HIGH MOLECULAR DIVERSITY IN CARABUS (HYGROCARABUS) VARIOLOSUS AND C. NODULOSUS

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The Carabus subgenus Hygrocarabus contains two taxa: C. variolosus and C. nodulosus, the species or subspecies status of which is handled far from uniform in the literature. Both taxa show a similar morphology, the shape of the tip of the aedeagus provides a reliable morphological marker for identification. We analysed two mitochondrial gene parts (COI-5' and COI-3') and a nuclear one (ITS2). High diversity was found showing specific geographical patterns. Introggressive hybridisation was detected but interpreted not as an argument for subspecies status because high genetic distances indicated that it must have taken place in former times. In a laboratory hybridisation experiment, the male did not accept the female of the other taxon, supporting the conclusion that these are separate species. A series of refuges was expected for the period of ice ages. Although only the taxon C. variolosus is listed in Annex II and IV of the EU Habitats Directive, C. nodulosus also falls under this listing, as at the time of including the species into the Annexes in 2004, the two taxa were considered subspecies and hence the listing would include both, independent of later taxonomic revisions.

Keywords: diversity, COI, ITS2, species versus subspecies, introgression, refuges, Carabus (Hygrocarabus) variolosus, Carabus nodulosus.

INTRODUCTION

The genus Carabus (Coleoptera, Carabidae) has high variability within species, which often causes problems with the species assignment. The Hygrocarabus taxa were described as Carabus variolosus Fabricius, 1787 and C. vari-
**oolosus nodulosus** Creutzer, 1799. In contrast to other *Carabus* spp., they show minimal morphological differences and a few slightly different characters that lead Casale *et al.* (1982) to establish them as separate species. Additionally, natural and experimental hybrids (e. g. Puissegur 1964, Allemande & Malusa 1984, Deuve 2004) and introgression events are frequently found: *Chrysocarabus solieri* (Rasplus *et al.* 2000), *Ohomopterus* spp. (Sota *et al.* 2000, Sota & Vogler 2001, Sota *et al.* 2001, Ujji *et al.* 2005, Nagata *et al.* 2007), *Chrysocarabus splendens* – *C. rutilans* (Düring *et al.* 2000, 2006), *Chrysocarabus splendens* – *C. punctatouraurus* (Streiff *et al.* 2005), *Coptolabrus* spp. (Zhang *et al.* 2005), *Leptocarabus* spp. (Zhang & Sota 2007), *Mesocarabus* spp. (Andujar 2012, Andujar *et al.* 2012) and *Limmocarabus clathratus* (Mossakowski 2016). However, COI sequences show extremely low variability within and between German, Austrian and Slovenian *C. nodulosus* populations and moderate differences in *C. variolosus* (Matern *et al.* 2010).

The starting point of this study was the hypothesis that molecular data would reveal the subspecies status of the two taxa and give some insight into the phylogeography of their populations.

The subgenus name *Hygrocarabus* indicates a high adaptation to the water where larvae, as well as adults, hunt for prey (Sturani 1963). The structure of the elytra is unique and may be seen as an adaptation to this lifestyle (Fig. 1). This extreme hygrophilic species is adapted to moderately cold temperatures and can be found at the upper stretches of small rivers and creeks, from lowlands (*C. nodulosus*: old record from Hamburg 50 m, Arnsberg 250 m) to 1000 m a.s.l. (*C. variolosus*, Turin *et al.* 2003); adults overwinter close to water. A first map (Breuning 1926) shows a more or less Central European distribution area. *C. variolosus* is restricted to the Carpathians, *C. nodulosus* occurs from the Balkans to northern Germany and the Massif Central in France.

Our aim in this study is to test three hypotheses.

**H1:** The taxa *C. variolosus* and *C. nodulosus* constitute one species. This can be tested by looking for hybrids in nature, by crossbreeding laboratory experiments and by an analysis of DNA sequences of mitochondrial and nuclear genes.

**H2:** High genetic variability. Matern *et al.* (2010) found high variability of enzymes in *C. nodulosus* populations but very low one in their sequence data. We expected a higher sequence variability looking on the whole distribution area although the *Hygrocarabus* taxa were morphologically very unique.

**H3:** The analysis of genetic variability will reveal the existence of refuges during the Pleistocene: south of the Holdhaus line (Holdhaus 1954) and north or above of the Mediterranean vegetation zone of garrigue and macchia.

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MATERIAL AND METHODS

Crossbreeding experiments

Live specimens of *C. variolosus* adults (1 male, 2 females) were collected in the Zemplén Mountains (Nagyhuta, Rostalló-patak), and those of *C. nodulosus* (14 males, three females) in Eastern Bakony (Réde, Küllőfeji-ér), which is part of the Transdanubian Mountains, both in Hungary. The two locations are 280 km apart. The beetles were collected between 15 May and 3 June 2020 and stored separately. The male and females of the different taxa were put in the same box for about 30 min on June 5 and again on June 10 to test whether crossbreeding occurs. On the second date, males were put to females of the same taxon immediately after the crossbreeding test.

