DNA barcoding of Austrian snow scorpionflies (Mecoptera, Boreidae) reveals potential cryptic diversity in *Boreus westwoodi*

Lukas Zangl[1,2], Elisabeth Glatzhofer[1], Raphael Schmid[1], Susanne Randolf[3], Stephan Koblmüller[1]

[1] Institute of Biology, University of Graz, Universitätsplatz 2, 8010 Graz, Austria
[2] Universalmuseum Joanneum, Studienzentrum Naturkunde, Weinzöttlstraße 16, 8045 Graz, Austria
[3] Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria

Corresponding Author:
Lukas Zangl[1]
Universitätsplatz 2, Graz, 8010, Austria
Email address: Lukas.zangl@uni-graz.at

Abstract

Background. Snow scorpionflies (genus *Boreus*) belong to a family of Mecoptera, Boreidae, that has been vastly neglected by entomological researchers due to their shift in seasonality to the winter months. Their activity during this time is regarded as a strategy for predator avoidance and regular sightings on snow fields suggest that this also facilitates dispersal. However, many aspects about snow scorpionflies, especially systematics, taxonomy, distribution of species, phylogenetics and phylogeography have remained fairly unexplored until today. In this study, we fill some of these gaps by generating a reference DNA barcode database for Austrian snow scorpionflies in the frame of the Austrian Barcode of Life initiative and by characterising morphological diversity in the study region.

Methods. Initial species assignment of all 67 specimens was based on male morphological characters previously reported to differ between *Boreus* species and, for females, the shape of the ovipositor. DNA barcoding of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene was carried out for all 67 samples and served as a basis for BIN assignment, genetic distance calculations, as well as alternative species delimitation analyses (ABGD, GMYC, bGMYC, bPTP) and a statistical parsimony network to infer phylogenetic relationships among individual samples/sampling sites.

Results. Morphological investigations suggested the presence of both *Boreus hyemalis* and *Boreus westwoodi* in Austria. DNA barcoding also separated the two species, but resulted in several divergent clades, the paraphyly of *B. westwoodi* in Austria, and high levels of phylogeographic structure on a small geographic scale. Even though the different molecular species delimitation methods disagreed on the exact number of species, they unequivocally

40 suggested the presence of more than the tradiationally recognized two *Boreus* species in Austria,
41 thus indicating potential cryptic species within the genus *Boreus* in general and especially in *B.*
42 *westwoodi*.
43

Introduction

45 The holometabolous insect genus *Boreus* (Mecoptera: Boreidae), commonly known as snow
46 scorpionflies or winter scorpionflies (Ibrahimi et al., 2018), has a Holarctic distribution and is
47 most famous for its cold tolerance and activity on snow (Hågvar & Østbye, 2011). The imagines
48 occur from about October to March/April (Finch, 1997; Hågvar, 2001), a temporal niche, which,
49 among other hypotheses, has been attributed to predator avoidance and improved dispersal
50 (Hågvar, 2010). Snow scorpionflies predominantly feed on mosses, but are also known to
51 process decaying insects (Finch, 1997). Despite a basic understanding of their general biology
52 (Finch, 1997; Hågvar, 2001; Hågvar, 2010), gaps in the knowledge concerning their distribution
53 and species richness are yet to be overcome (e. g., Willmann, 1978; Hågvar & Østbye, 2011;
54 Ibrahimi et al., 2018). However, most of the existing literature unanimously reports *Boreus*
55 *hyemalis* (Linnaeus, 1767) and *Boreus westwoodi* Hagen, 1866 from Southwest Europe to
56 Northeast Scandinavia, and consequently also from Austria (Willmann, 1978; Devetak, 1988;
57 Finch, 1997; Raemakers & Kleukers, 1999, Kreithner, 2001; Hågvar & Østbye, 2011; Tillier et
58 al., 2011; Ibrahimi et al., 2018). Field studies suggested similar ecological preferences for these
59 two species (Hagvar, 2010 and references therein) and therefore some authors have also regarded
60 them as only one species (e.g. Saure, 2003). Other species like *Boreus lokayi* Klapálek
61 1901(Romania, Slovakia), *Boreus aktijari* Pliginskij, 1914 (Crimea) or *Boreus kratochvili*
62 Mayer, 1938 (Czech Republic) are only scarcely mentioned in the literature (Penny, 1977;
63 Willmann, 1978; Kreithner, 2001; Ibrahimi et al., 2018) and the latter one is even regarded a
64 synonym of *B. hyemalis* (Kreithner, 2001). *Boreus gigas* Brauer, 1876 is another ambiguous
65 taxon, which is currently also considered a synonym of *B. hyemalis* and even lacks a formal
66 species description at all (Willmann, 1978). In the past, descriptions of *Boreus* species were
67 based exclusively on morphological characters (Brauer, 1876; Mayer, 1938; Blades, 2002).
68 Morphological similarity, plasticity and overlapping ranges, though, have issued continuous
69 discussions about their validity (Willmann, 1978 and references therein; Kreithner, 2001) and
70 consequently the distribution of distinct species across Europe in general (Willmann, 1978;
71 Finch, 1997; Kreithner, 2001), but also for Austria in particular (Gepp, 1982; Kreithner, 2001;
72 Gruppe and Aistleitner, 2011). A detailed morphological study compared material from the Alps
73 (Austria, Switzerland, Slovenia, Italy and France) with specimens from Croatia and Sweden and
74 provided a set of morphological characters for species discrimination, spanning some of the
75 intraspecific and geographic morphological variation (Kreithner, 2001). However, no relevant
76 genetic information of European *Boreus* species has been available so far.
77 Since DNA barcoding was introduced as a method for biological species discrimination (Hebert
78 et al., 2003), several studies have shown that its delimiting powers also apply to various insect
79 groups (e.g., Raupach et al., 2016; Huemer et al., 2019; Zangl et al., 2019; Galimberti et al.,

