

1 **Low intraspecific genetic divergence and weak niche differentiation despite wide ranges**
2 **and extensive sympatry in two epigean *Niphargus* species (Crustacea: Amphipoda)**

3
4 Denis Copilaş-Ciocianu^{1*}, Cene Fišer², Péter Borza³, Gergely Balázs⁴, Dorottya Angyal^{5,6},
5 Adam Petrusek¹

6 **1** Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, 12844 Prague, Czech
7 Republic

8 **2** Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101,
9 SI-1000 Ljubljana, Slovenia

10 **3** Danube Research Institute, MTA Centre for Ecological Research, Karolina út 29-31, H-1113 Budapest,
11 Hungary

12 **4** Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117
13 Budapest, Hungary

14 **5** Department of Zoology, Hungarian Natural History Museum, Baross 13, 1088 Budapest, Hungary

15 **6** Doctoral School of Animal-and Agricultural Environmental Sciences, Department of Animal Sciences and
16 Animal Husbandry, Georgikon Faculty, University of Pannonia, Deák Ferenc 16, 8360 Keszthely, Hungary

17 * Corresponding author. E-mail: denis.copilas@yahoo.com

18 Running title: Comparison of two epigean *Niphargus* species

19

20 **Abstract**

21 The amphipod genus *Niphargus* comprises hundreds of eyeless and depigmented species with
22 narrow ranges in Western Palaearctic subterranean freshwaters. Two morphologically and
23 ecologically similar species, *N. hrabei* and *N. valachicus*, are atypical due to their epigean
24 lifestyle and large ranges. Given their wide and largely sympatric distributions, we explored
25 their potential ecological niche overlap by comparing morphological functional traits, patterns
26 of co-occurrence and habitat selection, and tested for cryptic diversity by examining variation
27 of mitochondrial and nuclear markers in selected populations. Mitochondrial sequences and

28 the presence of shared nuclear alleles among individuals collected along the study area did not
29 support the existence of cryptic lineages; two other *Niphargus* species described in the past
30 from Hungary seem to be their younger synonyms. Except for body size, both species were
31 morphologically similar and seem only weakly differentiated ecologically. Though smaller in
32 size, *N. hrabei* is ecologically more tolerant, while *N. valachicus* might be a superior
33 competitor due to its larger size. Despite large-scale sympatry, co-occurrence of both species
34 was rare. Their present distributions may result from a dynamic relationship between dispersal
35 ability and competitive strength. These widespread and euryoecious *Niphargus* species
36 demonstrate that the genus may be successful not only in the subterranean realm but also in
37 various surface waters.

38

39 **Keywords**

40 Co-occurrence - COI - dispersal - functional traits - ITS - *Niphargus hrabei* - *N. valachicus* -
41 range size - taxonomy - zoogeography

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46 **Introduction**

47 The genus *Niphargus* Schiödte, 1849 is the most diverse genus of freshwater amphipods,
48 comprising more than 330 described taxa distributed throughout the Western Palaearctic
49 (Väinölä et al., 2008; Esmaeili-Rineh et al., 2015). All *Niphargus* species are blind and
50 depigmented and they inhabit virtually all types of subterranean habitats, whereas only a few
51 are known to occur in surface waters (Sket, 1981; Fišer et al., 2006). Therefore, the genus
52 represents a considerable part of the European groundwater animal biodiversity (Fišer, 2012;

53 Zgmajster et al., 2014). The overall morphological variability of *Niphargus* is greater than
54 that of most amphipod genera or even families, with several ecomorphs having arisen
55 repeatedly through convergent evolution (Fišer et al., 2009a; Trontelj, Blejec & Fišer, 2012).
56 Usually, *Niphargus* species have small, endemic ranges (less than 200 km) and most taxa that
57 have been considered widespread actually consist of highly divergent cryptic lineages that are
58 sometimes not even closely related (Lefébure et al., 2006, 2007; Trontelj et al., 2009).

59 However, there are two species that stand out from this general pattern: *N. hrabei*
60 Karaman, 1932 and *N. valachicus* Dobreanu & Manolache, 1933. They are atypical in having
61 apparently very large geographical ranges (>1300 km across) and inhabiting surface (epigean)
62 waters, only occasionally having been reported from subterranean (hypogean) habitats
63 (Karaman 1950; Cărauşu, Dobreanu & Manolache 1955; Straškraba, 1972; Sket, 1981;
64 Meijering, Jazdzewski & Kohn, 1995; Neemann, Pöckl & Wittmann 1995; Copilaş-Ciocianu
65 et al., 2014). These species had been formerly classified within the obsolete subgenus
66 *Phaenogammarus* Dudich, 1941 because they share several morphological characteristics
67 such as stout bodies, quadrate gnathopods, acutely produced postero-inferior corners of the
68 epimeral plates, and the presence of a spoon-shaped appendage on the first uropods of males
69 (Straškraba, 1972; Sket, 1981). Both species inhabit densely vegetated areas with muddy
70 substrate of lowland springs, streams, canals and rivers with slow water current, as well as
71 ponds, lakes and temporary water bodies (Straškraba, 1972; Copilaş-Ciocianu et al., 2014).
72 *Niphargus hrabei* is restricted to the Danube floodplains and lowlands and can be encountered
73 from south-eastern Germany to south-eastern Romania (Cărauşu et al., 1955; Neemann et al.,
74 1995; Copilaş-Ciocianu et al., 2014) (Fig. 1A). *Niphargus valachicus* follows a roughly
75 similar pattern but reaches the lowlands of the Black Sea in Bulgaria, Romania, Ukraine and
76 Turkey and even the shores of the Caspian Sea in Iran (Akbulut et al., 2001; Dedju 1980;
77 Karaman 1998; Fišer, Çamur-Elipek & Özbek, 2009b; Copilaş-Ciocianu et al., 2014) (Fig.

78 1B). Their geographical ranges seem to substantially overlap in the middle and lower Danube
79 lowlands, although the extent of this overlap and their fine-scale geographical distribution is
80 not sufficiently known. Because their ranges are large and fragmented, it seems possible that
81 these taxa actually consist of independently evolving cryptic lineages (Fišer et al., 2009b).
82 Indeed, ranges greater than 200 km are considered the exception rather than rule in niphargids
83 (Trontelj et al., 2009).

84 The aims and hypotheses of our study were: 1) using molecular tools, to test whether
85 both of these widespread epigeal *Niphargus* taxa indeed represent only two species or are
86 comprised of cryptic lineages; 2) to compile high resolution distribution maps to gain insights
87 into their biogeographical patterns; 3) to test for niche differentiation between them (as
88 suggested by their apparent large-scale sympatry) using a comparative functional
89 morphological approach and by recording the frequency of their co-occurrence and presence
90 in different habitat types. Moreover, we provide morphological and molecular evidence that
91 *N. thermalis* Dudich, 1941 may be a younger synonym of *N. hrabei*, and further molecular
92 evidence that supports the synonymy of *N. mediodanubialis* Dudich, 1941 with *N. valachicus*.