DNA isolation

We obtained specimens from almost all regions of the distribution area except the westernmost part (Jura Mts and Massif Central, France). Besides fresh or recent specimens collected in the monitoring programmes for the species, we analysed dry specimens from collectors and museums. The latter samples gave results also, but in many cases, only a shorter part of the sequence and only mitochondrial data. A leg or in some cases thorax muscles were sent to the Advanced Identification Methods (AIM) Laboratory, in Munich, Germany, for DNA extraction and sequencing. For further details, see Mossakowski (2016).

Molecular methods

We analysed parts of the mitochondrial genes cytochrome oxidase subunit 1 (COI-5’ and COI-3’) and NADH dehydrogenase subunit 5 (ND5) as well as the nuclear internal transcribed spacer 2 (ITS2) and the *wingless* gene. The ND5 and *wingless* genes gave in-

Fig. 1. *Carabus variolosus* male from Zemplén Mts, Hungary. (Photo: Sándor Bérces)
Table 1. Patterns of sequence COI variability in *Carabus variolosus* and *C. nodulosus*. All variable positions included, except such only with singletons. HTG: Haplotype groups. Yellow: singletons or ambiguity; grey: missing data; dark green: exclusive for a group; light green: exclusive but with a few exceptions; dark blue: exclusive; pale blue: in different groups, orange-coloured: in contrast to green.

| Var COI -S' | HTG       | Pos | T | C | T | G |
|-------------|-----------|-----|---|---|---|---|
| var168 TI Tisza | 1 nod | 3 | G | C | C | C | A | A | T | C | G |
| var75 HR Trebica | 1 nod | 3 | G | C | C | C | A | A | T | C | G |
| var65 HR Blatna | 1 nod | 3 | G | C | C | C | A | A | T | C | G |
| var136 Sokolovek Gdzt. | 1 nod | 3 | G | C | C | C | A | A | T | C | G |
| var139 SI Krakovsk Gdzt. | 1 nod | 3 | G | C | C | C | A | A | T | C | G |
| var20 AT B N Slagerab | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var31 AT B N Slagerab | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var57 AT B N Slagerab | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var185 AT K W Meierag | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var189 AT ST E Amfoles | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var51 AT ST F Rosie | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var270 AT W Rede | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var222 AT HU W Creznek | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
| var63 HR Jakovac | 2 nod | 3 | T | C | C | C | A | A | T | C | G |
Table 2. Mean COI-5’ Kimura-2-distances (%) within and between groups of Carabus variolosus and C. nodulosus. HTG: Haplotype groups.

| Distances COI-5’ | 1-3 | 4   | 5   | 6   | CE  | SE  | W   | CN  | OUT  |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|------|
| HTG              |     |     |     |     |     |     |     |     |      |
| 1,2,3 nod nod    | 16  | 0.60| 1.19| 1.89| 3.06| 2.26| 2.23| 3.34| 2.75 |
| 4 nod nod        | 3   | 1.19| 0.66| 1.93| 3.14| 2.28| 2.33| 3.30| 2.45 |
| 5 nod var        | 6   | 1.89| 1.93| 0.63| 3.02| 2.87| 2.85| 3.58| 3.52 |
| 6 both both      | 10  | 3.06| 3.14| 3.02| 1.49| 4.21| 4.55| 5.44| 4.29 |
| CE var var       | 1   | 2.26| 2.28| 2.87| 4.21| –   | 1.14| 2.85| 2.31 |
| SE var var       | 7   | 2.23| 2.33| 2.85| 4.55| 1.14| 0.52| 2.17| 1.80 |
| W var var        | 4   | 3.34| 3.30| 3.58| 5.44| 2.85| 2.17| 0.37| 2.45 |
| CN var var       | 10  | 2.75| 2.45| 3.52| 4.59| 2.31| 1.80| 2.45| 0.71 |
| OUT C. depressus | 1   | 8.74| 8.40| 8.93| 10.4| 8.2 | 7.39| 9.80| 9.23 |
| OUT C. irregularis | 1  | 10.80| 10.95| 11.02| 12.1| 10.7| 9.96| 12.15| 11.95|

Fig. 2. Mating behaviour of Carabus variolosus and C. nodulosus in the laboratory. A: No interest to mate between C. variolosus male (left) and C. nodulosus female (right). B: Copulation took place immediately after putting together male and female C. variolosus. (Photos: Sándor Bérces)
sufficient data and were omitted in the analyses. Editing and alignment of the sequences were done using CHROMAS (2008), BLAST (Altschul et al. 1990) and the Seqotron (Fourment & Holmes 2016) programs. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version X (Kumar et al. 2018, Stecher et al. 2020), trees and distances were calculated with the clock function, the Kimura 2-parameter and the GTR + \Gamma + I model. We analysed branch support using bootstrap analyses (Felsenstein 1985). We looked for compensatory base changes in ITS2 with 4SALE (Seibel et al. 2006).

Phylogenetic trees are an abstract representation of results and sometimes not so easily correctly understood by people untrained to read them. Therefore, we display our sequence data in detail: All base positions with differences were shown and arranged in clusters (Table 1 & 3).