80 2020). However, DNA barcoding also has well known limitations with respect to recently
81 diverged species, large population sizes retaining divergent haplotypes and
82 hybridization/introgression (e.g., van Velzen et al., 2012; Ermakov et al., 2015; Cong et al.,
83 2017; Zangl et al., 2020; Paill et al., 2021), and species delimitation therefore benefits from
84 additional sources of data (e.g., Trewick, 2008; Liu et al., 2017). Conducted in the framework of
85 the Austrian Barcode of Life initiative (ABOL, www.abol.ac.at; Haring et al., 2015), the present
86 study aims at i) contributing DNA barcodes of Austrian *Boreus* species to the Barcode of Life
87 database (BOLD; www.boldsystems.org; Ratnasingham & Hebert, 2007), ii) investigating their
88 genetic diversity, iii) validating the two proposed Central European species with genetic data and
89 iv) testing whether genetic results mirror the morphological variability displayed by both *B.*
90 *hyemalis* and *B. westwoodi*.
91

Materials & Methods
93 All specimens investigated in the present study were collected in concordance with state
94 conservation laws and under following permits granted by the Amt der Steiermärkischen
95 Landesregierung, Abt. 13 Umwelt und Raumordnung and the Amt der Kärtner Landesregierung,
96 Abt. 8 Umwelt, Energie und Naturschutz, respectively: ABT13-53S-7/1996-156, ABT13-53W-
97 50/2018-2, 08-NATP-845/1-2019(007/2019), N-2018-326688/8-Pin). From 2017 to 2020, 67
98 individuals from 18 Central and Eastern Austrian localities were caught by hand and stored in 2
99 ml Eppendorf tubes in pure Ethanol at -20°C (information on species determinations, collection
100 and storage is available on BOLD (dx.doi.org/10.5883/DS-BOREUS), Table S1). Morphological
101 species discrimination followed Penny (1977) and Kreithner (2001). Primarily, the shape of
102 tergal apophyses (TA), gonostyles (GS), epiandrum (EA) and hypandrum (HA) of males was
103 used to assign specimens to species, as these have been identified as the most reliable
104 discriminating characters in previous studies (Kreithner, 2001 and references therein). Due to the
105 unexpected genetic diversity recovered by the DNA barcoding (see below), further
106 morphological investigation included the properties of the caput, the number of antennal
107 segments and the number of bristles on the front wing. These characters, however, have been
108 regarded as questionable or even unsuited for species discrimination in previous studies
109 (Kreithner, 2001 and references therein) but we wanted to check if they show any
110 correspondence to the genetic results. For females, Kreithner (2001) suggested the shape of the
111 ovipositor and especially of the gonocoxosternites which we also used as the primary distinctive
112 character. A Keyence digital microscope was used to assess TA, EA and HA in males and to
113 capture the general appearance of all specimens.
114 For DNA analyses, total genomic DNA was extracted from three legs using the NucleoSpin
115 Tissue XS Micro kit (Macherey-Nagel) following the manufacturer's instructions. PCR
116 amplification, purification and chain termination sequencing using the primer set C_LepFolF and
117 C_LepFolR (Hernández-Triana et al., 2014) followed Koblmüller et al. (2011) and Duftner et al.
118 (2005). Sequences were visualized on a 3500xl capillary sequencer (ABI) and aligned by Muscle
119 in MEGA 6.06 (Tamura et al., 2013). Clustering analysis based on a Neighbor-Joining (NJ) tree

120   was performed using the "Taxon ID Tree" tool implemented on BOLD (www.boldsystems.org)
121   based on a muscle alignment and employing the pairwise deletion option. Genetic distances
122   within and between main lineages/species were calculated using the "Barcode Gap Analysis"
123   tool, also provided on BOLD. Furthermore, we estimated divergence times by translating COI
124   distances under the assumption of a general arthropod divergence rate of 1.5% (Quek et al.
125   2004). Sequences of *Boreus borealis* (KU874461.1, KU874462 (Sikes et al., 2017)), a North
126   American representative of the genus, were downloaded from GenBank and used as outgroup.
127   For molecular species delimitation, BIN assignment on BOLD (Ratnasingham & Hebert, 2013),
128   the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2011), the Bayesian Poisson
129   Tree Processes (bPTP) model (Zhang et al., 2013), the Generalized Mixed Yule Coalescent
130   (GMYC) (Zhang et al., 2013, and the Bayesian GMYC (bGMYC) (Reid & Carstens, 2012) were
131   used. ABGD was performed via the web version
132   (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using default settings and each of the
133   three distance models Kimura (K80) TS/TV, Jukes-Cantor (JC69) and Simple Distance (results
134   are reported for the Kimura (K80) TS/TV model here as they did not vary between the different
135   models). As input tree for the bPTP analysis, a Maximum Likelihood (ML) tree was inferred via
136   the web-version of PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/; Guindon et al., 2010),
137   employing the HKY model (selected by SMS in PhyML based on the Bayesian Information
138   Criterion (BIC); Lefort et al., 2017), no preset starting tree and 1000 bootstrap pseudo-replicates
139   to assess nodal support. bPTP was run on the web server (https://species.h-its.org/ptp/) using the
140   default settings (100,000 MCMC generations, thinning = 100, burn-in value = 0.1, seed = 123).
141   For the GMYC analysis, an ultrametric tree was inferred in BEAST v.2.6.3 (Bouckaert et al.,
142   2019). The MCMC chain was run for 10 million generations (sampling frequency = 5000)
143   employing the HKY model, a strict molecular clock and a birth-death tree prior. ESS values (all
144   > 200) were checked with Tracer v1.7 (Rambaut et al., 2018). TreeAnnotator v2.6.3 (part of the
145   BEAST2 package) was used to infer a maximum clade credibility tree from the set of posterior
146   trees. GMYC was run on the web server (https://species.h-its.org/gmyc/) with the single
147   threshold option. The bGMYC analysis was conducted on 501 posterior trees from the BEAST
148   analysis and run (MCMC = 50,000; burnin = 40,000; thinning = 100) in R v3.6.0 (R Core Team,
149   2013) using the package bGMYC v.1.0.2 (Reid & Carstens, 2012). We used a rather
150   conservative posterior probability threshold (posterior probability: 0.5 < P < 0.9) to identify
151   putative species, compared to higher thresholds that might overestimate the species' number
152   (Kornilios et al., 2020).
153   Furthermore, we calculated the number of haplotypes (h), the haplotype diversity (Hd) and the
154   nucleotide diversity (PiT) for the whole dataset using DnaSP v6 (Rozas et al., 2017)
155   Finally, a statistical maximum parsimony network was inferred using TCS (Clement et al., 2002)
156   with default settings as implemented in PopART v.1.7 (Leigh & Bryant, 2015) to visualize
157   phylogeographic relationships. The input file was created using a custom-made python script
158   (available on https://github.com/maxwagn/popart_popprep).
159

