of different adults captured amounted to 63 (Site 1) and 161 (Site 2) individuals, corresponding to a maximum of 150 (Site 1) and 215 (Site 2) individuals when corrected using the Jolly–Seber estimate (Jolly 1965; Seber 1965). Population densities, however, estimated at 1.75 (Site 1) and 0.85 (Site 2) individuals per 10m², were normal to fairly high in comparison to more common species of *Carabus*
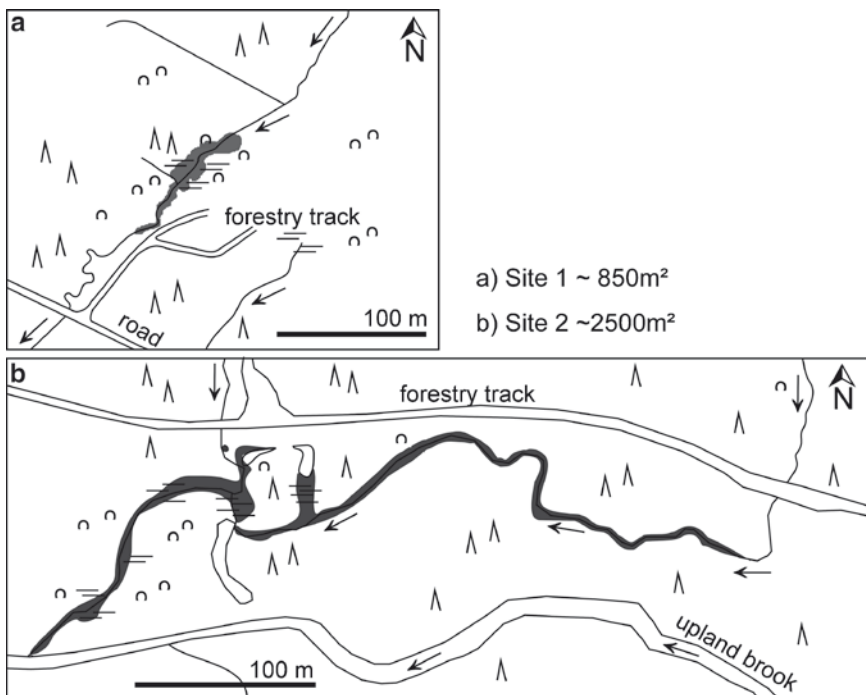


**Fig. 1** Habitat preference of two *C. variolosus nodulosus* populations in the Forest of Arnsberg, Northwestern Germany. Occupied habitat is represented in grey. Figure 2 shows the location of these sites. (**a**) Population 1, (**b**) Population 2

(Matern et al. 2008). Hence, the total population sizes appear to be limited by the extent of suitable habitat, rather than low density. This illustrates the imminent threat to population survival from mechanisms operating in small habitat patches (extinction vortex, Gilpin and Soulé 1986). However, adults were found to be long-lived and almost 30% of the reproductively active individuals were at least in their second breeding period (Matern et al. 2008). This reduces risks of reproductive failure from climatic variation or other stochastic factors in any given year.

## 3    Genetic Population Structure According to Nuclear Genes

Genetic analysis using cellulose acetate electrophoresis was performed on 12 populations of *C. v. nodulosus* throughout most of its distributional range, and including those used for mark-recapture studies above. A total of 308 specimens were included in the analysis for a mean sample size of 25 individuals and 16 allozyme loci (Matern et al. 2009; Fig. 2). Despite small population sizes (inferred from small areas of suitable habitats at most sampling sites), we found no evidence for inbreeding, as populations did not deviate significantly from Hardy–Weinberg equilibrium (GENEPOP 3.4, Raymond and Rousset 1995). However, allele numbers and the amount of polymorphism per population were low, with only eight of the investigated loci found to be polymorphic and a maximum of seven polymorphic loci per population, indicating genetic impoverishment.

Population differentiation and correlation between genetic and geographical distances between samples were quantified with the Arlequin package (Excoffier et al. 2005). Despite the low number of private alleles, populations were highly differentiated (overall $F_{ST} > 0.45$) owing to locally differentiated allele frequencies among populations, as exemplified by Fig. 2. This finding supports the possibility that populations were affected by genetic drift due to small population sizes. Neither clinal variation nor a directional loss of alleles that could hint at any postglacial recolonization scenario according to the allele elimination hypothesis (Reinig 1938; Hewitt 1999) could be detected. Generally, geographic distance only had a marginal influence (<10%) on the isolation of populations (Mantel test). A neighbor-joining tree based on genetic distances among all *C. v. nodulosus* populations confirms this result, showing poor agreement between genetic and geographical distance across the entire range (not shown). Even neighboring populations at distances of only 2–3 km in the same forest and drainage system were found to be virtually independent of each other as shown, for example, by significant pairwise $F_{ST}$ values of 0.27 and 0.14 between populations 1 and 2 and populations 5 and 6, respectively.