### RESULTS

#### Crossbreeding experiments

Neither males nor females of either taxa tried to mate with the other one (Fig. 2A). The individuals collected were sexually mature because *C. variolosus* males and females started copulating immediately after being put together; the same happened with *C. nodulosus* (Fig. 2B). A *C. nodulosus* male even tried to copulate with another male, a phenomenon also observed in the field (Vértes Mts, Hungary, S.B.).

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**Table 3.** Bases at all positions that show differences in *Carabus variolosus* (var) and *C. nodulosus* (nod) ITS2 sequence data. Colours indicate base distribution. N: Total number of specimens with identical sequences. Top lines: Position numbers.

| Position | Carabus variolosus (var) | Carabus nodulosus (nod) |
|----------|---------------------------|-------------------------|
| 1        | T                        | C                       |
| 2        | C                        | T                       |
| 3        | A                        | G                       |
| 4        | T                        | A                       |
| 5        | C                        | T                       |
| 6        | A                        | G                       |
| 7        | T                        | A                       |
| 8        | C                        | T                       |

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Fig. 3. Maximum likelihood tree of *Carabus variolosus* and *C. nodulosus* COI-end. Only specimens with differences in their sequences (G-H) model, with normal distribution of clock under K2P and Gamma distributed with invariant sites (G+I) model. Numbers indicate haplotype groups of *C. nodulosus*, letters those of *C. variolosus*. CE: central-east, SE: southeast, W: west, CN: central north. Compare map in Fig. 4.
Molecular sequence data

Mitochondrial COI-5’ end (barcode sequence) – Although the bootstrap values were low, *C. variolosus* and *C. nodulosus* were separated in the Maximum Likelihood tree. Ten haplotype groups (htg) were established (Fig. 3 & Table 1), seven in *C. nodulosus* and four in *C. variolosus*. The most remarkable result was the occurrence of *C. nodulosus* haplotypes (HTG 5 and 6) in populations of *C. variolosus*. This is an indication that *C. nodulosus* specimens migrated up to the region of the Carpathian Arc and that an introgression took place in former times (see discussion).

The data of the COI-3’ end were fewer and displayed lower variability than those of COI-5’end but showed a pattern consistent with them. They were also more variable than those of Matern *et al.* (2010) due to the larger geographical area under study (data not shown).

![Fig. 4. Distribution of COI-5’ haplotype groups of *Carabus variolosus* (triangles) and *C. nodulosus* (circles). *C. variolosus* occurs in the Carpathian Mountains (east of dividing line), *C. nodulosus* west of it. Circles in the *C. variolosus* area indicate *C. variolosus* specimens with a *C. nodulosus* haplotype indicating former introgression events. Line adapted from the map of Breuning (1926). Different haplotype groups were marked by numbers or direction of the triangle. Open triangles indicate probable extra refuges. AT: Austria, BA: Bosnia and Herzegovina, CZ: Czech Republic; DE: Germany, HR: Croatia, HU: Hungary, IT: Italy, PL: Poland, RO: Romania, RS: Serbia, SI: Slovenia, SK: Slovakia, UA: Ukraine](image-url)
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Table 4. Numbers of ITS2 sequence differences in *Hygrocarabus* compared to other *Carabus* spp.

| Comparison                          | No. specimens | Numbers of ITS2 sequence differences |
|-------------------------------------|---------------|--------------------------------------|
|                                     |               | base-base | base-gap | total  |
| *Macrothorax morbillosus* – *Chrysocarabus* spp. | 1–11          | 16        | 30       | 46     |
| *C. (Hygrocarabus) variolosus* – *C. nodulosus* | 13–18         | 17        | 13       | 30     |
| *Chrysocarabus rutilans* – *C. hispanus*    | 2–1           | 7         | 14       | 21     |
| *C. problematicus* – *C. difouri*        | 8–22          | 6         | 11       | 17     |
| *C. problematicus* – *C. lusitanicus*    | 20–66         | 10        | 2        | 12     |
| *C. problematicus* – *C. macrocephalus*   | 20–29         | 11        | 3        | 14     |
| *C. lusitanicus* – *C. macrocephalus*     | 66–29         | 3         | 12       | 15     |

Nuclear gene: ITS2 – There was a clear separation between *C. variolosus* and *C. nodulosus* with sufficient bootstrap support (Fig. 5). Two dot-marked specimens of *C. variolosus* had a mitochondrial sequence of *C. nodulosus*. They showed a hybrid character but belonged to *C. variolosus* by distribution area, morphology and confirmed by a sequence of a nuclear gene. They did not represent recent hybridisation events (see discussion).

Many base differences showed the same pattern (Table 3). A clear differentiation occurred between the two taxa. The ITS2 substitutions were classified

Fig. 5. ITS2 tree of *C. variolosus* (var) and *C. nodulosus* (nod) specimens. Reduced data set without identical sequences of the same or different locations. The tree was rooted by *C. irregulares*. Black dots indicate *variolosus* specimens with a *nodulosus* mitochondrion. Compare data in Table 4.