93

94

95 **Methods**

96 *Sampling and data collection*

97 Material was collected between 2001 and 2015 from 247 localities spanning the European
98 distribution area of both species (Supplementary Table S1). Specimens were collected with
99 the help of a hand net that was swept through the dense riparian/submerged vegetation and
100 stored in 96% ethanol (if used for molecular analyses) or either 70% ethanol or 4%
101 formaldehyde solution. In order to ecologically characterize the species, information on the
102 general habitat type (groundwater, spring, stream, river and pond/lake) and altitude was

103 recorded. Lotic water bodies with a width of more than 20 meters were classified as rivers.
104 We additionally performed a literature review to assemble distribution and habitat data from
105 relevant studies where the exact localities were provided (Karaman S, 1950; Căraușu et al.,
106 1955; Straškraba, 1959; Motaș, Botoșăneanu & Negrea, 1962; Berczik, 1966; Karaman GS,
107 1973; 1998; Dedju, 1980; Sket, 1981; Lantos, 1986; Nesemann, 1993; Akbulut et al., 2001;
108 Paraschiv et al., 2007; Fišer et al., 2009b; Borza, Nosek & Oertel, 2010; Copilaș-Ciocianu &
109 Pârvulescu 2012; Copilaș-Ciocianu et al., 2014; Flot et al., 2014; Csabai et al., 2015). The
110 number of co-occurrences of both taxa was assessed from both our data and reliable literature
111 records.

112 Furthermore, we obtained samples of *N. thermalis* from its type locality (Malom Lake)
113 in Budapest for the analysis of the status of this taxon, which should be closely related to *N.*
114 *hrabei* according to Balázs, Angyal & Kondorosy (2015). Samples of *N. hrabei* for molecular
115 analyses were obtained from ca. 30 km west of Parkan-Nána (presently Štúrovo), its type
116 locality in southern Slovakia, and five additional localities across its range (Table 1, Fig.2).
117 We obtained samples of *N. valachicus* from three localities in close proximity (ca. 20-30 km)
118 to where its presumed junior synonym, *N. mediodanubialis*, was mentioned (Balázs et al.,
119 2015 and references therein). We also collected individuals ca. 40 km south-west of the type
120 locality of *N. valachicus* in Bucharest, Romania, and from three other sites throughout its
121 whole range, including one locality in northern Turkey (Table 1, Fig. 1). Altogether,
122 individuals from seven localities per species (including their presumed synonyms) were
123 analysed molecularly.

124

125 *Molecular methods and analyses*

126 To test for cryptic diversity within *N. hrabei* and *N. valachicus* and to verify the taxonomic
127 status of *N. thermalis* and *N. mediodanubialis*, we analysed the variation of the mitochondrial

128 gene for the cytochrome *c* oxidase subunit I (COI), and of the internal transcribed spacer
129 (ITS) region in the nuclear ribosomal DNA (containing ITS1, gene for 5.8S rRNA, and ITS2).
130 Genomic DNA was extracted from the head or from the entire animal (if smaller than 1 cm)
131 using the Genomic DNA Mini Kit for tissue (Geneaid Biotech Ltd, Taipei) according to the
132 manufacturer's protocol. For samples older than ca. 5 years, the ground tissue was incubated
133 overnight during the lysis step of the procedure. A 618 bp fragment of the COI gene was
134 amplified using the primer pair UCOIF/UCOIR (Costa et al., 2009). Polymerase chain
135 reactions (PCR) were performed according to Copilaş-Ciocianu and Petrussek (2015). The ITS
136 marker was usually sequenced from individuals that had distinct COI haplotypes using the
137 primers and PCR protocols of Flot et al. (2010b) for its amplification. For *N. hrabei*, we
138 sequenced several more individuals in order to obtain a complete haplotype interconnection in
139 the haploweb analysis (see below). The ITS alignment had a length of 2684 and 1565 bp in *N.*
140 *hrabei* and *N. valachicus*, respectively. Amplicons were sequenced in both directions using an
141 ABI PRISM 3130XL DNA Analyser. Sequences were deposited in GenBank with the
142 accession numbers KU948611–KU948631 for COI and KU948711–KU948724 for ITS
143 (Table 1).

144 Sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 6
145 (Tamura et al., 2013) and the COI alignment was translated to amino acids to check for the
146 potential presence of stop codons that would indicate pseudogenes. For individuals showing
147 intragenomic variation in the ITS region (i.e. with double peaks in chromatograms, indicating
148 either heterozygosity or presence of multiple ITS variants across the genome), haplotypes
149 were phased using SeqPHASE (Flot, 2010) and PHASE (Stephens, Smith & Donnelly, 2001);
150 this concerned four *N. hrabei* and five *N. valachicus* individuals. The sequences of individuals
151 that had numerous double peaks in the chromatograms (indicating length-variant
152 heterozygotes) were unravelled using the online program Champuru (Flot, 2007; <http://www.>

153 mnhn.fr/jfflot/champuru). Haplotype networks were produced using the integer neighbour-
154 joining (IntNJ) method in POPART 1.7 (Leigh & Bryant, 2015). The ITS network was turned
155 into a haploweb by connecting the haplotypes co-occurring in heterozygous individuals (Flot,
156 Couloux & Tillier, 2010a). Phylogenetic analyses were performed only on unique COI
157 haplotypes in MEGA 6 using a maximum-likelihood (ML) algorithm with a GTR+I+ Γ
158 evolutionary model (selected by MEGA 6) and 500 bootstrap pseudoreplicates. Numbers of
159 haplotypes as well as nucleotide (π) and haplotype diversity (Hd) were calculated with DnaSP
160 5 (Librado & Rozas, 2009).

161

162 *Morphological analyses*

163 To further elucidate the status of *N. thermalis*, we also performed a thorough morphological
164 comparison with *N. hrabei*. Cleared and stained exoskeletons of six *N. thermalis* specimens
165 from the type locality (Malom Lake) were dissected under a Leica MZ75 and a Leica M125
166 stereomicroscope. Measurements were made using the AnalySIS Program Package, on a
167 computer connected to a Zeiss Axioscope II light microscope. In total, 230 morphological
168 characters were examined on each specimen following Fišer et al. (2009a) in the DELTA
169 program package (Dallwitz, 1974) and were recorded in an Excel data matrix. The
170 measurements were compared with the *N. hrabei* online dataset available at
171 <http://niphargus.info/morpho-database/>. Since some of the entries in the database can vary
172 subjectively (e.g. descriptions of the epimeral plate shape), the respective characters were
173 compared with the original *N. hrabei* slides of the dataset collection, thus validating the
174 comparison with the online data.

175 We also characterized *N. hrabei* and *N. valachicus* using ecologically relevant
176 morphological traits. In the absence of experimental data on population dynamics needed for
177 disentangling whether or not two species co-occur or coexist (stable co-occurrence)

178 (Siepielski & McPeck, 2010), an analysis of ecologically relevant morphology may provide
179 indirect evidence for stable coexistence (*sensu* Chesson, 2000). However, a direct translation
180 of statistically significant differences among morphological traits into ecologically
181 meaningful differences needs to be made with caution (Fišer et al., 2015b). A comparison
182 with a non-related but co-occurring taxon may clarify whether or not the magnitude of
183 differences among focal species can be considered as ecologically relevant. The geographical
184 ranges of several amphipod taxa overlap to some extent with the ranges of both *Niphargus*
185 species in the studied area. However, only *Synurella ambulans* Müller, 1846 (family
186 Crangonyctidae) is broadly sympatric with our focal species while the other taxa (several
187 species or species complexes of Gammaridae) exhibit only small scale-sympatry (Căraușu et
188 al. 1955; Copilaș-Ciocianu et al., 2014). Furthermore, the available published records and our
189 own observations indicate that gammarids and niphargids tend to be spatially separated, while
190 *Synurella ambulans* and epigean niphargid species live in in the same type of habitats and
191 frequently co-occur (Sket, 1981; Lantos, 1986; Nesemann, 1993; Akbulut et al., 2001;
192 Sidorov & Palatov, 2012; Copilaș-Ciocianu et al., 2014). For this reason, we included *S.*
193 *ambulans* as the third taxon into the comparison of morphological traits.