Strongly separated were two populations from Southern Slovenia (sites 11 and 12) which showed deviating allele frequencies and some private alleles compared to the other investigated populations. This is confirmed by Bayesian structure analysis (STRUCTURE, Pritchard et al. 2000) which generally assigns the individuals of these two populations to one distinct genetic cluster if no a priori assumptions of geographical origin are made (Matern et al. 2009). This pattern
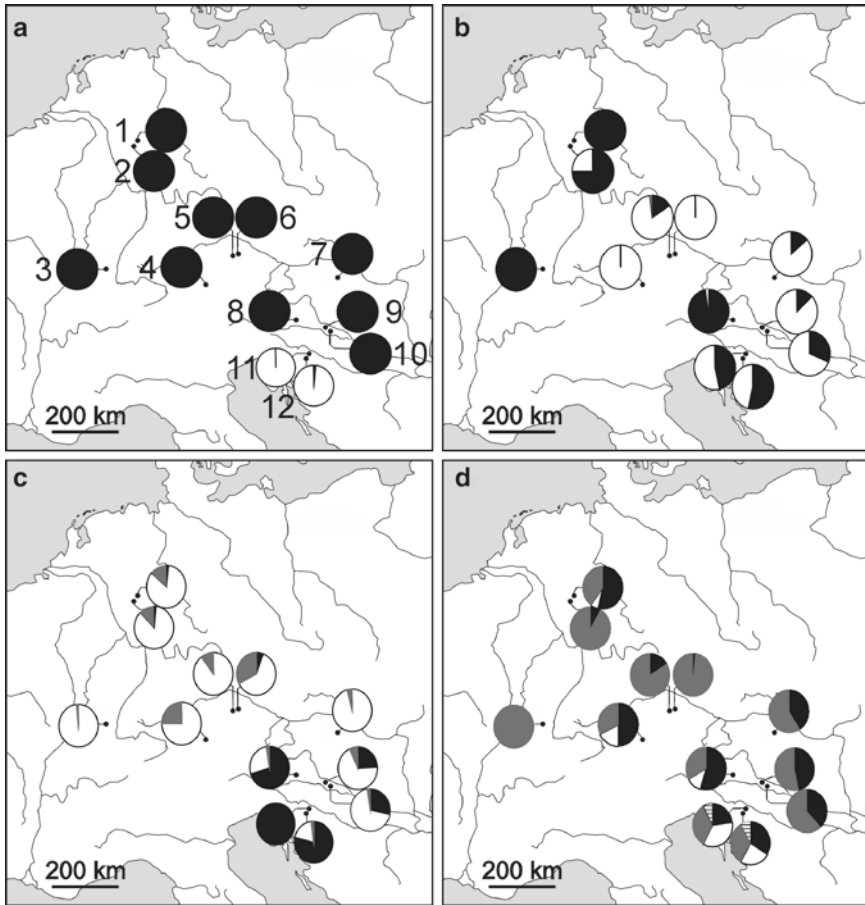
**Fig. 2** Allele frequencies of (**a**) alcohol dehydrogenase, (**b**) glucose-6-phosphate isomerase, (**c**) aspartate aminotransferase, and (**d**) mannose-6-phosphate isomerase in the investigated populations of *C. variolosus nodulosus*. Different colours indicate various alleles at the respective locus

may be caused by genetic drift and an efficient present isolation between the Southern Slovenian samples and the remaining Central European populations that might have existed since the survival of these populations in different glacial refugia (cf. Chaps. 4 and 5.1, this contribution).

# 4   Mitochondrial DNA Data

Mitochondrial DNA sequences largely confirmed the results from allozyme studies, also indicating minimal within-population variation and the separation of the South Slovenian samples. We amplified a fragment of the cytochrome oxidase I (COI)