160

Results

162 Morphological determination resulted in one *B. hyemalis* and 28 *B. westwoodi* males and one *B.*
163 *hyemalis* and 37 *B. westwoodi* females (Fig. 1, Fig. 2, Table 1).
164 The investigated morphological characters in males showed different degrees of variation
165 between the specimens. TAs, which have been regarded as reliable for species discrimination,
166 were anvil-shaped in all except one (294) *Boreus* males, resembling one of the 12 forms
167 presented in Fig. 1T-EE. Among the different anvil shapes, little to no geographical pattern
168 became obvious as for example all males from Hochwechsel share the same TA shape, but this
169 particular shape was also recovered from males from Weinebene and Gösting. Some of the
170 shapes recovered from Austrian samples also resembled the forms 35-39 (Fig. 1O-S), which
171 Kreithner (2001) referred to as taxonomically uncertain. Similar results were also obtained for
172 the shape of the GS, which generally appeared much more slender in *B. westwoodi*. Only the *B.*
173 *hyemalis* male (294) showed a distinctly different form of the GS with a bulkier appearance and
174 a longer medituberculus (Table 1), all *B. westwoodi* males regardless of their sampling location
175 shared the same GS shape. The number of antennal segments, the number of bristles on the outer
176 side of the front wing and the shape of the EA, all considered as unreliable characters (Kreithner
177 2001), did neither correlate with the two species nor with geographical origins. Characters of the
178 ovipositor like the ventral membranous part or the lateral tapering were remarkably homogenous
179 among all *B. westwoodi* females and similar to *B. hyemalis*. The only difference between *B.*
180 *westwoodi* and *B. hyemalis* females was the breadth of the proximal part of the
181 gonocoxosternites, which was broader in *B. hyemalis*. However, since we only have one nominal
182 *B. hyemalis* female included in our study, we are cautious to rely on this character as we cannot
183 estimate the extent of plasticity.
184 DNA barcodes of the partial COI gene ranging from 649 to 657 bp in length were generated for
185 67 specimens (sequences are available on BOLD (dx.doi.org/10.5883/DS-BOREUS) and
186 GenBank (MW627590 - MW627656). These sequences were grouped into seven BINs (Fig. 3,
187 4), four of which were newly created (BOLD:AEF6177, BOLD:AEF6178, BOLD:AEF6179 and
188 BOLD:AEF8503). The different BINs, however, cannot be distinguished morphologically from
189 each other, except for the BOLD:ACT2769, which comprises *B. hyemalis*. Based on the results
190 of the DNA barcoding, *Boreus westwoodi* turned out to be paraphyletic with respect to *B.*
191 *hyemalis* (Fig 3, Suppl. Fig. 1). As we only had two *B. hyemalis*, the actual extent of intraspecific
192 K2P distance within this species cannot be discussed here. However, since both specimens were
193 from the same sampling site, the observed intraspecific distance was expectedly low.
194 Intraspecific distances within *B. westwoodi* (up to 7.21%) in part considerably exceeded
195 interspecific distances (2.53 -5.9%). Species delimitation analysis results differed considerably
196 among the four alternative methods. While ABGD inferred four species in the recursive
197 approach including the outgroup (three species in the initial approach, Suppl. Fig. 2) and GMYC
198 suggested six species, bPTP estimated 19 to 44 (mean 28) and bGMYC resulted in 10 species,