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into three categories (Table 3). Bases – base and base – gap substitutions added up to 30 informative differences between *C. variolosus* and *C. nodulosus*, a high value when compared to species differences of other *Carabus* subgenera (Table 4).

### DISCUSSION

**Are the *C. variolosus* and *C. nodulosus* one species?**

The taxonomic status of *Carabus variolosus* and *C. nodulosus* is not consistent. Several authors classify them as two species (*Casale et al.* 1982, *Deuve* 2004, 2019, *Szél et al.* 2008, *Lorenz* 2019), most, however, consider them subspecies (*Breuning* 1926, *Guéorguiev & Guéorguiev* 1995, *Vreczec* 2007, *Matern* 2010, *Curcic & Stankovic* 2011, *Březina et al.* 2017, *Tallósi & Mesaroš* 2020; for a review see *Müller-Kroehling* 2006). *Casale et al.* (1982) classified them as semi-species from an evolutionary viewpoint.

Natural and experimental hybrids – The genus *Carabus* is well known for natural hybridisation (c.f. *Deuve* 2004), and numerous experimental hybrids are described (*Puissegur* 1964, *Allemand & Malaula* 1984). The question, whether this occurs between *C. nodulosus* and *C. variolosus*, however, is hampered by the difficulties of keeping them in captivity. Mario *Sturani* (1962) could rear larvae but no adults. Other Italian colleagues were not successful, either, in rearing *C. nodulosus* (A. Casale, personal communication). This difficulty may be due to the very specialised biology of the species. These beetles need shallow waters and swampy ground (*Sturani* 1963, *Koth* 1974, *Matern et al.* 2008). Only *Lassalle* and *Renaut* (2008) succeeded by using a complicated technique of circulating water.

Species concepts are numerous (*Mayden* 1997). We followed the one defined by *Dobzhansky* (1970: 374): ‘A biological species is an inclusive Mendelian population; it is integrated by the bonds of sexual reproduction and parentage’.

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**Table 5.** Calibration of COI-5’ in *Carabus variolosus* and *C. nodulosus*, respectively. We took the split distance between south-eastern populations of *C. variolosus* (SE) and the probably oldest group (htp 6) of *C. nodulosus* as basis: 4.55 was set as 1.00 (K2P distances; pairwise rate of 2.26%/my). Splits correspond to those in Fig. 3.

| Splits       | Parts | Mya  |
|--------------|-------|------|
| *variolosus* | *nodulosus* | 1.00 | 2.01 |
| CE – Rest    | 4 – 1–6 | 0.45 | 0.91 |
| SE – CN, W   | 6 – 1–5 | 0.30 | 0.60 |
| CN – W       | 5 – 1–3 | 0.25 | 0.50 |
| Within SE    | within 1–3, 5 | 0.20 | 0.39 |
No hybrids between *C. nodulosus* and *C. variolosus* are known either from nature or from laboratory experiments. Although our experiments were of short duration, we could not observe mating behaviour between *C. nodulosus* and *C. variolosus*; however, the same specimens copulated immediately when put together with conspecifics. This is a strong argument for species status of the two taxa although the mechanisms behind this behaviour remain unclear.

**Indirect method: DNA sequences of mitochondrial and nuclear genes**

Sequence patterns – Mitochondrial and nuclear patterns differed clearly. The nuclear ITS2 sequences gave significant differences between the two taxa (Fig. 5 and Tables 3 & 4) as expected for different species. Both mitochondrial sequences exhibited divergent patterns of variability. The barcode region of COI had a few positions with deviating bases between the taxa, but this pattern was not strict, a base widespread in *C. variolosus* occurred also in *C. nodulosus* in one or a few cases; and vice versa. Both parts of COI showed a high variability indicating a high diversity within the taxa. The mitochondrial data (Table 1) support a subspecies status, which is in line with the conclusions drawn by *Matern et al.* (2010).

The data of the ITS2 sequences were compared with data of other *Cara-bus* species to get a basis for the evaluation. The numbers of ITS2 substitutions between *C. variolosus* and *C. nodulosus* were counted and compared to data on *Chrysocarabus, Macrothorax* (*Düring et al.* 2006) and *Mesocarabus* (*Andujar* 2012) (Table 4).

Very few substitutions were found within and between *Chrysocarabus* spp., a subgenus well known for its crossbreeding ability (*Allemand & Malausa* 1984). Numerous base substitution existed between the subgenera *Chrysocarabus* and *Macrothorax*. At the species – species level, we also found a high degree of differentiation between *C. variolosus* and *C. nodulosus*, lower than between the subgenera *Chrysocarabus vs. Macrothorax* (Table 4) but higher than those between the *Mesocarabus* spp.

These data support species status of *C. (Hygrocarabus) variolosus* and *C. nodulosus*.

**Distances and temporal aspects**

A high genetic distance between two taxa may indicate a species status, but there is no general limit. *Raupach et al.* (2010) found a minimum Kimura 2-parameter interspecific distance of 3.14% among a wide range of carabid species. *Pentisaari* (2014) found a mean of 2.2% (min. 0, max. 3.76%) within-and 7.27% (0–13.45%) between-species distance in carabids. The carabid ge-
nus Amara has three species pairs with a COI Kimura 2-parameter distance of 2.2% lower as normal for species (Raupach et al. 2018).

