

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. Introgressive 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-*

olosus 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. PUISSEGUIR 1964, ALLEMANDE & MALAUSA 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. punctatoauratus* (STREIFF *et al.* 2005), *Coptolabrus* spp. (ZHANG *et al.* 2005), *Leptocarabus* spp. (ZHANG & SOTA 2007), *Mesocarabus* spp. (ANDUJAR 2012, ANDUJAR *et al.* 2012) and *Limnocarabus 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.

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 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	mt	morph	N	16	3	7	10	1	7	4	10	
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	<i>C. depressus</i>		1	8.74	8.40	8.93	10.4	8.2	7.39	9.80	9.23	4.60
OUT	<i>C. irregularis</i>		1	10.80	10.95	11.02	12.1	10.7	9.96	12.15	11.95	

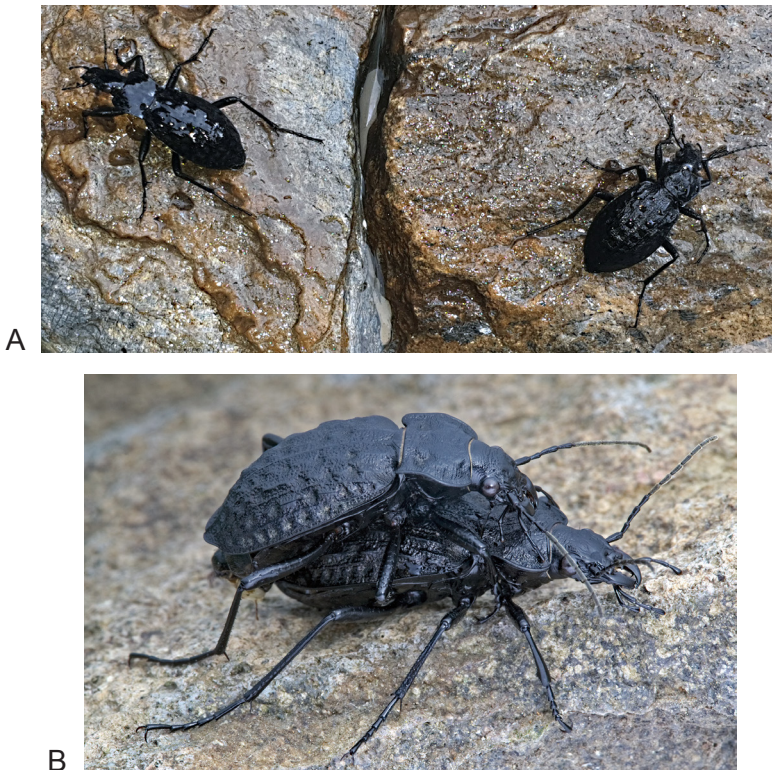
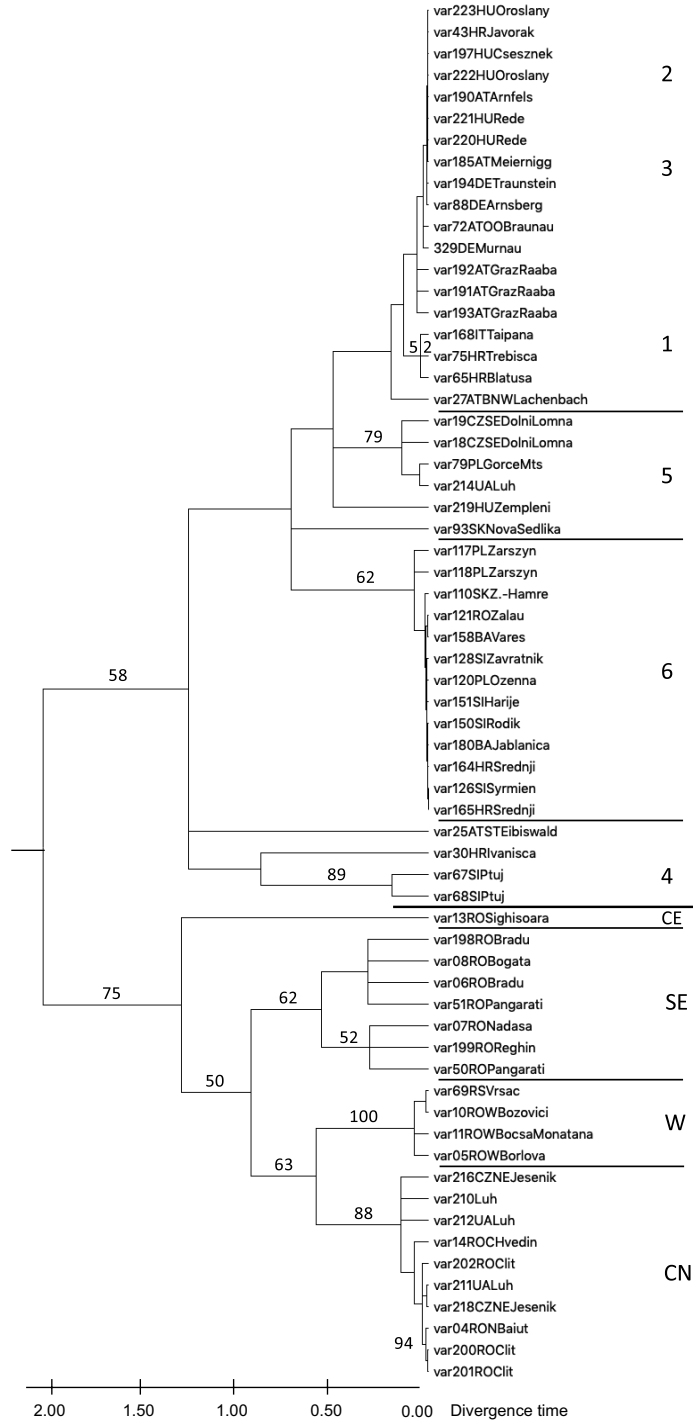
**Fig. 2.** Mating behaviour of *Carabus variolosus* and *C. nodulosus* in the laboratory. A: No interest to mate between *C. variolosus* male (left) and *C. nodulosus* female (right). B: Copulation took place immediately after putting together male and female *C. variolosus*. (Photos: Sándor Bérces)