199   when assuming a threshold 0.5 < P < 0.9 for conspecificity (Fig. 3). Overall, we found 55
200   haplotypes (h) across the whole dataset (Hd = 0.99394, PiT = 0.03362).
201   The statistical maximum parsimony network revealed some phylogeographic structure and very
202   little haplotype sharing among and even within sampling sites. Even though geographically close
203   sampling sites often group together in the network, there are some exceptions to this pattern (Fig.
204   5). Thus, the samples from Hochwechsel resulted in a part of the network (and tree, Fig. 3) that
205   otherwise comprises samples collected west of the Mur River. On the other hand, all samples
206   from Gösting, which is geographically close to Thal (distance <3 km) and west of the Mur River,
207   grouped with samples north and east of the Mur River, even though they formed a quite distinct
208   cluster there. Interestingly, the *B. westwoodi* lineage most closely related to *B. hyemalis* showed
209   a pattern different from the rest of *B. westwoodi*, i.e., haplotype sharing was found among
210   geographically distant sampling sites and the overall genetic diversity in this clade was low.
211
212   **Discussion**
213   In this study, we provide 67 new DNA barcodes representing the first genetic insights into the
214   snow scorpionfly diversity of the genus *Boreus* from Austria and thus also Europe. This apparent
215   lack of genetic information may be attributed to a certain characteristic of the boreids' biology.
216   Due to their shift in seasonality of the imaginal stage to the winter months, few entomologists
217   ever collect them as bycatch from passive stationary traps, let alone actively pursue them
218   (Hågvar and Østbye, 2011). Consequently, contemporary literature about Boreidae almost
219   exclusively only covers new records (Tillier et al., 2011; Ibrahimi et al., 2018), re-evaluates
220   national distribution of species (Devetak, 1988; Finch 1997; Raemakers & Kleukers, 1999;
221   Tillier et al., 2011; Hågvar & Østbye, 2011) and conducts morphological comparison of already
222   available material (Kreithner 2001). However, phenotypic plasticity has been found to be
223   extensive both within species and across larger geographic distances and has fueled debates
224   about the validity and exact distribution of extant species (Willmann, 1978; Kreithner 2001).
225   Nonetheless, certain morphological traits have been reported to hold sufficient discriminative
226   power and suggested the presence of *Boreus hyemalis* and *Boreus westwoodi* throughout Central
227   Europe (Kreithner, 2001, Hågvar & Østbye 2011; Ibrahimi et al., 2018) and consequently also in
228   Austria. However, examination of these characters on material from Austria also recovered a
229   high degree of morphological variation at least within *B. westwoodi* (Fig. 1, Table 1).
230   Comparison of the Austrian material with morphological characteristics reported for European
231   *Boreus* by Kreithner (2001) showed that some of the different morphotypes are very similar
232   between the Austrian and the European samples, indicating a large diversity even within Austria.
233   Furthermore, the resemblance of some of the Austrian TA shapes with the forms regarded as
234   taxonomically uncertain by Kreithner (2001) potentially hints at the existence of cryptic species.
235   The morphological variation, though, is not perfectly consistent with the distribution of genetic
236   haplotypes as samples from the same location might share similar DNA barcodes but show
237   different morphologies or vice versa (Table 1, Fig. 1, Fig. 5), similar to patterns previously
238   reported for some other arthropods, such as scorpions of the genus *Buthus* in the Atlas Mountains

239   or North African darkling beetles (Habel et al., 2012; Husemann et al., 2012; Rangel López et al.
240   2018). Since only one single male (sample 294) could be assigned to *B. hyemalis* based on
241   synoptic inspection of all morphological characters, phenotypic plasticity cannot be evaluated
242   here. However, the shape of TA recovered for sample 294 matches form 34 of Kreithner (2001)
243   almost perfectly (Fig. 1N) and for the first time links this particular morphotype with a DNA
244   barcode and a particular BIN (BOLD:ACT2769). In females, morphological variability, i.e.
245   ovipositor shape, was virtually non-existent. The sole exception was the female individual
246   collected at Zirbitzkogel, the locality where we also collected a *B. hyemalis* male. Though
247   morphologically similar to other females, this individual had a broader ovipositor than other
248   specimens and, unlike any other specimen, lateral extensions at the proximal part of the
249   gonocoxosternites (compare with Kreithner, 2001). These few findings of *B. hyemalis* are also in
250   line with Kreithner (2001), who reported *Boreus* populations across Austria being predominantly
251   *B. westwoodi* with only a few reports of *B. hyemalis* from Eastern Austria. Kreithner (2001) also
252   indicated, that reports of sympatric occurrences may reflect cases of misidentification and that
253   additional species could be present, e.g. in alpine regions.
254   Furthermore, the results of the DNA barcoding and species delimitation analyses suggest that
255   there might be more than two species of *Boreus* present in Austria (Fig. 3). As we had only two
256   nominal specimens of *B. hyemalis* included in our dataset, no inferences can be drawn about
257   intraspecific genetic diversity or the presence/absence of a barcoding gap. In *B. westwoodi*,
258   maximum intraspecific distances are significantly higher than the distance to their nearest
259   neighbor (Table 2), which has also been reported for e.g. ground beetles, butterflies and aphids
260   (Raupach et al., 2016; Janzen et al., 2017; Lee et al., 2017) and may be an indication for cryptic
261   diversity. Furthermore, interspecific distances of two to three percent separating good species
262   have previously been found in e.g., aphids and mosquitos as well (Lee et al., 2017; Wang et al.,
263   2012). While distance-based species delimitation methods like ABGD are prone to lump many
264   species together (da Silva et al. 2018; Dellicour & Flot, 2018; Galimberti et al., 2020) and
265   Dellicour and Flot (2015) even report ABGD and GMYC as unable to correctly delimit species
266   in scenarios involving only one or two species, ABGD suggested one additional species for
267   Austrian boreids. Tree-based methods on the other hand are known to have a tendency for
268   oversplitting, both in cases with few and many species (Dellicour and Flot, 2018), which would
269   explain the large number of species suggested by bGMYC and bPTP in our case. However,
270   despite a general incongruence and a large range in the number of potentially recovered species
271   with the different methods (Fig. 3), they all concur in suggesting that more than the two
272   previously reported species exist in Austria. Limitations for species delimitation inferences based
273   on a single gene are obvious and well discussed in literature but still can pinpoint ambiguous
274   cases (da Silva et al., 2018; Galimberti et al., 2020). However, in the case of Austrian *Boreus*, the
275   patterns obtained from DNA barcoding, species delimitation, statistical maximum parsimony
276   network and morphological analyses do indicate potential cryptic diversity.
277   Besides potential cryptic diversity, we found a remarkable geographic structure, with distinct
278   haplogroups present in geographically close populations, sometimes only a few kilometers apart.