194 Several morphological characters in amphipods are apparently related to species' functional
195 ecology (Dahl, 1977; Trontelj et al., 2012; Fišer, Zagamajster & Zakšek, 2013). We estimated
196 functional differences between species in four complexes of functional morphological traits
197 (further details in Fišer et al., 2009a) using nine measurements detailed below.

198 i) Body size is commonly considered as a surrogate of species' trophic niche (e.g., Vergnon et
199 al., 2013). This is due to the fact that this parameter is related to feeding behaviour or
200 metabolic rates (Allen et al., 2006).

201 ii) Body shape, i.e., slender versus stout, is a result of a trade-off between the need for
202 crawling through the crevices and the need for swimming and breathing (Fišer et al., 2013;

203 but see Fišer et al., 2015a). Body shape is mainly influenced by the shape of the coxal plates.
204 Amphipod coxal plates I-IV are flattened and elongated ventrally, and form a ventral channel
205 with gills and a brooding chamber. The movements of pleopods generate water currents that
206 deliver oxygenated water to gills and brood, and can also be used as jet propulsion (Dahl,
207 1977). As a proxy for body shape, we thus measured the depth and the width of coxa III, a
208 coxal plate that has the most rectangular shape in niphargids.

209 iii) Appendage lengths may be involved in crawling through the tiny voids (in particular
210 pereopods V–VII, see Coineau 2000), but also used for sensory perception (in particular
211 antennae, Dahl, 1977). In subterranean species, appendages are frequently elongated (Culver
212 & Pipan, 2009). We measured the total lengths of antenna I and pereopod VII.

213 iv) Gnathopods are involved in feeding, grooming, and antennae cleaning. Unlike many other
214 gammarid amphipods, *Niphargus* species do not form precopula (Ginet, 1967); hence we
215 assumed that the shape of gnathopods is not subject to sexual selection but rather linked to
216 food collection. We called this trait ‘grip ability’, and assessed it by measuring four
217 parameters on gnathopod II: carpus length, propodus length, palm length, and the distance
218 between palmar corner and joint between carpus and propodus.

219 We measured 33 individuals of *N. valachicus*, 28 individuals of *N. hrabei* (including 9
220 individual named as *N. thermalis*) and 10 individuals of *S. ambulans*; details on their
221 populations of origin are available in Table 2. We did not discriminate between sexes because
222 sexual dimorphism in these taxa is small (Sket, 1981). Specimens were partly dissected in
223 glycerol. Appendages were mounted on slides, and photographed using the camera
224 ColorView III mounted on an Olympus SZX9 stereomicroscope. Measurements were
225 performed in the program cellB (Olympus) from the photographs; landmarks are described in
226 Fišer et al. (2009a).

227

228 *Statistical analyses*

229 Differences in the proportions of habitat types between species were tested using a two-tailed
230 Fisher's exact test and differences in altitude were tested with a Mann-Whitney U test. We
231 analysed two sets of ecological data (altitude and habitat), one that encompassed all the data
232 for both species, and one in which we included only localities from the regions of sympatry
233 (i.e. the Pannonian Basin, excluding the Small Hungarian Plain, and the lower Danube
234 lowlands, see Supplementary Table S1). This was done to disentangle variation caused by
235 differences in geographical ranges from that arising from distinct habitat preferences of the
236 study species. For morphological comparisons, we tested for differences between the three
237 species, i.e., *N. hrabei*, *N. valachicus* and *S. ambulans*. Differences in body size and
238 appendage lengths were tested using analysis of variance (ANOVA), or, in the case of non-
239 normally distributed data, a Kolmogorov-Smirnoff test. In the latter, pairwise comparisons
240 were made using a Mann-Whitney U test, with alpha level adjusted for multiple comparisons.
241 Four measures on gnathopods summarized a single functional trait, the grip ability, and two
242 measures from coxal plates were related to another single trait we were interested in, the
243 shape of the ventral channel. Hence, the differences between species were assessed using a
244 multivariate analysis of variance (MANOVA). To account for the effect of body size, the
245 lengths of appendages and the measures on coxae and gnathopods were regressed onto body
246 size and residuals were used in all data analyses. All tests were made in PASW Statistics 18.

247

248 **Results**

249 *Distribution and ecology*

250 The total number of records obtained from our own data and literature was 345 (138 for *N.*
251 *hrabei* and 207 for *N. valachicus*), out of which 247 originated from our field surveys (111 for
252 *N. hrabei* and 136 for *N. valachicus*) (Table S1).

253 The presence of *N. hrabei* has been confirmed for Germany (first mentioned by
254 Nesemann et al., 1995 but without any further details), and we provide, for the first time,
255 evidence for its presence in Serbia and western Romania. Thus, the range of this species
256 extends for more than 1300 km along the Danube lowlands, from Bavaria in south-eastern
257 Germany to Dobrogea in south-eastern Romania (Fig. 1A). Likewise, the presence of *N.*
258 *valachicus* in Bulgaria (first reported by Karaman, 1973) has been confirmed after more than
259 40 years by an additional record. The range of this species extends for ca. 3200 km, from the
260 Pannonian Basin, through the lower Danube lowlands, along the shores of the Black Sea and
261 finally reaching the southern shore of the Caspian Sea in northern Iran (Fig. 1B). It is
262 noteworthy that *N. valachicus* is apparently absent from the Little Hungarian Plain (north-
263 west of the Pannonian Basin) and further upstream along the Danube, where *N. hrabei* was
264 the sole species found (Fig. 1). However, the ranges of these species substantially overlap
265 throughout the Pannonian Basin (south of the Transdanubian Mountains) and in the lower
266 Danube lowlands (Fig. 1).

267 In the complete dataset, the altitudes at which the species were collected differed
268 significantly (Mann-Whitney U, $p < 0.0001$, $n = 344$) as well as the proportion of habitats
269 (Fisher's exact test, $p = 0.001$, $n = 265$) (Fig. 2A-B). *N. hrabei* had a wider altitudinal
270 variability which ranged from 0 to 472 m above sea level (mean 133, median 115 ± 70 m),
271 while *N. valachicus* ranged from 0 to 192 m (mean 86, median 90 ± 38 m), with a single
272 record from literature data at 360 m (Fig. 2A). Both species had the highest proportion of
273 occurrences in streams (and canals), although *N. valachicus* was more frequently found in
274 lotic habitats (42 % of all observations of *N. hrabei* vs. 60 % of *N. valachicus*). *N. hrabei* was
275 more frequently encountered in large rivers than *N. valachicus* (35 % vs. 14 %) (Fig. 2B).
276 Both species were recorded in similar proportions in groundwater (*N. hrabei* 2 % and *N.*
277 *valachicus* 1.8 %) and ponds/lakes (*N. hrabei* 20 % and *N. valachicus* 18 %), while *N.*

278 *valachicus* was slightly more common in springs (*N. hrabei* 2 % and *N. valachicus* 4.9 %)
279 (Fig. 2B). However, both altitude and proportion of habitats did not differ significantly
280 between the sympatric localities of both species (Mann-Whitney U, $p = 0.7$, $n = 236$; Fisher's
281 exact test, $p = 0.3$, $n = 189$) (Fig. 2C-D). The number of localities in which we recorded the
282 co-occurrence of both species was very low, only 10 sites (3 from the literature and 7 from
283 this study) out of the total of 345 (<3%) (Fig. 1B).