Fig. 3. Maximum likelihood tree of *Carabus variolosus* and *C. nodulosus* COI-5'end. Only specimens with differences in their sequences were included. Relative time tree calculated with normal distribution under K2P and Gamma distributed with invariant sites (G+I) model. Calibration of clock see discussion. Bootstrap values $\geq 50\%$ are shown. 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).

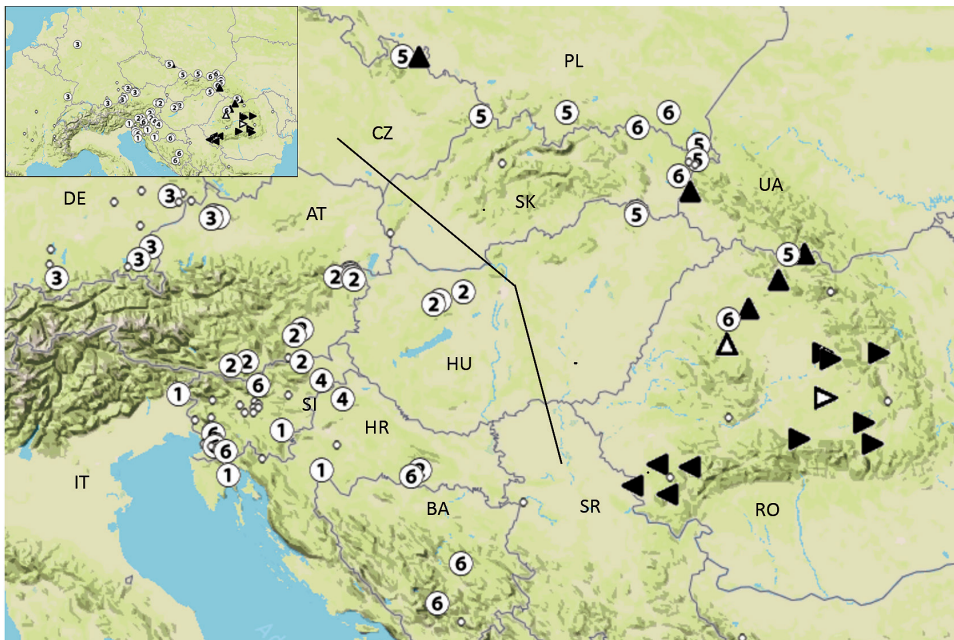
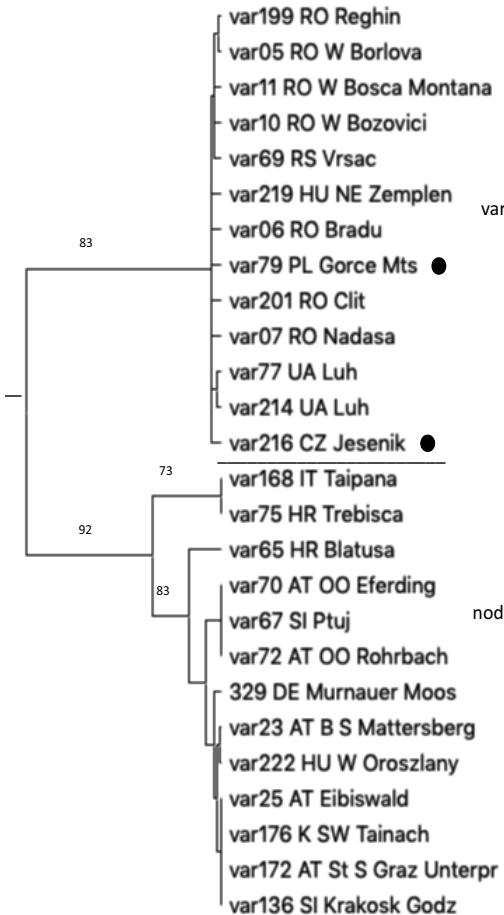