gene using primers C1-J-2183 (Jerry) and TL2-N-3014 (Pat) (Simon et al. 1994) and successfully sequenced 608 bp for 114 specimens of *C. v. nodulosus* and six individuals from three Romanian populations of *C. v. variolosus*. Only nine different haplotypes were identified (Fig. 3). There was hardly any haplotype variation within populations of *C. v. nodulosus*. With the exception of two individuals deviating by one and three nucleotide changes, respectively, all remaining 92 individuals
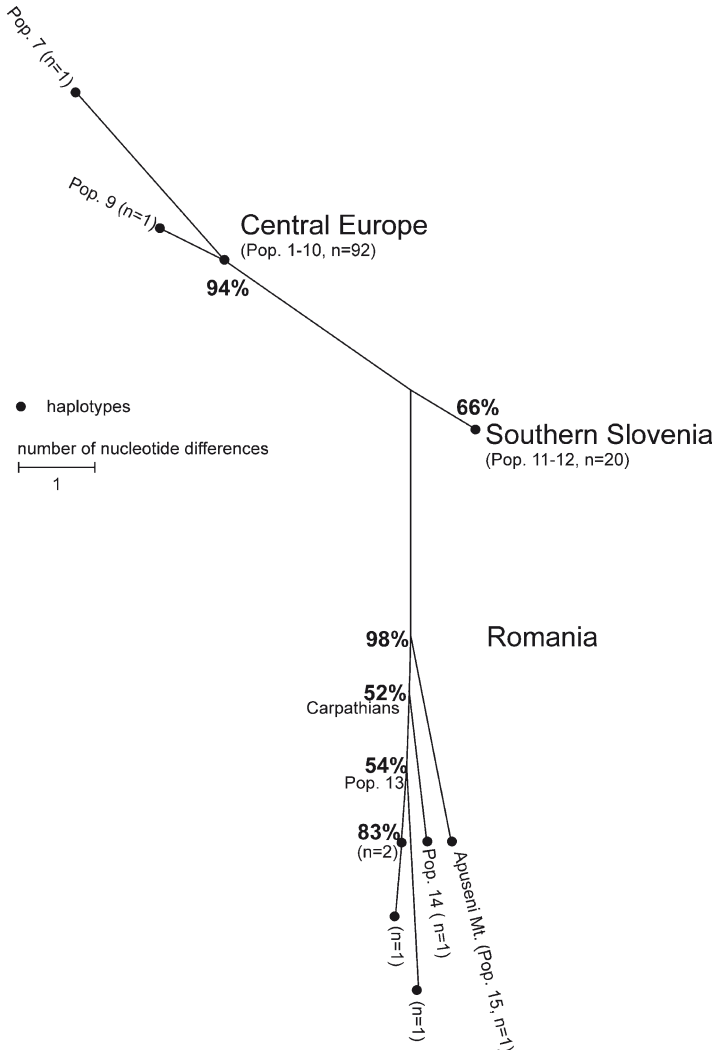


**Fig. 3** Neighbor-joining tree (Saitou and Nei 1987) based on the number of nucleotide changes between 120 sequences of COI fragment. Numbers at nodes refer to support from 1,000 bootstrap replicates. Central European and Southern Slovenian specimens: *C. v. nodulosus*, Romanian specimens: *C. v. variolosus*. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

in the Central European populations (1–10) shared the same haplotype. The 20 individuals from Southern Slovenian (populations 11 and 12) shared a single haplotype that differed from the former by four nucleotide changes. All of these were separated from *C. v. variolosus* haplotypes by at least four nucleotides (Fig. 3). This finding corroborates that the Southern Slovenian populations of *C. v. nodulosus* survived in a different glacial refugium than the remaining European samples (cf. Chap. 5.1, this contribution).

# 5   Synthesis

## 5.1   *C. variolosus: A Relict Species*

In contrast to other weak dispersers that survived the ice ages in Southern refugia and remained largely confined to these areas ever since, *C. variolosus* expanded its range subsequent to postglacial climate changes despite being flightless. As a stenotopic woodland species specialised on water margins, *C. variolosus* must have suffered considerably from the climate changes of the glacial periods. Given the species' current habitat preference (e.g. Sturani 1962), its confinement to lowland or lower montane altitudes (e.g. Breuning 1926; Barloy et al. 2004) and its strict stenotopy established by the habitat models (Matern et al. 2007), it is likely that populations survived the ice ages in sheltered, wet deciduous woodlands. These habitats may have persisted during the Last Glacial Maximum in Iberia, Italy and the Balkans (Zagwijn 1992), and in the Carpathians (Kotlík et al. 2006). Deffontaine et al (2005) furthermore suggest the existence of mixed coniferous-deciduous forests in the river systems present near the Alps and in the Hungarian plain and Slovakia. Paleobotanical and genetic evidence also corroborates the survival of beech populations as far North as Southern France, the Eastern Alps, Slovenia, Istria and possibly Southern Moravia-Bohemia (Magri et al. 2006), but so far no refuges of forest-forming tree species are known from further North. Recent faunistic records of *C. variolosus*, however, exist from numerous regions North of the Alps such as the presence of *C. v. variolosus* at the foothills of the Sudetic Mountains, Upper Silesia and the Lublin Upland in Southern Poland (Pawlaczyk et al. 2004) and as far as the Hamburg area, where *C. v. nodulosus* occurred until about 100 years ago (Breuning 1926).