The COI-5’ distances found in our study depended on the population group compared. Within C. nodulosus mean Kimura 2-parameter distances were <2% except for haplotype group 6 that underlaid introgression into C. variolosus. Between the C. variolosus groups high differences in these scores occur, the highest within species value was 2.85 between central-east and the western populations of the Southern Carpathian Arc. Only small glaciers covered the Carpathian Mts during the ice age period, except a bigger one on the Fagaras Mts and another one in the northern parts (Theowald & Mannheims 1962), the former isolated the western populations form those in more eastern regions for a more extended period.

The differences between C. variolosus and C. nodulosus ranged from <1.0% to 5.4%, with the highest values between geographically close populations. Calibration of a time scale was performed using the rate published by Andujar et al. (2012) that was based on various Carabus species of diverse subgenera (2.26%/my). This is a rate comparable to the standard for insects (Brower 1994). Papadopoulou et al. (2010) discussed the problems of rate calibration in detail and documented high variation in published rates. They got a rate of 3.36 or 3.54, respectively, calibrating their data on tenebrionid beetles by the Mid-Aegean trench.

ITS2 substitution rates were much lower than those of the mitochondrial genes (1.14%/my, Andujar et al. 2012). Applying this to the 3.5% ITS2 distance between C. variolosus and C. nodulosus, the split between them occurred 3.07 myBP. A partial branch with more or less constant values had a mean distance of 5.2, and a single maximum was 6.3 in another branch. This is an indication for an early split.

In another EU protected species, the cerambycid Morimus funereus, the ITS2 data show only a 7-position gap between two haplotype groups and hybrids also occur (Solano et al. 2013). At the other hand, Audisio et al. (2009) found high ITS2 distances (mean 4.4–18.6%) between Osmoderma spp., another Annex II taxon, the data of which are unfortunately not available in GenBank.

At first sight, our data suggest a lower evolutionary rate in COI-3’ than in COI-5’. We found a higher diversity in the latter gene part in the same specimens. A much earlier split of C. variolosus and C. nodulosus was the result when considering ITS2 data. In consequence, the split must be dated between 1–3 myBP. The most probable date seemed to be that indicated by COI-5’.

In plant ITS2 data, CBC indicate different species with high certainty (Seibel et al. 2006). We found neither CBCs in our data nor those of the Carabus subgenus Chrysocarabus (data used from Düring et al. 2001) but given that
very few data exist on insects, we cannot conclude that this indicates that the two taxa are subspecies.

High variability

Both *Hygrocarabus* taxa show low or very low morphological variability and only a few small differences could be found to establish their separate species status, except the form of the aedeagus’ tip (Casale et al. 1982).

*Carabus nodulosus* colonised Central Europe after the last glaciation from a refuge, located southeast of the Alps. Such a low variability is an indication for a fast recolonisation, a phenomenon also found in *Carabus intricatus* (Prüser 1996) and *Abax parallelepipedus* (Düring 2004) in Central Europe and *Cychrus caraboides* in Scandinavia (Düring & Mossakowski 1995).

Matern et al. (2010) found a very low sequence variability of COI-3’ in their *C. nodulosus* populations. That was the consequence of the regions under study, which cover mainly the areas of postglacial recolonisation (our HTP3 and partly HTP2).

Refuges

For a long period, the discussion on refuges during the Pleistocene was dominated by the paradigm of Mediterranean refuges, first based on morphological and biogeographical (e. g. compilation by De Lattin 1967), later on, molecular data (Hewitt 1996, 2000, Taberlet 1998). The three sample cases are the grasshopper (Balkan Peninsula), the hedgehog (all three peninsulas) and the brown bear (E and W). With the growing evidence for refuges north of the Mediterranean area, Stewart et al. (2010) suggested the existence of cryptic refuges.

However, concepts of refuges existed long before the modern debate (Drees et al. 2010). Holdhaus and Lindroth (1939) are the pioneers in this field, and they pointed to the Holdhaus line (Holdhaus 1954) that describes a northern limit of the European occurrence of blind edaphic and troglobiotic beetles. There are three refuges south of the Holdhaus line for *Carabus auroni-tens* and possibly others north of it (Drees et al. 2010). Homburg et al. (2013) found refuges north of the Holdhaus line in a study on *Carabus irregularis* in the eastern and the southern Carpathian Arc, but they did not analyse material from the westernmost part of the southern arc.
We hypothesise several refuges for both taxa:

**C. nodulosus**
- SE edge of the Alps (haplotype group (htg 1); the starting point of migration to the North and West (htg 2, 3);
- E Slovenia, N Croatia (htg 4);
- S Balkan Peninsula (htg 6); migration into the **C. variolosus** area and introgression into **C. variolosus**;
- Unknown; introgression into **C. variolosus** (htg 5).