Fig. 4. Distribution of COI-5' haplotype groups of *Carabus variolosus* (triangles) and *C. nodulosus* (circles). *C. variolosus* occurs in the Carpathian Mountains (east of dividing line), *C. nodulosus* west of it. Circles in the *C. variolosus* area indicate *C. variolosus* specimens with a *C. nodulosus* 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

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
<i>Macrothorax morbillosus</i> – <i>Chrysocarabus</i> spp.	1–11	16	30	46
<i>C. (Hygrocarabus) variolosus</i> – <i>C. nodulosus</i>	13–18	17	13	30
<i>Chrysocarabus rutilans</i> – <i>C. hispanus</i>	2–1	7	14	21
<i>C. problematicus</i> – <i>C. dufouri</i>	8–22	6	11	17
<i>C. problematicus</i> – <i>C. lusitanicus</i>	20–66	10	2	12
<i>C. problematicus</i> – <i>C. macrocephalus</i>	20–29	11	3	14
<i>C. lusitanicus</i> – <i>C. macrocephalus</i>	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. irregularis*. Black dots indicate *variolosus* specimens with a *nodulosus* mitochondrion. Compare data in Table 4

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
<i>variolosus</i>	<i>nodulosus</i>	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

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, VREZEC 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 (PUISSEUR 1964, ALLEMAND & MALAUSA 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'.

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 *Carabus* 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 (ALLEMANT & 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 from 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 auronitens* 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*