279 Even though our sample comprises only a few animals per location, the general lack of haplotype
280 sharing among most sampling sites (with a few exceptions) is striking, and the large number of
281 singletons indicates large (effective) population sizes. Assuming a general range of arthropod
282 COI divergence rates of 1.0-2.5% per MY (e.g. Brower 1994; Quek et al., 2004; Papadopoulou
283 et al., 2010; Pons et al., 2010), the observed pairwise distances among main lineages of ~2.5 –
284 7.2% translate into divergence times of ~1 - 2.5 to 2.9 - 7.2 MY. As *Boreus* spp. are flightless,
285 some phylogeographic structure was expected, even considering the rather small geographic
286 scope of our study. Yet, the extent of structure is surprising and unexpected, even though in
287 general, flightless and/or less mobile taxa show higher levels of population genetic
288 differentiation than good dispersers (Papadopoulou et al., 2009). Previous studies on small
289 flightless arthropods with alleged low dispersal ability found varying patterns, from little
290 phylogeographic structure with haplotype sharing across long distances (e.g., oribatid mites of
291 the genus *Cymbaeremaeus*, Schäffer et al., 2019) to deeply divergent genetic lineages without
292 gene flow, potentially representing cryptic species, across distances of only tens of kilometers
293 (e.g., springtails of the genus *Lepidocyrtus*, Ciccionardi et al., 2010). The patterns observed in
294 Austrian *Boreus* fit this latter extreme (disregarding the *B. westwoodi* haplogroup most closely
295 related to *B. hyemalis* that was shared among some samples from distant localities). In addition,
296 the factors and processes underlying the peculiar phylogeographic pattern observed in Austrian
297 *Boreus* remain unclear, but it appears that *Boreus* are indeed rather stationary and do not
298 generally disperse over larger distances.
299

Conclusions

301 In conclusion, this study presents the first genetic information on the genus *Boreus* in Austria,
302 and consequently also Europe. Furthermore, it provides several new localities from which
303 boreids have not been reported within Austria so far and thus augments their known distribution
304 range in Austria. DNA barcodes linked to different morphotypes prove the presence of *Boreus*
305 *westwoodi* and *Boreus hyemalis* in Austria, high levels of phylogeographic structure on small
306 geographic scales, and indicate the potential presence of further cryptic species. The phenotypic
307 plasticity previously reported for these two species is confirmed in the present study as several
308 morphological characters show a large variation that does not correlate with genetic variation.
309 Seven distinct BINs were recovered by BOLD and several, albeit inconsistent, potential species
310 were suggested across the alternative species delimitation analyses. This potential cryptic
311 diversity probably also extends to other European populations of *Boreus* but disentangling the
312 exact number of species, possible (ancient) hybridization/introgression, (lack of) gene flow
313 among localities and the precise distribution of these species will require further multilocus or
314 genomic as well as morphological investigations and a pan-European sampling of boreids.
315

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321
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Morphological characterization of Austrian male *Boreus* spp.

Morphological characterization of male *Boreus* spp. according to Kreithner (2001). Numbers in Field ID correspond with supplementary table 1. Tergal apophyses (TA), epiandrum (EA), gonostylus (GS). Numbers in GS correspond to figures in Kreithner (2001). Forms of TA correspond with figure 1(T-EE).

Field ID	Caput	No. of antennal segments	TA	No. of bristles on outer side of front wing (external/internal)	EA	GS
200	Corrugated	24	Form Z	8/36	Lateral lobes shorter than septum, septum broad triangular	41
208	Corrugated	25	Form T	2/33	Lateral lobes shorter than septum, septum broad triangular	41
209	Corrugated	24	Form U	9/31	Lateral lobes same length as septum, septum broad triangular	41
210	Corrugated	25	Form W	9/10	Lateral lobes shorter than septum, septum broad triangular	41
211	Corrugated	23	Form Z	9/29	Lateral lobes shorter than septum, septum broad triangular	41
212	Corrugated	24	Form Z	10/30	Lateral lobes shorter than septum	41
214	Corrugated	23	Form V	10/33	Lateral lobes shorter than septum, septum broad triangular	41
215	Corrugated	23	Form Z	6/23	Lateral lobes shorter than septum, septum broad triangular	41
216	Corrugated	16	Form X	2/33	Lateral lobes shorter than septum, septum	41

					broad triangular	
265	Corrugated	25	Form AA	11/33	Lateral lobes shorter than septum, septum broad triangular	41
266	Smooth, pilose		Form AA	9/28	Lateral lobes shorter than septum, flat, septum broad triangular	41
267	Corrugated	23	Form AA	7/29	Lateral lobes shorter than septum, flat, septum broad triangular	41
268	Corrugated	25	Form AA	13/36	Lateral lobes shorter than septum, flat, septum broad triangular	41
269	Corrugated	23	Form AA	8/30	Lateral lobes shorter than septum, flat, septum broad triangular	41
274	Corrugated	25	Form CC	12/32	Lateral lobes shorter than septum, septum broad triangular	41
275	Corrugated	24	Form AA	9/32	Lateral lobes longer than septum, septum broad triangular	41
276	Corrugated	23	Form AA	13/31	Lateral lobes shorter than septum, septum broad triangular	41
277	Corrugated	24	Form X	11/26	Lateral lobes longer than septum, septum broad triangular	41
278	Corrugated	23	Form BB	8/20	Lateral lobes shorter	41

					than septum, septum broad triangular	
280	Corrugated	24	Form CC	8/34	Lateral lobes same length as septum, septum broad triangular	41
282	Corrugated	24	Form X	10/32	Lateral lobes same length as septum, septum broad triangular	41
284	Corrugated	24	Form EE	10/34	Lateral lobes same length as septum, septum broad triangular	41
285	Corrugated	n.a.	Form EE	8/25	Lateral lobes same length as septum, septum broad triangular	41
287	Corrugated	n.a.	Form EE	8/32	Lateral lobes shorter than septum, flat, septum broad triangular	41
290	Corrugated	n.a.	Form DD	8/24	Lateral lobes shorter than septum, septum broad triangular	41
291	Corrugated	24	Form DD	8/27	Lateral lobes same length as septum, septum broad triangular	41
292	Corrugated	n.a.	Form W	8/27	Lateral lobes shorter than septum, septum broad triangular	41
294	Corrugated	23	Form U	2/33 Hind wings with 2 diffuse rows of 23	Lateral lobes longer than septum, septum pointed	45