**C. variolosus**
- Western part of S Carpathian Mts (htg W);
- SE part of Carpathian Mts (htg SE);
- Central and northern part of Carpathian Mts? (htg CN);
- Separate refuge in the Sighisoara region? (htg CE).

The different haplotype groups in **C. variolosus** show a pattern of at least three separated regions that we interpret as refuges: the western part of the southern Carpathian Mts, the SE of the Romanian Carpathian Arc, and a Romanian north-central area. The early split of beetles from Sighisoara may indicate an additional one that must be corroborated. It is striking that **C. variolosus**

*Fig. 6. Holdhaus line (black) and the northern limits of garigue and macchia vegetation (dotted). Based on Drees *et al.* (2010), line of northern garrique and macchia limits from Cadenas (2020), and two glaciers in the Carpathians from Theowald and Mannheims (1962)*
did not expand markedly after the last glaciation except along the mountain arc to the north to the Sudeten Mts. The reason for this may be its low power of dispersal. On the other hand, C. variolosus can be found down to the foothill of lower mountains where alder (Alnus glutinosa) trees occur, but not on the plains at the foot of the mountain.

It is not sufficient to divide refuges into Mediterranean and cryptic ones. Additional ecological aspects should complement this geographical aspect.

A zone of moderate Mediterranean climate can be distinguished between the Holdhaus line and the region with more extreme conditions indicated by the occurrence of garigue and macchia vegetation (Fig. 6).

The hypothesised refuges for both Hygrocarabus taxa showed a position in this moderate Mediterranean zone.

**CONCLUSIONS**

We started with the hypothesis that the taxa C. variolosus and C. nodulosus are subspecies, as this was the common viewpoint in the literature (Müller-Kroehling 2006). The data of mitochondrial genes and the occurrence of hybrids between the taxa under study seemed to corroborate that. Nevertheless, three arguments contradicted this hypothesis: (i) no mating attempts between the two taxa; (ii) The ITS2 data between both taxa showed remarkable differences that were comparable with those between species of three other Carabus subgenera that include well-established species; (iii) At first glance, the detection of hybrids between C. variolosus and C. nodulosus seems to be an argument for subspecies status. However, the high number of differences of these groups against other C. variolosus as well C. nodulosus must be interpreted as the consequence of an early hybridisation introgression, relatively close in time to the basal split of the taxa.

Although these three arguments are supporting the species status, it is necessary to complement our study by additional crossbreeding experiments with specimens from other locations and by sequence data in particular from Slovakia, the southern Balkan and the Carpathian Basin in order to get a better insight into regions where early splits happened.

Although the taxa differ sparsely in morphology, the sequence data show high diversity. A relatively uniform morphology may be interpreted as a consequence of the extreme adaptation of their common ancestor to life under water.

A series of refuges existed during the ice ages southeast of the Alps, on the Balkan Peninsula and in the Carpathian region. All are located south of the Holdhaus line in a region with a moderate Mediterranean climate north or above the garrigue or macchia vegetation.
Although only the taxon C. variolosus is listed in Annex II and IV of the EU habitats directive, the results reported here do not put in question the general consensus that C. nodulosus also falls under this listing, as at the time of amendment of the Annexes to include the species in 2004, the majority of sources treated both taxa as subspecies (Müller-Kroehling 2006), and hence the listing would include both, independent of later taxonomic revisions, very much like the situation in Osmoderma eremita, which is now split into five different species, and which all are Osmoderma eremita sensu the Habitats Directive (Audisio et al. 2009). Hence, no matter how the taxonomic decision finally turns out, both should be considered as Annex Species of the Habitats Directive (Müller-Kroehling et al. 2019).

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### Appendix I. Material of *Carabus variolosus* and *C. nodulosus* used for the analyses. CC = Country Code