284

285 *Phylogeographic structure*

286 A total of six COI haplotypes were detected in 13 analysed individuals from seven localities
287 of *N. hrabei* (including *N. thermalis*) and eight haplotypes were observed in 11 individuals
288 from seven localities of *N. valachicus* (including *N. mediodanubialis*). For ITS, we observed
289 five haplotypes in ten analysed individuals of *N. hrabei* (six homozygous and four
290 heterozygous) and five haplotypes in eight individuals of *N. valachicus* (three homozygous
291 and five heterozygous).

292 The ML and network analyses revealed that COI haplotype H1 was shared between *N.*
293 *thermalis* and two individuals of *N. hrabei* from northern Serbia and two individuals from
294 north-eastern Austria (Fig. 3). The sample collected near the type locality of *N. hrabei*
295 (haplotype H2) differed by a single mutational step from haplotype H1 (Fig. 3B). Overall, the
296 genetic variation of the COI gene in this taxon was small ($\pi = 0.0043$, $SD = 0.0006$; $Hd =$
297 0.821 , $SD = 0.082$), the greatest distance observed among haplotypes was four mutations, i.e.
298 0.6% p distance (Fig. 3B). Little genetic variation was also observed for the ITS region ($\pi =$
299 0.0005 , $SD = 0.0001$; $Hd = 0.769$, $SD = 0.076$), the greatest distance observed between
300 different haplotypes was 5 mutational steps (0.2% p distance) (Fig. 3C). Some individuals
301 separated by more than 700 km shared the same haplotype and all haplotypes were
302 interconnected.

303 COI sequences of *N. valachicus* obtained near the regions where *N. mediodanubialis*
304 was reported differ by 10-13 mutations (1.8-2.3% *p* distance) from the sequences obtained
305 from specimens near the type locality of *N. valachicus* (Fig 3B). The overall genetic variation
306 of this clade (i.e., *N. valachicus* and *N. mediodanubialis* combined) was greater than in *N.*
307 *hrabei* ($\pi = 0.0138$, SD = 0.0015; Hd = 0.945, SD = 0.054), the maximum divergence
308 detected among haplotypes was 13 mutations, i.e. 2.3% *p* distance (Fig. 3B). The ITS marker
309 had similar variation as for *N. hrabei* ($\pi = 0.0038$, SD = 0.0007; Hd = 0.628, SD = 0.143)
310 with a maximum divergence between haplotypes of 14 steps (0.4 % *p* distance) (Fig. 3C). One
311 of the haplotypes was found in all the individuals despite more than 1000 km between the
312 respective populations and, therefore, all individuals share a common gene pool.

313

314 *Morphology*

315 All of the examined morphological characters of *N. thermalis* fell within the range indicated
316 for *N. hrabei*. This morphological similarity is in agreement with the patterns observed at the
317 studied mitochondrial marker, i.e., shared COI haplotypes between the two taxa.

318 The body sizes of *N. hrabei*, *N. valachicus* and *S. ambulans* were significantly
319 different from each other (Kolmogorov-Smirnoff, $p < 0.01$; Mann-Whitney U tests for all
320 pairwise comparisons, $p < 0.001$; Fig. 4A). By contrast, appendage lengths after correction for
321 the body size did not differ significantly between these three species (ANOVA, $p = 0.43$
322 [antenna I]; $p = 0.211$ [pereopod VII]; Fig. 4B). All species differed significantly in grip
323 ability (Table 3). Univariate analyses of each of the four parameters on gnathopods indicated
324 that differences between species are due to differences in three measures taken from propodus
325 but not carpus length. Pairwise comparison of the three species revealed that the significant
326 differences between gnathopods are mainly due to difference between *N. valachicus* and *S.*
327 *ambulans* (Table 3, Fig. 4C); *N. valachicus* has much larger propods. The gnathopods of both

328 *Niphargus* species are in general aspect similar to each other; the propodus of *N. valachicus* is
329 slightly (but significantly) longer than *N. hrabei*. Similarly, the three species differ in the
330 shape of their ventral channel (Table 3). The difference is due to coxal depth, not width. The
331 pairwise comparison revealed that *S. ambulans* is much stouter (i.e. has much deeper coxae)
332 than both *Niphargus* species; the latter two, however, did not differ from each other (Table 3,
333 Fig. 4D).

334

335 **Discussion**

336 Our results reveal that both *N. hrabei* and *N. valachicus* exhibit a shallow intraspecific genetic
337 divergence despite their wide and fragmented ranges. They are sympatric on a large scale,
338 even though the frequency of their co-occurrence is apparently very low. Except for body
339 size, they seem to be quite similar morphologically. Furthermore, their altitudinal distribution
340 and habitat preferences did not differ significantly in the areas of sympatry. We consider that
341 the overall evidence rejects our hypothesis that their large-scale sympatry is facilitated by
342 substantial niche differentiation.

343

344 *Morphology and ecology*

345 The morphological differences between the two *Niphargus* species are small when compared
346 to differences between *Niphargus* and co-occurring *S. ambulans*. This suggests that niche
347 differentiation between the two *Niphargus* species is weaker than between *Niphargus* and *S.*
348 *ambulans*, and might explain why *S. ambulans* is frequently found together with both
349 *Niphargus* species (Motaş et al., 1962; Akbulut et al., 2001; Copilaş-Ciocianu et al., 2014),
350 but co-occurrence between the two *Niphargus* is rare (less than 3% of the total number of our
351 and literature records). Due to its larger body size, *N. valachicus* may be a stronger competitor
352 (Macneil et al., 1997) while *N. hrabei* may have a broader tolerance to environmental

353 conditions; it can inhabit thermal and sulphidic waters (Dudich, 1941; Flot et al., 2014) and
354 has a wider altitudinal range. Although both species seem to prefer similar types of habitats,
355 *N. hrabei* was more frequently found in rivers while *N. valachicus* was more common in
356 smaller streams and canals. This suggests a dynamic relationship between dispersal,
357 ecological tolerance and competitive strength (e.g. Leibold & McPeck, 2006). In the dynamic
358 process of colonization-extinction, common to many amphipods (Macneil & Dick, 2014;
359 Fišer et al., 2015b), *N. hrabei* may be a superior competitor in habitats with extreme
360 environmental conditions, whereas in less extreme habitats it is likely to be outcompeted by
361 the larger *N. valachicus*. Surprisingly, it appears that both species are more tied to the epigeal
362 rather than the hypogean environment, an observation strengthened by a seasonal life-cycle in
363 *N. valachicus* (Copilaş-Ciocianu & Boroş, 2016). This may suggest a reversal from a
364 troglobitic life style and a secondary colonization of surface environments – an exceptional
365 phenomenon that has been reported only in scorpions so far (Prendini, Francke & Vignoli,
366 2010). Both *Niphargus* species apparently prefer dimly lit habitats covered by dense submerge
367 vegetation (D. Copilaş-Ciocianu, personal observation). This light-avoidance behaviour is
368 consistent with the findings of Fišer et al. (2016) that eyeless *Niphargus* species are able to
369 use light as an important factor affecting habitat choice. If our focal species are indeed
370 photophobic, they may thrive in shallow subterranean habitats (i.e. the boundary between
371 surface and subterranean ecosystems) (Culver & Pipan, 2014; Fišer, Pipan & Culver, 2014),
372 rather than in groundwater itself. Additional phylogenetic data, experimental, and fine-scale
373 ecological observations are needed to test the hypothesis of a secondary colonization of the
374 surface environments, and uncover any adaptation that may have been associated with this
375 shift.