				bristles		
296	Corrugated	23	Form AA	11/26	Lateral lobes shorter than septum, septum broad triangular	41
297	Corrugated	23	Form AA	14/32	Lateral lobes shorter than septum, septum broad triangular	41
299	Corrugated	24	Form Z	8/33	Lateral lobes same length as septum, flat, septum broad triangular	41
301	Corrugated	25	Form X	10/39	Lateral lobes shorter than septum, septum broad triangular	41
303	Corrugated	24	Form CC	12/39	Lateral lobes shorter than septum, septum broad triangular	41
307	Corrugated	24	Form CC	10/34	Lateral lobes shorter than septum, septum broad triangular	41
308	Corrugated	24	Form BB	12/23	Lateral lobes shorter than septum, septum broad triangular	41
312	Corrugated	25	Form Y	10/34	Lateral lobes shorter than septum, septum broad triangular	41
314	Corrugated	25	Form BB	11/34	Lateral lobes shorter than septum, septum broad triangular	41
318	Corrugated	24	Form CC	13/39	Lateral lobes shorter	41

	than septum, septum broad triangular

Genetic distances of *Boreus* spp. based on COI sequences.

Maximum intraspecific K2P distances (I_{max}) and distances (DNN – distance to nearest neighbor) to nearest neighbor (NN) are listed.

Species	I_{max}	DNN	NN
Boreus hyemalis	0.15	2.53	*Boreus westwoodi*
Boreus westwoodi	7.21	2.53	*Boreus hyemalis*

Figure 1

Images and drawings of boreids and their morphological characters.

Images of a copulation (A) and a single male (B) *Boreus westwoodi*, as well as digital microscopy images of the anvil-shaped tergal apophyses (TA) of *B. westwoodi* (C) and pointed TA of *B. hyemalis* (D) from Austria (indicated by black arrows). (E-EE) Drawings of types of TA of *B. westwoodi* (forms E-L), *B. hyemalis* (forms M-N) as well as forms of uncertain taxonomic status (O-S) from across Europe (edited from Kreithner (2001)) and Austrian *B. westwoodi* (T-EE). Drawings of the main shapes of the genital segments (GS) with the epiandrum (EA) (FF-GG, see table 1) retrieved and edited from Kreithner (2001). © Photos by Elisabeth Glatzhofer.
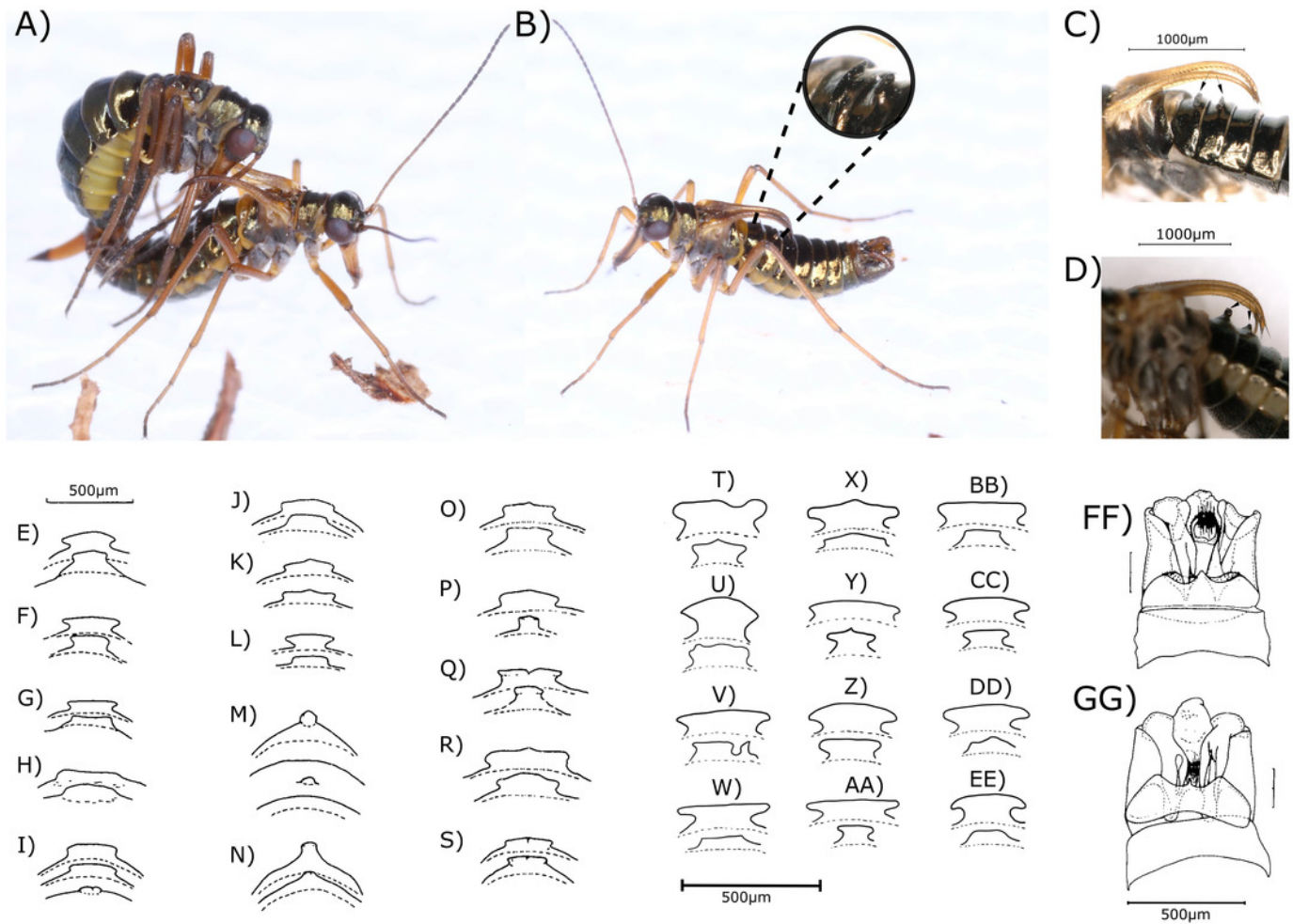
Figure 2

Images of *Boreus* females and their ovipositors.