| Specimen | Taxon | CC  | Location | HTG | Med | N  | N  | N  | Latitude | Longitude |
|----------|-------|-----|----------|-----|-----|----|----|----|-----------|-----------|
| var03-04 | var   | RO  | Baiut    | CN  | Eth | 2  | 2  | -  | 47°38'06"N | 23°55'35"E |
| var05    | var   | RO  | Borlova  | W   | Eth | 1  | 1  | 1  | 45°21'26"N | 22°22'16"E |
| var06    | var   | RO  | Bradu    | SE  | Eth | 1  | 2  | 1  | 45°42'42"N | 24°18'28"E |
| var07    | var   | RO  | Nadasa   | SE  | Eth | 1  | 1  | 1  | 46°41'11"N | 24°51'29"E |
| var08    | var   | RO  | Bogata   | SE  | Eth | 1  | 1  | 1  | 45°54'11"N | 25°26'02"E |
| var09-10 | var   | RO  | Bocrovici| W   | Eth | 2  | 1  | 1  | 45°00'16"N | 21°58'02"E |
| var11    | var   | RO  | Bocsa    | W   | Eth | 1  | 1  | 1  | 45°22'35"N | 21°47'53"E |
| var12-13 | var   | RO  | Sightsoara| CE | Eth | 2  | 2  | -  | 46°13'N    | 24°47'E    |
| var14    | var   | RO  | Hvedin   | CN  | Eth | 1  | 1  | 1  | 46°52'N    | 23°01'E    |
| var18,19 | var   | RO  | SE Dolni Lomna| 5 | Eth | 2  | 2  | -  | 49°32'6"N  | 18°40'10"E |
| var20    | nod   | AT  | B NW Siegraben| 2 | Eth | 1  | 2  | -  | 47°40'11"N | 16°21'32"E |
| var21    | nod   | AT  | B N Siegraben| 2 | Eth | 3  | 2  | -  | 47°39'36"N | 16°22'53"E |
| var23    | nod   | AT  | B S Mattersberg| 2 | Eth | 1  | 1  | 1  | 47°41'45"N | 16°22'20"E |
| var27    | nod   | AT  | B NW Lachenbach| 2 | Eth | -  | 1  | 1  | 47°38'12"N | 16°26'29"E |
| var28    | AT    | B S_Mattersburg| Eth | -  | 1  | 1  | -  | 47°42'33"N | 16°24'49"E |
| var30    | nod   | HR  | Ivanisca | 4   | Dry | 1  | -  | 46°11'35"N | 16°14'32"E |
| var39    | var   | PL  | Bleschady| 5   | Dry | 1p | -  | -  | 49°13'N    | 22°31'E    |
| var43    | nod   | HR  | Javorak  | 2   | Dry | 1  | -  | 45°18'59"N | 17°35'57"E |
| var50    | var   | RO  | Pangarati| SE  | Eth | 1  | -  | 46°45'20"N | 25°42'20"E |
| var51    | var   | RO  | Pangarati| SE  | Eth | 1  | -  | 46°45'20"N | 25°42'20"E |
| var65    | nod   | HR  | Blatusa  | 1   | Eth | 1  | 1  | 1  | 45°18'59"N | 15°51'33"E |
| var67-68 | nod   | SI  | Ptuj     | 4   | Eth | 2  | 2  | 1  | 46°25'N    | 15°52'E    |
| var69    | var   | RS  | Vrsac    | W   | Eth | 1  | 1  | 1  | 45°07'N    | 21°21'E    |
| var70-71 | nod   | AT  | OO Rohrbach| 3 | Eth | 1  | 1  | 2  | 48°23'14"N | 13°56'52"E |
| var72    | nod   | AT  | OO Braunau| 3   | Eth | 1  | 4  | 1  | 48°01'02"N | 12°51'14"E |
| var73    | nod   | AT  | OO Eferding| 3 | Eth | 1  | 2  | -  | 48°21'46"N | 13°54'17"E |
| var75    | nod   | HR  | Trebsica | 1   | Eth | 1  | 1  | 1  | 45°15'29"N | 14°12'37"E |
| var76    | var   | UA  | Luh      | CN  | Eth | 2  | 1  | 2  | 47°56'14"N | 24°06'25"E |
| var77-78 | var   | UA  | Luh      | CN  | Eth | 1  | 2  | 2  | 47°56'14"N | 24°06'25"E |
| var79    | var   | PL  | Gorce Mts| 5   | Eth | 1  | -  | 1  | 49°35'01"N | 20°12'00"E |
| var80    | var   | PL  | Gorce Mts| Eth | Eth | -  | 1  | 1  | 49°35'01"N | 20°12'00"E |
| var81    | var   | SK  | Nova Sedilika| 3 | Eth | 1  | 1  | -  | 51°28'N    | 08°07'E    |
| var93    | var   | SK  | Arnsberg | 3   | Dry | 1p | -  | -  | 49°02'N    | 22°30'E    |
| var110   | nod   | SK  | Z Hamre  | 6   | Dry | 1p | -  | 48°51'N    | 22°10'E    |
| var118   | var   | PL  | Zarszyn  | 6   | Dry | 2p | -  | 49°35'N    | 22°08'E    |
| var120   | var   | PL  | Ozenna   | 6   | Dry | 1p | -  | 49°25'N    | 21°27'E    |
| var121   | var   | PL  | Zalau    | 6   | Dry | 1p | -  | 47°10'N    | 23°03'E    |
| var126   | nod   | SI  | Symrmi   | 6   | Dry | 1p | -  | 46°05'N    | 14°40'E    |
| var127   | nod   | SI  | Symrmi   | 6   | Dry | 1p | -  | 46°05'N    | 14°40'E    |