The homogeneous mtDNA in the Central European populations of *C. v. nodulosus* is consistent with a recent colonization of the region North of the Alps. Under the standard molecular clock for insect mtDNA of approximately 2.3% divergence per million years (Brower 1994), all Central European populations of *C. v. nodulosus* and the Southern Slovenian lineage separated no more than 0.3 Myrs ago (0.7% pairwise average sequence divergence). Likewise, the Central European populations and haplotypes of the subspecies *C. v. variolosus* separated approximately 0.5 Myrs ago (1.2% divergences). The existence of these lineages which likely predate

the most recent glacial cycles may suggest the existence of more than one glacial refuge or source area within or near to the investigated area.

Post glacial colonization processes are not reflected in allozyme variability. We did not observe a directional loss of alleles according to the allele elimination hypothesis (Reinig 1938; Hewitt 1999) nor any regionally specific sets of allozymes and their combination in intermediate populations, which could suggest potential contact zones. The largely random geographic structure of allozyme variants, the lack of private alleles, and the lack of correlation in allele frequencies with geographic distance (Matern et al. 2009) suggests that the variation in Central European populations was more similar at first, but that populations lost variability subsequently due to stochastic processes. Hence, the case of *C. variolosus* could represent an example where postglacial colonization, in combination with subsequent geographic isolation, produced genetic differentiation of a similar magnitude as that resulting from survival in different glacial refuges. For example, the postglacial *C. variolosus* populations from Germany and the Vosges display an $F_{ST}$ of 0.45 (7 polymorphic enzyme loci). This is similar to values obtained for 29 populations of *Carabus punctatoauratus* in the Pyrenees that survived in different glacial refugia (mean $F_{ST}$ of 0.23, enzyme loci, Assmann and Weber 1997) or to values found in *C. solieri*, another flightless silvicolous ground beetle, that remains largely confined to its glacial refuges in the Southwestern Alps close to the border between France and Italy ($F_{ST}$ of 0.34, microsatellite markers, Garnier et al. 2004). Although $F_{ST}$ values obtained with different makers are not directly comparable, the latter example also supports the depth of population separation within the Northwestern lineage of *C. variolosus*.

It is likely that the present interpopulation differentiation of *C. v. nodulosus* is only partly due to low dispersal power of these flightless beetles, but was exacerbated by small population sizes. Ecological knowledge helps to understand better what affects the recent population history of *C. v. nodulosus*: its high habitat specificity not only increases the barrier function of the landscape structure surrounding suitable habitat patches but may also result in very small populations, as demonstrated for the above two populations in Northwestern Germany (Matern et al. 2008). Descriptions of patch sizes in additional populations of *C. v. nodulosus* suggest that these may exist or have existed under similar spatial conditions (e.g. Gersdorf and Kuntze 1957; Perraudin 1960; Morati and Huet 1995). While the investigated populations do not appear to be inbred, they are genetically impoverished in comparison with other carabid beetles (Matern et al. 2009). This renders them highly susceptible to genetic stochasticity that is associated with extinction vortices, in particular in the face of expected climatic changes.

While *C. v. nodulosus* was distributed much more widely until about a century ago, it has since suffered from a marked decline and nowadays only persists in very localised relict populations (Breuning 1926; Turin et al. 2003; Matern and Assmann 2004). Intensive human land use with its massive impact on the natural hydrological budget is thought to be responsible for the decline (e.g. Perraudin 1960; Weber and Weber 1966; Pavicevic and Mesaros 1997; Turin et al. 2003). The resulting destruction and degeneration of suitable habitat has aggravated the geographical separation of headwater areas and pristine forest swamps that already affected

*C. variolosus* populations under natural conditions but is now increasing the effect of habitat fragmentation. Currently, *C. v. nodulosus* is considered endangered throughout its entire range (Turin et al. 2003), and its highly restricted distribution is further contracting. Therefore, it is unquestionably a relict in the sense of Fryxell (1962) meeting also further specified criteria such as a reduced variability and narrow ecological tolerance, which potentially indicate a reduction of adaptability, and may ultimately be a candidate for extinction. While the beginning of the decline of *C. v. nodulosus* cannot be clearly identified, the relict status of *C. v. nodulosus* in Central Europe, however, is relatively recent, as the beetle successfully colonized this area post glacially. While the situation of *C. v. variolosus* apparently is less alarming (Turin et al. 2003; Pawlaczyk et al. 2004), this subspecies is also characterized by a narrow ecological tolerance and a restricted range that is further declining in some regions (Turin et al. 2003).