Digital microscopy images of (A) *Boreus westwoodi* and (B) *B. hyemalis* females as well as the dorsal (C and E) and ventral (D and F) view on their ovipositor, respectively.
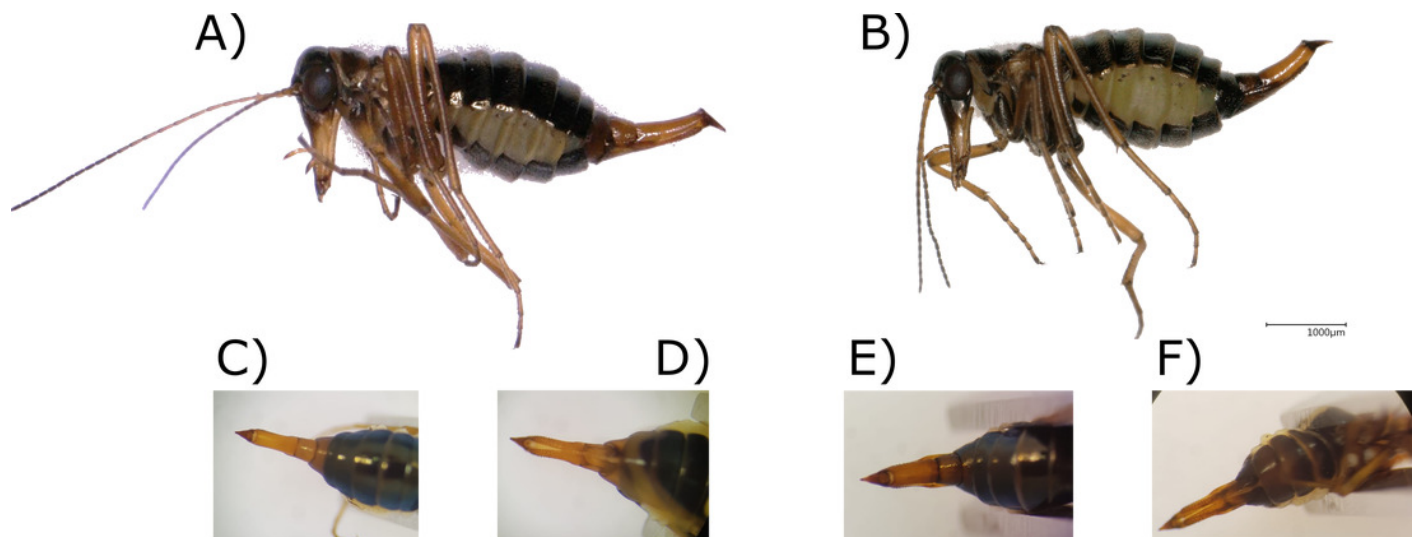
Figure 3

Bayesian inference phylogeny and species delimiation.

Bayesian Inference (BI) phylogeny based on the DNA barcoding region (part of the mitochondrial COI gene). Tip numbers correspond with table S1 and represent specimen IDs. Colored dots represent sample origin. Colored branches indicate initial morphological species assignment. Dots near nodes represent posterior probability categories. Boxes and heatmap to the right indicate the number of putative species inferred by different molecular species delimitation methods.
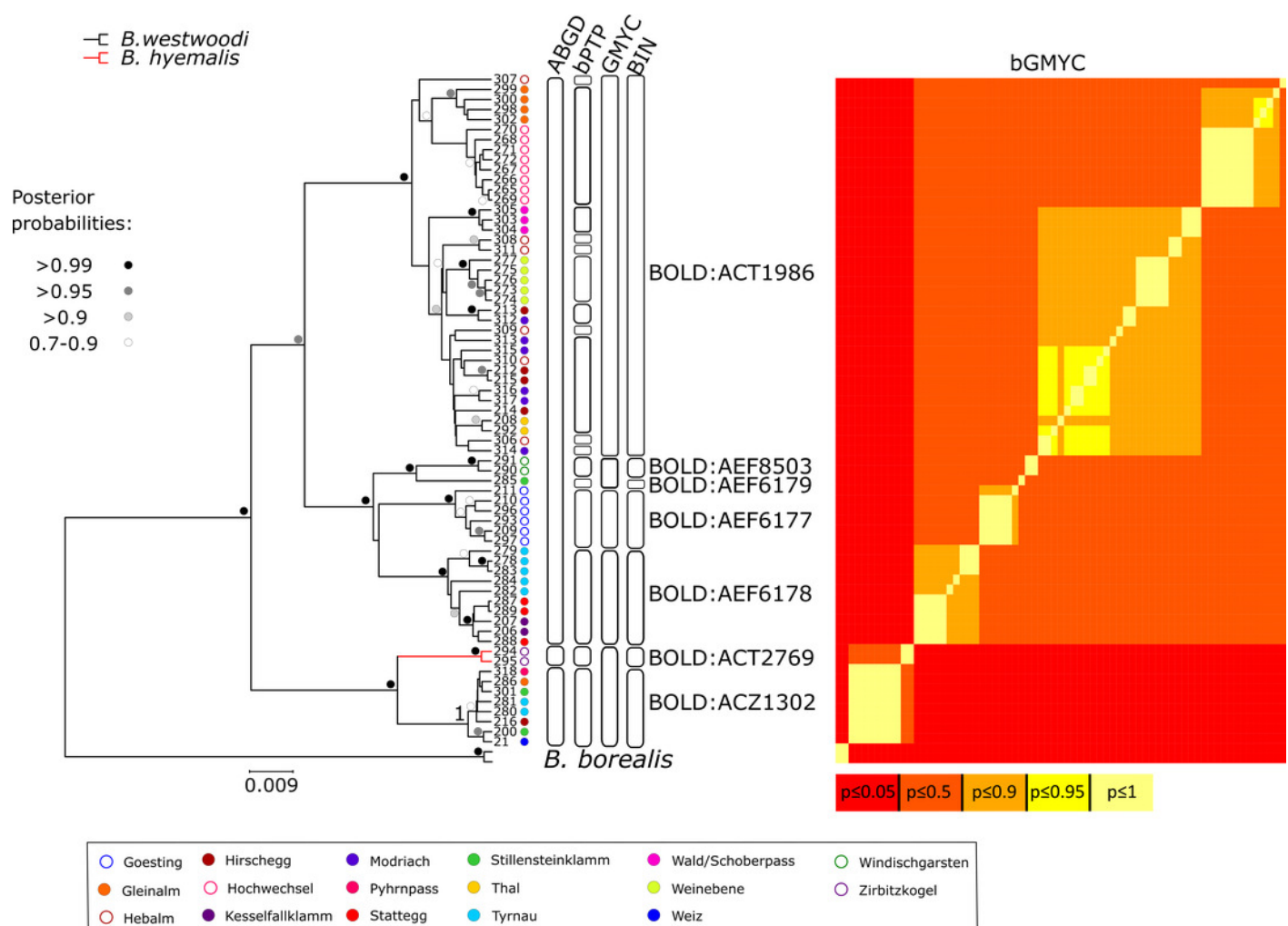
Figure 4