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| Specimen | Taxon | CC | Location | HTG | Med | N | N | N | Latitude | Longitude |
|----------|-------|----|----------|-----|-----|---|---|---|-----------|-----------|
| var128   | nod   | SI | Zavratnik | 6   | Dry | 1p| - | - | 46°22′N | 14°45′E   |
| var136   | nod   | SI | Krakovsk Godz | 1 | Dry | 2 | 4 | 2 | 45°49′N | 15°10′E  |
| var139   | nod   | SI | Krakovsk Godz | 1 | Dry | 1p | 1 | - | 45°49′N | 15°10′E  |
| var150   | nod   | SI | Rodik     | 6 | Dry | 1p| - | - | 45°37′29″N | 13°58′53″' |
| var151   | nod   | SI | Harije    | 6 | Dry | 1p | 1 | - | 45°33′N | 14°11′E  |
| var153   | nod   | SI | Vrbace    | 6 | Dry | 2p | - | - | 45°46′N | 13°57′E  |
| var158   | nod   | BA | Vares     | 6 | Dry | 1p | - | - | 44°09′N | 18°19′E  |
| var164   | nod   | HR | Srednja  | 6 | Dry | 1 | 1 | - | 45°15′N | 17°22′E  |
| var165   | nod   | HR | Srednja  | 6 | Dry | 1p | - | - | 45°15′N | 17°22′E  |
| var168   | nod   | IT | Taipana  | 1 | Eth | 2 | 2 | 2 | 46°15′N | 13°20′E  |
| var170-73| nod   | AT | ST S Graz Unterpr. | 2 | Eth | 4 | 3 | 3 | 46°57′58″N | 15°23′10″E |
| var174-77| nod   | AT | K SW Tainach | 2 | Eth | 4 | 4 | 3 | 46°38′35″N | 14°32′59″E |
| var180   | nod   | BA | Jablanica | 6 | Dry | 1p | - | - | 43°39′N | 17°45′E  |
| var185   | nod   | AT | K W Meierigg | 2 | Eth | 2 | 2 | - | 46°36′17″N | 14°11′5′E |
| var188-89| nod   | AT | St E-Arnfels | 2 | Eth | 2 | 2 | - | 46°36′17″N | 15°31′10″E |
| var191-92| nod   | AT | ST S Graz Raaba | 2 | Eth | 2 | 2 | - | 47°02′08″N | 15°30′38″E |
| var193   | nod   | AT | ST S Graz Raaba | 3 | Eth | 1 | - | - | 47°02′08″N | 15°30′38″E |
| var194-95| nod   | DE | Traunstein | 3 | Eth | 1 | 1 | - | 47°49′47″N | 12°30′04″E |
| var197   | nod   | HU | W Cesznek | 2 | Eth | 1 | 1 | - | 47°20′08″N | 17°50′22″E |
| var198   | var   | RO | Bradu    | SE | Eth | 1 | - | - | 45°42′42″N | 24°18′28″E |
| var199   | var   | RO | Reginhin  | SE | Eth | 1 | 1 | 1 | 46°45′17″N | 24°34′0″E  |
| var200   | var   | RO | Clit     | CN | Eth | 2 | 3 | - | 47°17′13″N | 23°24′31″E |
| var201-02| var   | RO | Clit     | CN | Eth | 1 | 1 | 2 | 47°17′13″N | 23°24′31″E |
| var203   | var   | RO | Clit     | CN | Eth | 1 | - | - | 47°17′13″N | 23°24′31″E |
| var204-6 | var   | CZ | Jesenik  | 5 | Eth | 3 | 3 | 3 | 50°12′54″N | 17°17′55″E |
| var213   | var   | UA | Luh      | CN | Eth | 1 | 1 | - | 47°56′14″N | 24°06′25″E |
| var214-15| var   | UA | Luh      | CN | Eth | 2 | 2 | 2 | 47°56′14″N | 24°06′25″E |
| var216   | var   | CZ | Jesenik  | 5 | Eth | 1 | 2 | 5 | 50°12′54″N | 17°17′55″E |
| var217   | var   | CZ | Jesenik  | CN | Eth | 1 | 1 | 1 | 50°12′54″N | 17°17′55″E |
| var218   | var   | CZ | Jesenik  | CN | Eth | 1 | 1 | 1 | 50°12′54″N | 17°17′55″E |
| var219   | var   | HU | NE Zemplen | 5 | Dry | 1 | 1 | 1 | 48°25′30″N | 21°49′0″E |
| var220   | nod   | HU | W Rede   | 2 | Eth | 2 | 1 | 2 | 47°22′52″N | 17°56′51″E |
| var221   | nod   | HU | W Rede   | 2 | Eth | 1 | - | - | 47°22′52″N | 17°56′51″E |
| var222-23| nod   | HU | W Oroszlan | 2 | Eth | 2 | 2 | 2 | 47°29′27″N | 18°21′31″E |
| 329      | nod   | DE | Murnau   | 3 | Eth | 1 | - | 1 | 47°30′N | 11°11′E  |
| JQ646615 | nod   | FR | Vosges1  | 3 | GenB | 1 | - | - | 48°04′N | 07°10′E  |
| KU917778 | nod   | DE | Vilshofen2 | 3 | GenB | 1 | - | - | 48°37′21″N | 13°11′0″E |
| KM439236 | depres.| AT | Heiligenblat3 | - | GenB | 1 | - | - |  |
| JQ646593 | irregul.| HR | Kapela Vrh1 | - | GenB | 1 | - | - |  |
| JQ089919 | irregul.| RO | Resitai4 | - | GenB | 1 | - | - |  |

1 = Deuve et al. (2012); 2 = Kulik & Ahrens, GenB; 3 = Hendrich et al. (2015); 4 = Andujar et al. (2012)