## 5.2 *Implications for Conservation and Future Work*

The example of a species that survived past climate change in refuges and benefited from reverse climatic changes by range expansion despite low dispersal power may give hope for conservation. However, *C. v. nodulosus*, like many other postglacial populations, shows reduced genetic variability (Hewitt 2001), which implies certain risks for long-term survival. Hence, the maintenance of genetic diversity within species is one important goal of biodiversity conservation.

As the Southern Slovenian populations are a distinct genetic unit holding genetic diversity not found in Central European populations, they should consequently be granted special protection alongside the Central European samples. To conserve the remaining genetic diversity, as many of the relict populations as possible should be targeted, since more than 45% of the total allozyme variance of *C. v. nodulosus* is found among populations (Matern et al. 2009). Where possible, a cautious enlargement of the habitat patches may also be attempted.

If further populations within or close to the core of the subspecies range could be found that display high genetic diversity (owing to their location in glacial refuges, for example), these of course would have an especially high conservation priority. Locations of all current relict populations of *C. variolosus* (and of other flightless, sensitive species) may also be an indicator of unique environmental conditions not affected by disturbance and demonstrate the general continuity (stability) of habitats. These occurrences can therefore be used to identify valuable habitats for the conservation of entire species communities.

This study provides an example of increased knowledge and sensible conservation strategies that were derived from a combination of diverse methodological approaches offering different perspectives. The contribution of ecological knowledge is essential where knowledge on a species is scant. Details on the habitat preference and habitat use of *C. variolosus* are crucial for an assessment of factors influencing the incidence and distribution pattern of the species. In the case of

*C. variolosus* this information especially emphasises the urgency to assess population sizes and the necessity to enlarge populations. The analysis of COI provides valuable information on long-term population history that was not available from allozyme analysis. Moreover, it strongly supports the allozyme results which already suggested that the Southern Slovenian populations are a differentiated entity, with relevance for conservation (Moritz 1994). The identification of differentiated population groups is considered crucial for the conservation of the genetic diversity of species (e.g. Ryder 1986; Moritz 1995) and provides important data in rescue efforts of species by means of translocations (e.g. Avise 1996). While mtDNA sequence data contribute additional information on population differentiation and genetic diversity, they should not be used exclusively in the discussion of conservation priorities. It is the population genetic approach with nuclear markers that offers an advanced insight into the isolation of populations and the level and overall distribution of genetic diversity within and across populations. The allozyme results underline the need for recognition of distinct Management Units even at adjacent sites within the same drainage system.

In the context of the EU Habitats Directive and Natura 2000 Network it is now important to extend analyses to the Eastern subspecies *C. v. variolosus*. Further allozyme and DNA analyses to unravel the phylogeny and discover subspecific population structure will show whether the conservation measures established for *C. v. nodulosus* are also valid for *C. v. variolosus*. One important clue, however, is already given by the current COI analysis: sequence divergence between the two taxa is surprisingly low. As the amount of sequence divergence is extremely variable in different lineages, it is impossible to apply a general threshold value to justify taxonomic distinction at the species level (Vogler et al. 1993). Some molecular taxonomists have (controversially) used a 3 or 2% COI divergence for species delimitation across a wide range of organisms including insects (cf. Duckett and Kjer 2003; Hebert et al. 2003, 2004; Moritz and Cicero 2004). Comparisons of mtDNA studies find sequence divergence of less than 2% primarily on an intraspecific level (Vogler and DeSalle 1994; Avise 2000; Woodcock et al. 2007). This is also the case among populations of another ground beetle species, *Nebria rufescens*, which are not formally distinguished at the species or subspecies level and display a mean mtDNA-sequence divergence (ND1) of 1.3% (Schmitt et al. this book). Although the two taxa of *C. variolosus* are "diagnosable" under a phylogenetic species concept (Vogler and DeSalle 1994), their divergence is low and only slightly greater than the striking separation of the Central European and Southern Slovenian populations. This finding may support the older classification as subspecies (cf. Breuning 1926), settle the past discussion on the potential species rank of *C. v. nodulosus* and counteract taxonomic inflation (cf. Isaac et al. 2004).

Future genetic assessment of *C. variolosus* will be necessary to study the success of conservation recommendations such as population increase or population connectivity and thus augment ecological monitoring. Moreover, it may also enhance our knowledge on the relationship between population sizes and the maintenance of genetic diversity and probably their influence on the persistence of these populations.

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