Map of geographic BIN distribution.

Distribution map of Barcode Index Number (BIN) composition across Austrian sampling localities.
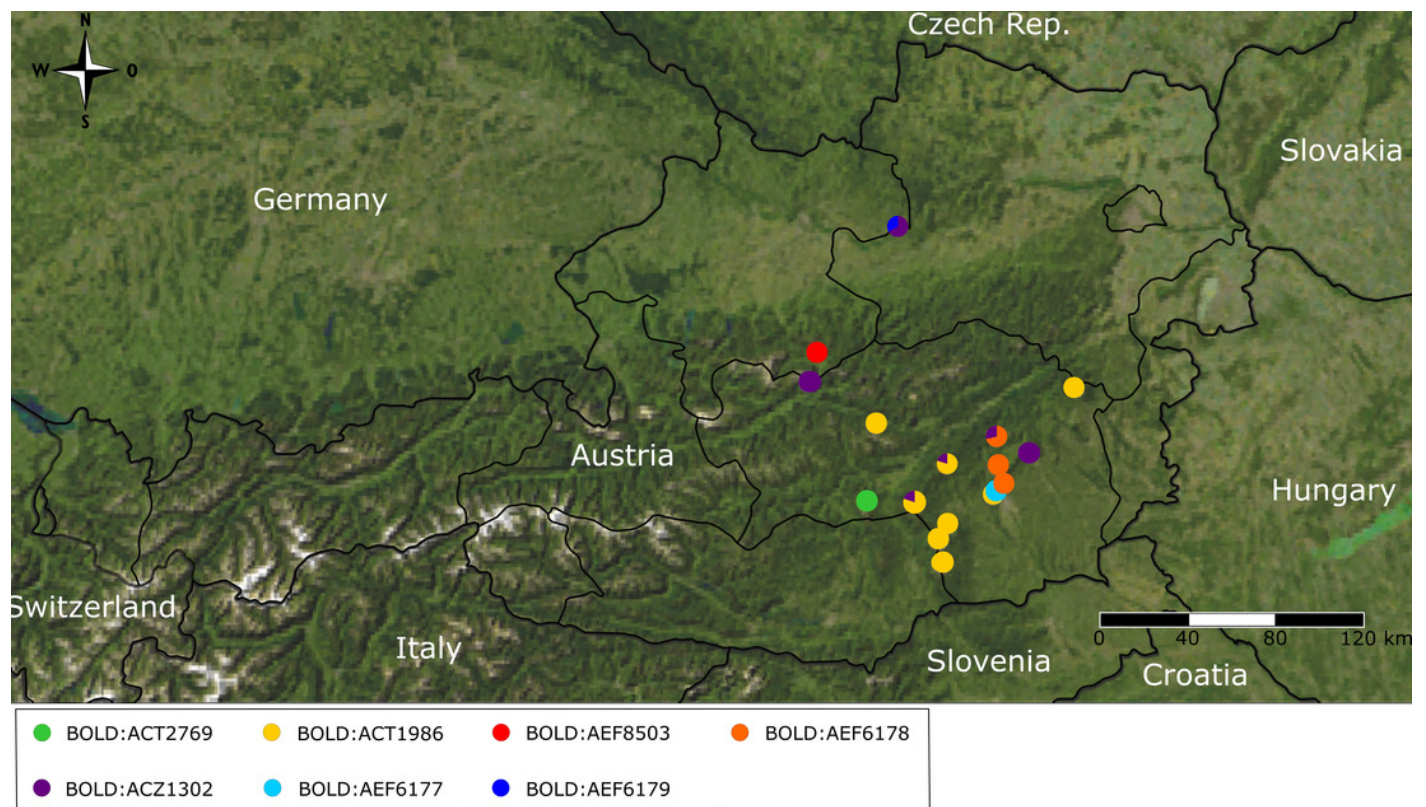
Figure 5

Sampling map and maximum parsimony network.

Map of Austria and surrounding countries including sampling localities (color coding matches insert in 5b). b) Statistical parsimony network based on COI sequences. Colors indicate the different sampling localities. Each circle corresponds to one haplotype and its size is proportional to its frequency. Single mutational steps up to five substitutions between haplotypes are indicated by black bars (substitutions > 5 are represented by numbers next to connective lines). Red frame outlines *Boreus hyemalis* specimens.