376

377 *Biogeographical patterns*

378 Both *Niphargus* species display low intraspecific COI genetic divergences across more than
379 1000 km, and distant populations even share identical ITS haplotypes, clearly indicating
380 conspecificity. This is exceptional among niphargids, or even among amphipods, which are
381 considered poorly vagile (Trontelj et al., 2009; Myers & Lowry, 2009), highlighting that
382 relatively rapid dispersal across wide distances is possible within the highly diverse niphargid
383 clade. It is possible that their dispersal was to a large extent mediated by floods (e.g. Van
384 Leeuwen et al., 2013), as both species are very common in the floodplains of the Danube.
385 Their low genetic divergence contrasts with *Gammarus* species from the region, which
386 comprise dozens of cryptic lineages (Copilaş-Ciocianu & Petrusek 2015, 2016), or with the
387 isopod *Asellus aquaticus* Linnaeus, 1758 that also exhibits substantial genetic diversity
388 (Verovnik, Sket & Trontelj, 2005; Sworobowicz et al., 2015). Although our sample size is
389 rather small and our insight into genetic differentiation of the studied taxa is limited, we
390 analysed representative samples across the ranges of both species, and the conclusion that
391 they may disperse over long distances is strongly supported. However, the possibility that
392 some regionally restricted cryptic lineages related to either *N. hrabei* or *N. valachicus* will be
393 found in the future cannot be ruled out.

394 The distribution of *N. hrabei* seems linked to the main channel of the Danube River
395 and its larger tributaries. This is further supported by its frequent occurrence in rivers, which
396 may act as corridors of rapid dispersal. *N. valachicus* is more evenly distributed across the
397 Danube lowlands due to its more frequent occurrence in smaller streams and canals. Its
398 presence in freshwaters along the southern Black and Caspian seas shores is intriguing from a
399 biogeographical perspective. Small genetic distances between European and Turkish
400 populations imply a recent dispersal. Thus, these scattered locations were reached recently,
401 possibly in the late Pleistocene when the water and salinity levels of the Black and Caspian
402 seas varied considerably and there were several connection episodes between them

403 (Badertscher et al., 2011). Those basins share a complex palaeogeographic history which has
404 greatly influenced the biogeography of their biotas (Cristescu, Hebert & Onciu, 2003;
405 Audzijonyte et al., 2015; Kotlik et al., 2008; Nahavandi et al., 2013; Maguire et al., 2014).
406 However, it should be noted that we did not genetically analyse material from the Caspian Sea
407 shores in Iran.

408 Straškraba (1972) and Sket (1981) indicated that the ranges of *N. hrabei* and *N.*
409 *valachicus* overlap with the extent of the Paratethys Sea during the Pliocene and hypothesized
410 that these species have invaded coastal freshwaters from the sea. There is, however, a dispute
411 about the origin of the genus *Niphargus*. On the one hand, some species tolerate brackish
412 water (Sket, 1977), including species living several tens of kilometres from the sea (Delić,
413 pers. com.); on the other hand, fossil niphargids indicate that the group might have colonized
414 freshwaters more than 30 Myr ago (Coleman & Myers, 2000; Jażdżewski & Kupryjanowicz,
415 2010). Moreover, some phylogenies suggest a Late Cretaceous, north-western European
416 origin of the genus (McInerney et al., 2014, Brad et al., 2015), although the deep phylogenetic
417 relationships within *Niphargus* remain weakly supported (Esmaeili-Rineh et al., 2015).
418 Regardless of the origin of the genus, both *N. hrabei* and *N. valachicus* exhibit a shallow
419 genetic structure, which implies a recent rapid dispersal (possibly including passive long-
420 distance dispersal events). Therefore, in the case of *N. hrabei*, its distribution pattern
421 apparently only coincidentally reflects the extent of the Pliocene Paratethys sea levels. The
422 distribution of *N. valachicus* seems indeed related to fluctuating sea levels, but this is more
423 likely due to the contraction/expansion of marginal freshwater habitats rather than a recent
424 colonization of freshwaters from the sea. Further molecular studies involving more
425 populations and multiple markers are required to critically test these biogeographical
426 hypotheses.

427

428 *Taxonomy*

429 Balázs et al. (2015) suggested a close relationship between *N. thermalis* and *N. hrabei*. Our
430 data showed that the variation of 230 analysed morphological traits of the former falls within
431 the boundaries of the latter, and both share the same COI haplotype. Therefore, we did not
432 find any evidence confirming the separate status of *N. thermalis* and *N. hrabei*. Furthermore,
433 although we did not analyse the ITS region of *N. thermalis*, and thus cannot rule out that the
434 shared COI haplotype results from mitochondrial introgression, unpublished data based on
435 additional mitochondrial and nuclear markers further support the conspecificity of both taxa
436 (Jorge Pérez-Moreno, pers. comm.).

437 Although S. Karaman (1950) synonymized *N. mediodanubialis* with *N. valachicus*
438 decades ago, many Hungarian authors have kept the name (Balázs et al., 2015). Our results
439 based on COI reveal that putative *N. mediodanubialis* individuals are very closely related to
440 *N. valachicus* specimens near the type locality and they even share the same ITS haplotype.
441 Such small genetic distances are considered as intraspecific for freshwater amphipod species
442 (Hou & Li, 2010; Sutherland, Hogg & Waas, 2010; Lagrue et al., 2014) and further support
443 Karaman's taxonomic decision.

444 The taxonomic status of presumed populations of *N. hrabei* or *N. valachicus* in the
445 north-eastern parts of the Black and Azov seas, indicated in Straškraba (1972), remains
446 unclear. Russian authors (Birstein 1954; Dedju 1980; Sidorov, 2014; Takhteev, Berezina &
447 Sidorov, 2015), however, do not report either species from that region, but two other
448 morphologically and ecologically similar taxa, *N. cubanicus* Birstein, 1954 and *N.*
449 *potamophilus* Birstein, 1954. Whether or not these two Russian species are related to or even
450 conspecific with *N. valachicus* or *N. hrabei* remains to be explored. Based on the data
451 presented here, at least the presence of *N. hrabei* seems unlikely in this region.

452 We conclude that due to their surprisingly low intraspecific genetic divergence, low
453 frequency of co-occurrences and overall ecological and morphological similarity, *N. hrabei*
454 and *N. valachicus* are two widespread species that are weakly differentiated ecologically.
455 Therefore, their wide ranges and broad ecological tolerance indicate that the success of
456 *Niphargus* is not limited to the subterranean realm, but is also apparent in surface waters.

457

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469

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719 **Figure legends**

720 Figure 1. Distribution patterns of the studied species. Dots with black outline are literature
721 data, white outline indicates data from this study, and squares/stars highlight the origin of

722 genetically analysed samples. The type localities of *N. hrabei* and *N. valachicus* are indicated
723 by green triangles. A) Distribution of *N. hrabei*. The type locality of *N. thermalis* is indicated
724 by a star. B) Distribution of *N. valachicus*. The locations of presumed *N. mediodanubialis* are
725 indicated by stars. Dark-blue dots indicate the localities where both *N. hrabei* and *N.*
726 *valachicus* were found co-occurring. The additional inset map indicates the easternmost
727 localities of *N. valachicus* from Turkey and Iran, respectively. The colours and codes of the
728 squares and stars correspond with those of the COI haplotypes in Fig. 3. Countries are
729 indicated by corresponding 2-letter ISO codes: AT—Austria, BG—Bulgaria, DE—Germany,
730 HR—Croatia, HU—Hungary, MD—Republic of Moldova, RO—Romania, RS—Serbia, SI—
731 Slovenia, SK—Slovakia, UA—Ukraine, TR—Turkey.