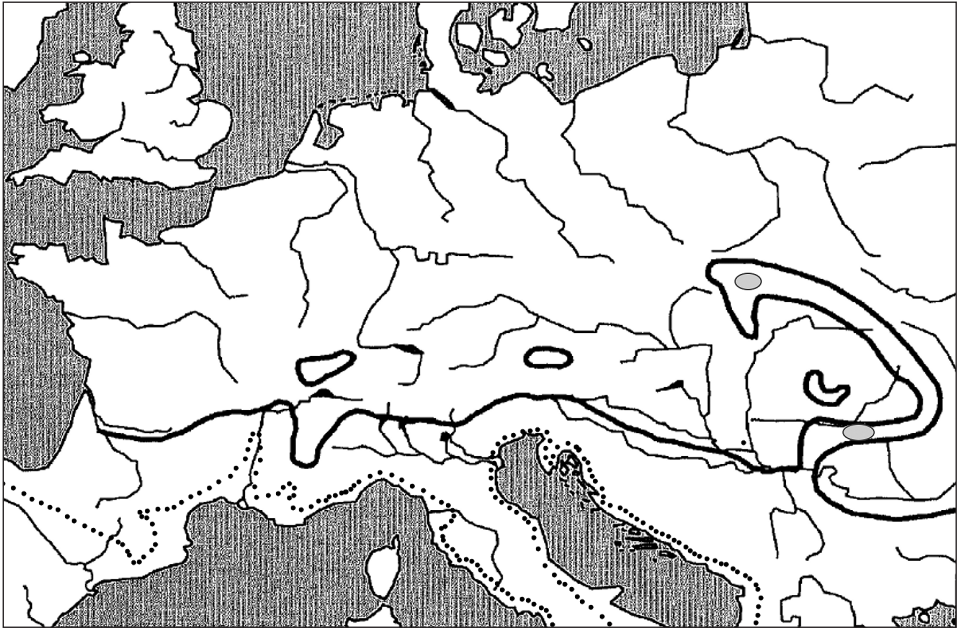


Fig. 6. Holdhaus line (black) and the northern limits of garigue and macchia vegetation (dotted). Based on DREES *et al.* (2010), line of northern garrigue and macchia limits from CADENAS (2020), and two glaciers in the Carpathians from THEOWALD and MANNHEIMS (1962)

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

Specimen	Taxon	CC	Location	HTG	Med	COI-5' COI-3' ITS2			Latitude	Longitude
						N	N	N		
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	-	45°54'11"N	25°26'02"E
var09-10	var	RO	Bozovici	W	Eth	2	1	1	45°00'16"N	21°58'02"E
var11	var	RO	Bocsa Montana	W	Eth	1	1	1	45°22'35"N	21°47'53"E
var12-13	var	RO	Sighisoara	CE	Eth	2	2	-	46°13'N	24°47'E
var14	var	RO	Hvedin	CN	Eth	1	1	-	46°52'N	23°01'E
var18,19	var	CZ	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	47°41'45"N	16°22'20"E
var27	nod	AT	B NW Lachenbach	2	Eth	-	1	-	47°38'12"N	16°26'29"E
var28		AT	B_S_Mattersburg		Eth	-	1	-	47°42'33"N	16°22'49"E
var30	nod	HR	Ivanisca	4	Dry	1	-	-	46°11'35"N	16°14'32"E
var39	var	PL	Bleszchady	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'
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	Trebisca	1	Eth	1	1	1	45°15'29"N	14°12'37"E
var76	var	UA	Luh	CN	Eth	2	2	1	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	-	1	1	49°35'01"N	20°12'00"E
var88	nod	DE	Arnsberg	3	Eth	1	1	-	51°28'N	08°07'E
var93	var	SK	Nova Sedlika	5	Dry	1p	-	-	49°02'N	22°30'E
var110	var	SK	Z Hamre	6	Dry	1p	-	-	48°51'N	22°10'E
var118	var	PL	Zarszyn	6	Dry	2p	-	-	49°35'N	22°00'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	Syrmien	6	Dry	1p	-	-	46°05'N	14°40'E
var127	nod	SI	Syrmien	6	Dry	1p	-	-	46°05'N	14°40'E

Specimen	Taxon	CC	Location	HTG	Med	COI-5' COI-3' ITS2			Latitude	Longitude
						N	N	N		
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"E
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	Srednji	6	Dry	1	1	-	45°15'N	17°22'E
var165	nod	HR	Srednji	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 Meiernigg	2	Eth	2	2	-	46°36'34"N	14°15'05"E
var188-89	nod	AT	St_E-Arnfels	2	Eth	2	2	-	46°39'17"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 Csesznek	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	Reghin	SE	Eth	1	1	1	46°45'17"N	24°43'40"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	-	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	5	Eth	2	2	2	47°56'14"N	24°06'25"E
var216	var	CZ	Jesenik	CN	Eth	1	2	5	50°12'54"N	17°17'55"E
var217	var	CZ	Jesenik	CN	Eth	1	1	-	50°12'54"N	17°17'55"E
var218	var	CZ	Jesenik	CN	Eth	1	1	-	50°12'54"N	17°17'55"E
var219	var	HU	NE Zemplen	5	Dry	1	1	1	48°25'50"N	21°24'59"E
var220	nod	HU	W Rede	2	Eth	1	2	1	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 Oroszlany	2	Eth	2	2	2	47°29'27"N	18°21'31"E
329	nod	DE	Murnau	3	Eth	1	-	1	47°39'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°11'40"E
KM439236	depres.	AT	Heiligenblut3	-	GenB	1	-	-		
JQ646593	irregul.	HR	Kapela Vrh1	-	GenB	1	-	-		
JQ689919	irregul.	RO	Resita4	-	GenB	-	1	-		

1 = DEUVE *et al.* (2012); 2 = KULIK & AHRENS, GenB; 3 = HENDRICH *et al.* (2015); 4 = ANDUJAR *et al.* (2012)