732

733 Figure 2. Altitudinal distribution of the studied *Niphargus* species (*N. hrabei* in light grey, *N.*
734 *valachicus* in dark grey), and their occurrence in different types of water bodies. A, C)
735 Frequency histograms and box-plots illustrating the altitudinal distribution of both species,
736 from all sites and from sympatric sites, respectively. B, D) Proportions in which the species
737 were found in each type of habitat, from all sites and from sympatric sites, respectively.

738

739 Figure 3. Phylogeny and haplotype network of *N. hrabei* and *N. valachicus* based on COI and
740 ITS. A) Habitus of *N. hrabei* and *N. valachicus*, scale bar is 5 mm (Photographs: D. Copilaș-
741 Ciocianu). B) Integer neighbour-joining networks based on COI. Black dots indicate
742 unsampled or extinct haplotypes. Hatch marks indicate mutational steps. The size of a circle is
743 proportional to the observed number of the corresponding haplotype and sample size is
744 indicated where $n > 1$. The colours and codes of particular haplotypes correspond to those in
745 Fig. 1. C) Maximum-likelihood phylogeny of the unique COI haplotypes, numbers near
746 branches indicate bootstrap support values. D) Haploneb based on ITS variation, haplotypes

747 from heterozygous individuals are connected by dashed curved lines. Individuals are colour-
748 coded according to their corresponding COI haplotype.

749

750 Figure 4. Body size and measurements of *N. valachicus* (V), *N. hrabei* (H) and *S. ambulans*
751 (S). A) Distribution of body sizes. All species significantly differ in body length from each
752 other. B) Distribution of antenna I (dark grey) and pereopod VII (light grey) lengths. C)
753 Distribution of four gnathopod II measures. From left to right: carpus length, propodus length,
754 palm length of propodus and diagonal length of propodus. D) Distribution of two measures on
755 coxa III: coxal depth (dark grey) and coxal width (light grey). Means grouped by a horizontal
756 line are not significantly different from each other. For measurements in B, C and D, we
757 corrected for body size using residuals (see Methods).

758

760 **Tables**

761 Table 1. List of samples used for molecular analyses. Sequences of specimens from Hagieni
 762 spring, Romania, originate from Flot et al. (2014), all other sequences were obtained in the
 763 present study.

Species	Locality	Coordinates	Country	<i>n</i>	Isolate code	COI haplotype	Accession number COI	Accession number ITS
<i>N. thermalis</i>	Budapest	N47.5180 E19.0358	Hungary	1	Nth1	H1	KU948612	-
<i>N. hrabei</i>	Lugovo	N45.7363 E19.1391	Serbia	2	1RS5H	H1	KU948615	KY310539
					2RS5H		KU948616	KU948713
<i>N. hrabei</i>	Freudenau	N48.1936 E16.4508	Austria	2	1AU1H	H1	KU948619	-
					2AU1H		KU948620	KU948716
<i>N. hrabei</i>	Dunaalmás	N47.7295 E18.3203	Hungary	1	4NH2	H2	KU948611	KU948711
<i>N. hrabei</i>	Šabac	N44.7441 E19.6691	Serbia	2	1RS9H	H3	KU948617	KU948714
					2RS9H	H4	KU948618	KU948715
<i>N. hrabei</i>	Hagieni spring	N43.8024 E28.4747	Romania	3	JFF_12.29	H5	KF290272	KF290201
					SS_11.06		KF290261	KF290186
					SS_11.18		KF290239	KF290147
<i>N. hrabei</i>	Movila Banului	N44.9683 E26.6630	Romania	2	1CR19H	H6	KU948613	KU948712
					2CR19H		KU948614	-
<i>N. valachicus</i> *	Doba	N47.7561 E22.7013	Romania	1	1SM5	V1	KU948621	KU948717
<i>N. valachicus</i> *	Cenad	N46.1152 E20.5936	Romania	2	1CENV	V2	KU948622	KU948718
					2CENV		KU948623	-
<i>N. valachicus</i> *	Balatonfenyves	N46.6551 E17.4985	Hungary	1	1HUN6V	V3	KU948624	KU948719
<i>N. valachicus</i>	Sela	N45.5060 E16.3184	Croatia	1	1BK13V	V4	KU948625	KU948720
<i>N. valachicus</i>	Schitu	N44.1588 E25.8358	Romania	2	2CR8V	V5	KU948628	KU948722
					3CR8V	V7	KU948629	KU948723
<i>N. valachicus</i>	Murighiol	N45.0460 E29.1416	Romania	2	1DD6V	V6	KU948626	KU948721
					2DD6V		KU948627	-
<i>N. valachicus</i>	Abalı	N42.0386 E35.0191	Turkey	2	1TRK	V8	KU948630	KU948724
					2TRK		KU948631	-

764 *n* – number of analysed individuals, * – presumed *N. mediodanubialis*

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Table 2. List of samples used in the morphological analysis.

Species	Locality	Coordinates	Country	<i>n</i>
<i>Niphargus valachicus</i>	Ghimpați	N44.6741 E25.8127	Romania	8
	Băbești	N47.9661 E23.0691	Romania	11
	Abalı	N42.0386 E35.0191	Turkey	7
	Tiszafüred	N47.6166 E20.7333	Hungary	7
<i>Niphargus hrabei</i>	Šabac	N44.7441 E19.6691	Serbia	11
	Hagieni spring	N43.8024 E28.4747	Romania	5
	W of Lupoglav, Zagreb	N45.7508 E16.3622	Croatia	3
<i>Niphargus thermalis</i>	Malom lake, Budapest	N47.5180 E19.0358	Hungary	3
	Molnár János cave, Budapest	N47.5180 E19.0358	Hungary	6
<i>Synurella ambulans</i>	Timișoara	N45.7555 E21.2727	Romania	10

769 *n* – number of analysed individuals

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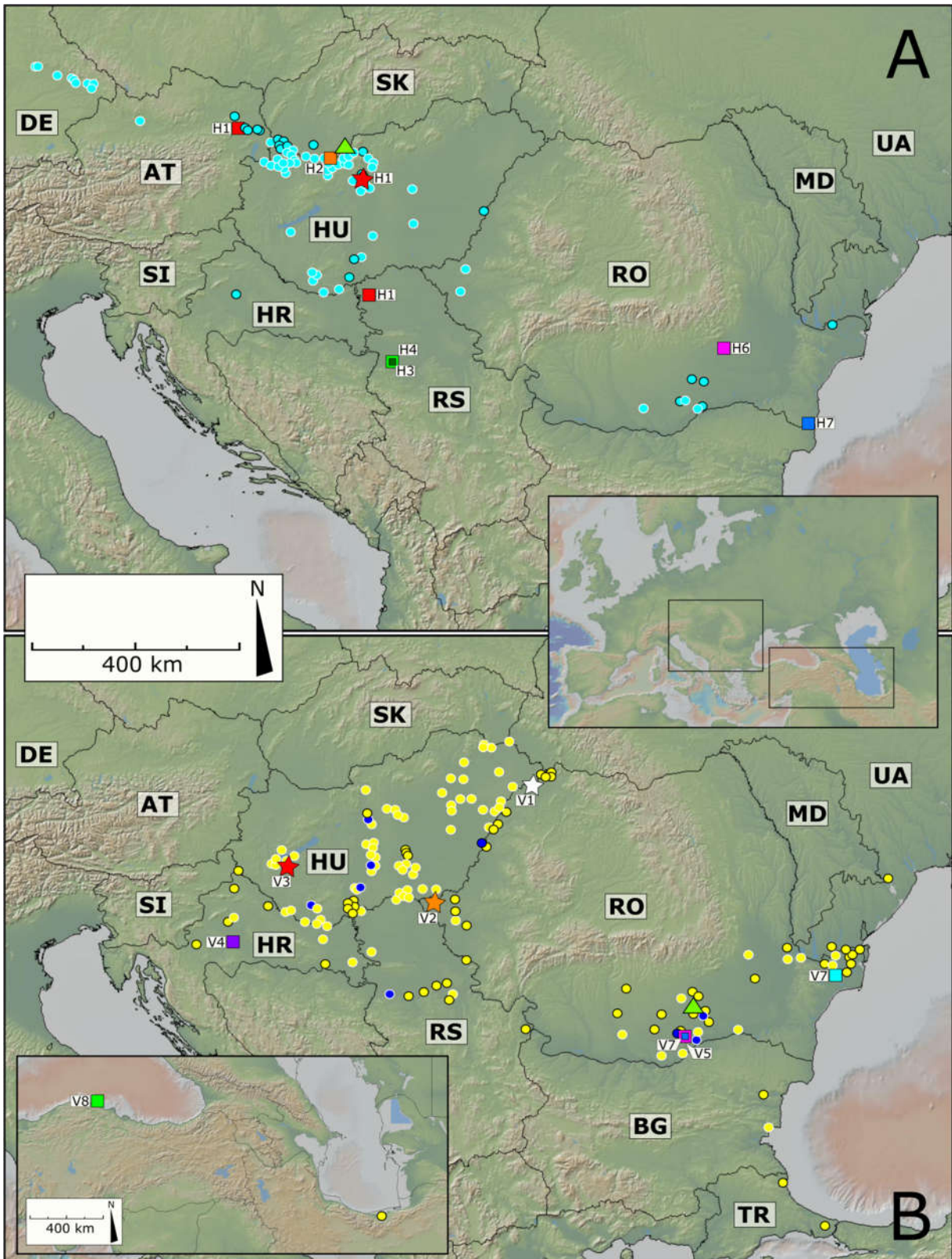
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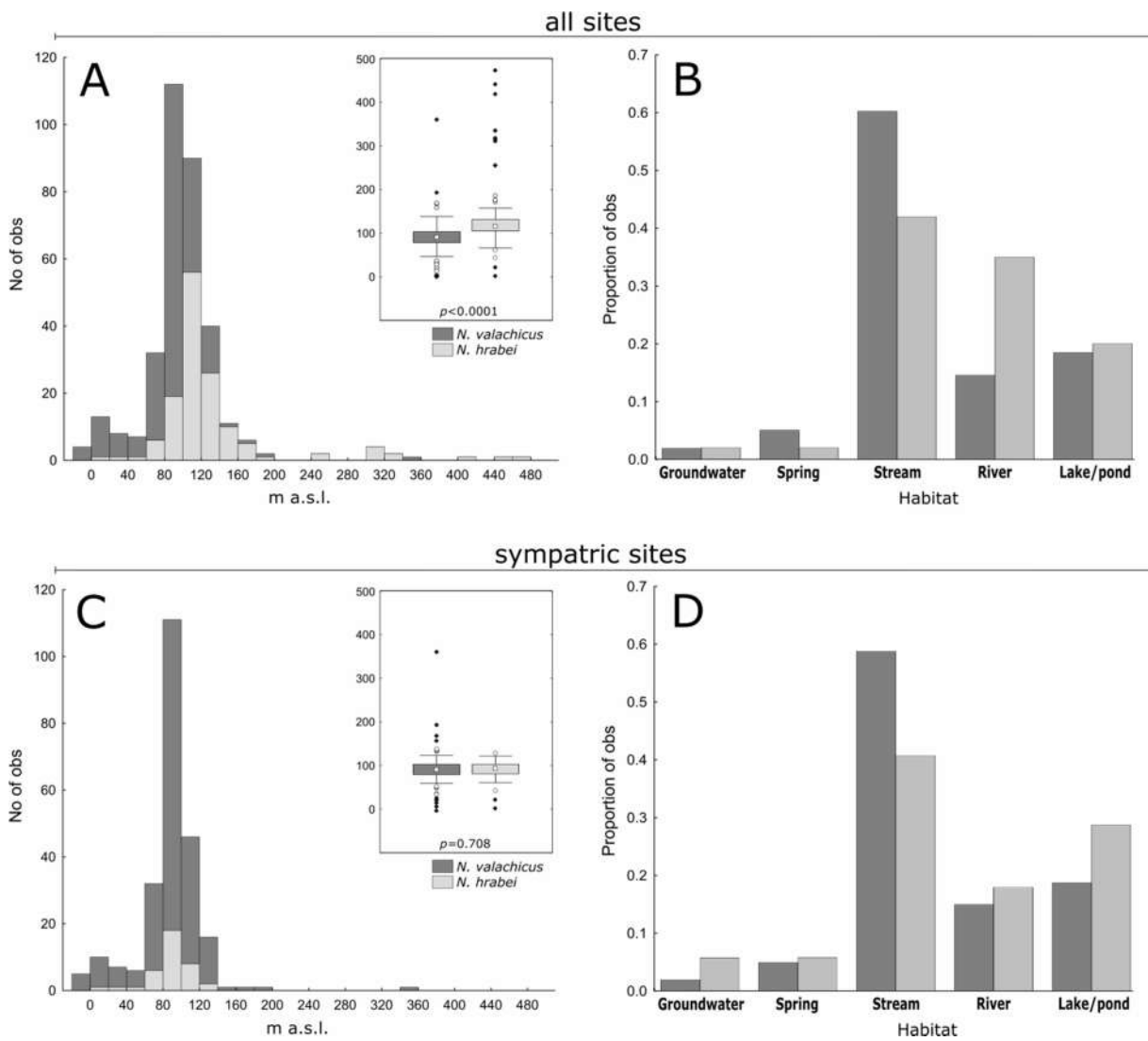
Table 3. Differences in body length and appendage length of the three species. Statistically significant values are depicted in bold fonts.

complex trait	MANOVA (p value for Wilks λ)	parameters describing complex*	ANOVA for parameters (p values)	pairwise comparisons (p values)		
				<i>N. valachicus</i> - <i>N. hrabei</i>	<i>N. valachicus</i> - <i>S. ambulans</i>	<i>N. hrabei</i> - <i>S. ambulans</i>
grip ability	< 0.001	carpus length	0.158	-	-	-
		propodus length	< 0.001	< 0.001	0.938	0.01
		palm length of propodus	0.002	0.266	0.002	0.075
		length of propodus diagonal	0.004	0.144	0.005	0.238
ventral channel	< 0.001	coxa depth	< 0.001	0.395	< 0.001	< 0.001
		coxa width	0.232	-	-	-

784 *All traits were corrected for body length by using residuals rather than raw values.

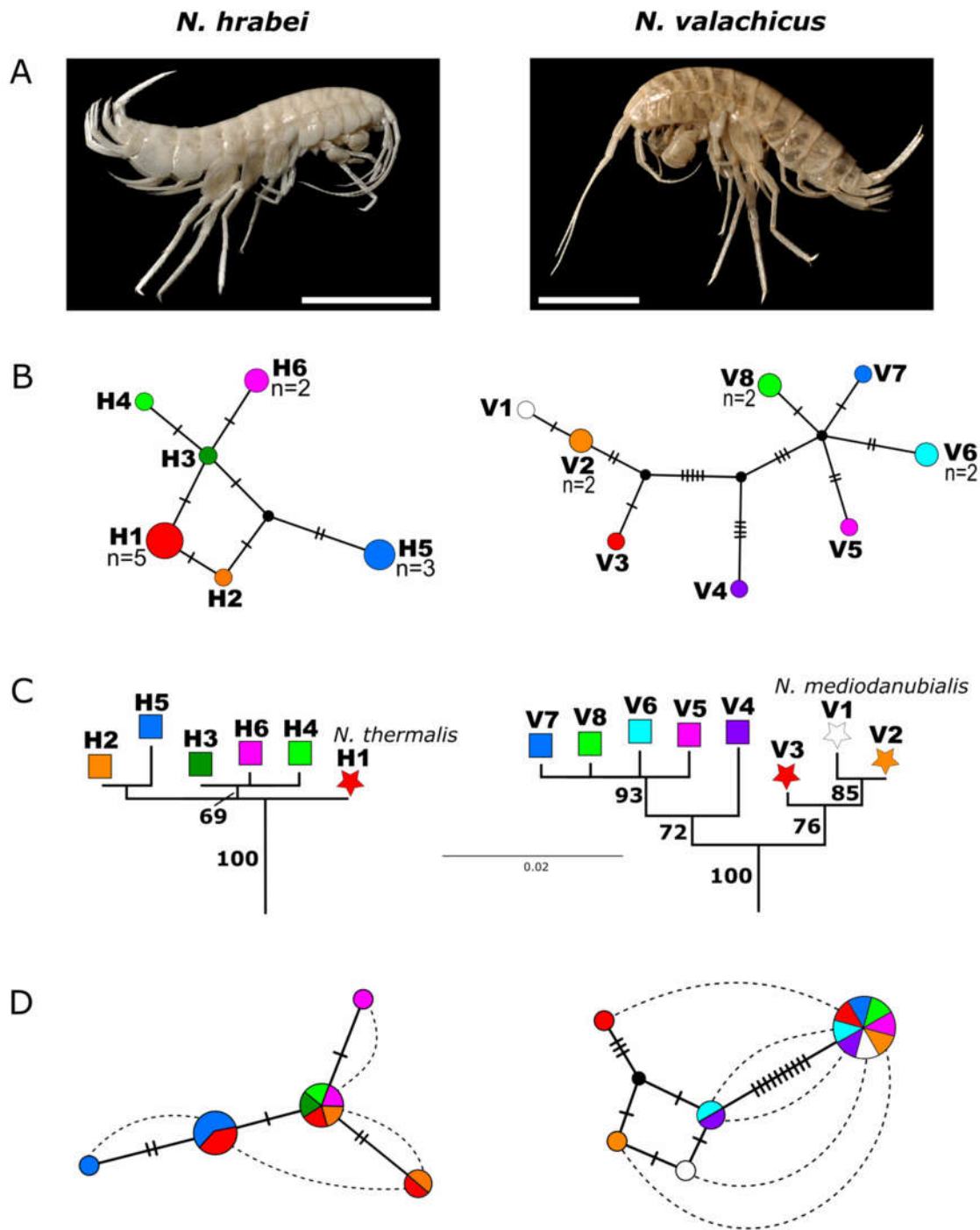
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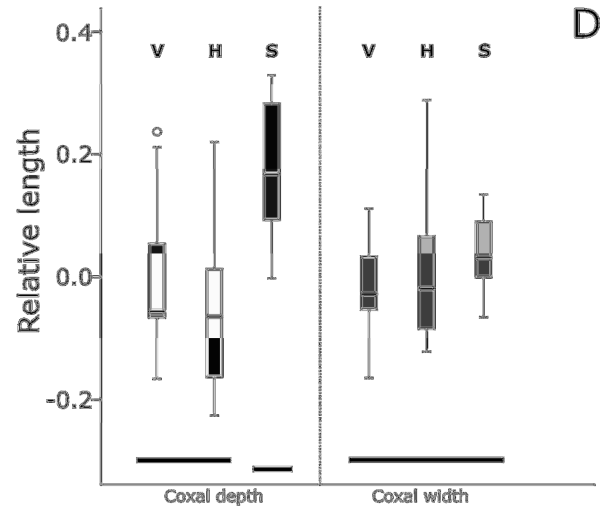
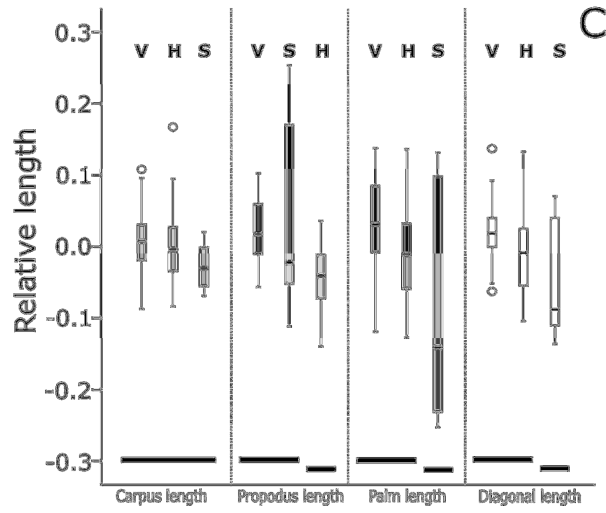
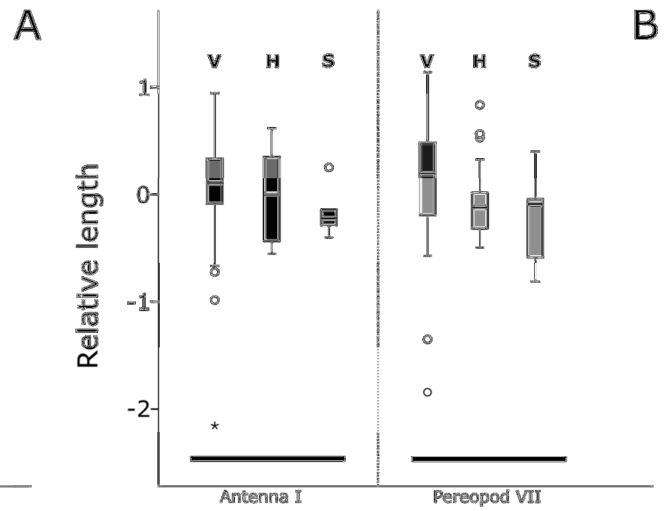
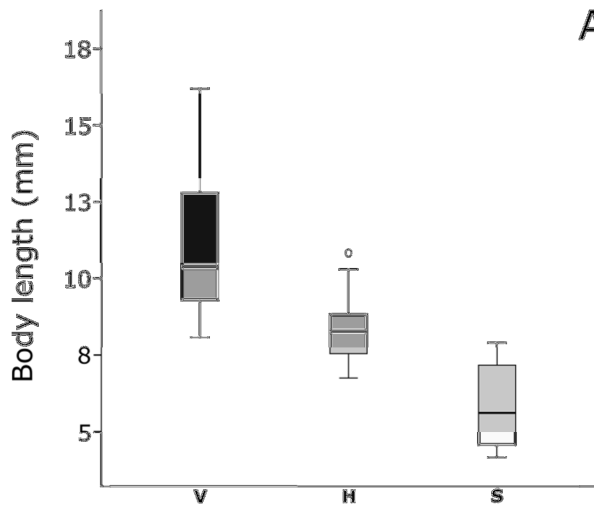




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