From water striders to water bugs: The molecular diversity of aquatic Heteroptera (Gerromorpha, Nepomorpha) of Germany based on DNA barcodes

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With about 5,000 species worldwide, the Heteroptera or true bugs are the most diverse taxon among the hemimetabolous insects in aquatic and semi-aquatic ecosystems. Species may be found in almost every freshwater environment and have very specific habitat requirements, making them excellent bioindicator organisms for water quality. However, a correct determination by morphology is challenging in many species groups due to high morphological variability and polymorphisms within, but low variability between species. Furthermore, it is very difficult or even impossible to identify the immature life stages or females of some species, e.g. of the corixid genus *Sigara*. In this study we tested the effectiveness of a DNA barcode library to discriminate species of the Gerromorpha and Nepomorpha of Germany. We analyzed about 700 specimens of 67 species, with 63 species sampled in Germany, covering more than 90% of all recorded species. Our library included various morphological similar taxa, e.g. species within the genera *Sigara* and *Notonecta* as well as water striders of the genus *Gerris*. Fifty-five species (82%) were unambiguously assigned to a single Barcode Index Number (BIN) by their barcode sequences, whereas BIN sharing was observed for 10 species. Furthermore, we found monophyletic lineages for 52 analyzed species. Our data revealed interspecific K2P distances with below 2.2% for 18 species. Intraspecific distances above 2.2% were shown for 11 species. We found evidence for hybridization between various corixid species (*Sigara*, *Callicorixa*), but our molecular data also revealed exceptionally high intraspecific distances as a consequence of distinct mitochondrial lineages for *Cymatia coleoptrata* and for the pygmy backswimmer *Plea minutissima*. Our study clearly demonstrates the

usefulness of DNA barcodes for the identification of the aquatic Heteroptera of Germany and adjacent regions. In this context, our data set represents an essential baseline for a reference library for bioassessment studies of freshwater habitats using modern high-throughput technologies in the near future. The existing data also opens new questions regarding the causes of observed low inter- and high intraspecific genetic variation and furthermore highlight the necessity of taxonomic revisions for various taxa, combining both molecular and morphological data.

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Abstract

With about 5,000 species worldwide, the Heteroptera or true bugs are the most diverse taxon among the hemimetabolous insects in aquatic and semi-aquatic ecosystems. Species may be found in almost every freshwater environment and have very specific habitat requirements, making them excellent bioindicator organisms for water quality. However, a correct determination by morphology is challenging in many species groups due to high morphological variability and polymorphisms within, but low variability between species. Furthermore, it is very difficult or even impossible to identify the immature life stages or females of some species, e.g. of the corixid genus *Sigara*. In this study we tested the effectiveness of a DNA barcode library to discriminate species of the Gerromorpha and Nepomorpha of Germany. We analyzed about 700 specimens of 67 species, with 63 species sampled in Germany, covering more than 90% of all recorded species. Our library included various morphological similar taxa, e.g. species within the genera *Sigara* and *Notonecta* as well as water striders of the genus *Gerris*. Fifty-five species (82%) were unambiguously assigned to a single Barcode Index Number (BIN) by their barcode sequences, whereas BIN sharing was observed for 10 species. Furthermore, we found monophyletic lineages for 52 analyzed species. Our data revealed interspecific K2P distances with below 2.2% for 18 species. Intraspecific distances above 2.2% were shown for 11 species. We found evidence for hybridization between various corixid species (*Sigara*, *Callicorixa*), but our molecular data also revealed exceptionally high intraspecific distances as a consequence of distinct mitochondrial lineages for *Cymatia coleoptrata* and for the pygmy backswimmer *Plea minutissima*. Our study clearly demonstrates the usefulness of DNA barcodes for the identification of the aquatic Heteroptera of Germany and adjacent regions. In this context, our data set represents an essential baseline for a reference library for bioassessment studies of freshwater habitats using modern high-throughput technologies in the near future. The existing

47 data also opens new questions regarding the causes of observed low inter- and high intraspecific
48 genetic variation and furthermore highlight the necessity of taxonomic revisions for various taxa,
49 combining both molecular and morphological data.

50 **Introduction**

51 Aquatic insects are the dominant invertebrate fauna element in most freshwater
52 ecosystems and are enormously variable in morphology, development, physiology, and ecology
53 (Lancaster & Downes, 2013; Dijkstra, Monaghan & Pauls, 2014). Among the hemimetabolous
54 insects, the Heteroptera or true bugs comprise a significant and diverse component of the world's
55 aquatic insect biota (Polhemus & Polhemus, 2007). They are unique as a group because they
56 comprise both aquatic and terrestrial species, whereas other taxa comprise only species that are
57 aquatic during some life stage (e.g. mayflies, stoneflies, or dragonflies), (Wesenberg-Lund, 1943;
58 Lancaster & Downes, 2013; Gullan & Cranston, 2014). Two infraorders, the Gerromorpha and
59 Nepomorpha, are considered as primarily aquatic (Polhemus & Polhemus, 2007; Lancaster &
60 Downes, 2013; Gullan & Cranston, 2014; Henry, 2017). With more than 4,400 described species
61 worldwide (Henry, 2017), aquatic Heteroptera are well-known for utilizing an exceptionally
62 broad range of habitats, from the marine and intertidal to the arctic and high alpine (Polhemus &
63 Polhemus, 2007). They may be found in almost every freshwater biotope. Approximately 120
64 species of the Gerromorpha and 230 species of the Nepomorpha are known from the Palearctic
65 region (Polhemus & Polhemus, 2007). For Germany, 47 species of the Nepomorpha and 22
66 species belonging to the Gerromorpha have been recorded so far (Wachmann, Melber & Deckert,
67 2006; Strauss & Niedringhaus, 2014).

68 Species of the Nepo- and Gerromorpha exhibit numerous morphological and ecological
69 adaptations to their aquatic environment. For instance, nepomorphan true bugs have a
70 streamlined body, natatorial legs and short antennas, whereas gerromorphan species are well-

known for their long slender legs which operate as motive (middle leg) and rudder (hind legs), allowing them to operate on the water surface (e.g. Wesenberg-Lund, 1943; Andersen, 1982; Lancaster & Downes, 2013; Gullan & Cranston, 2014) (Fig. 1). Furthermore, a reduction, loss, and/or polymorphism of wings can be observed in many taxa, which is controlled by environmental conditions and genetic factors (e.g. Zera, Innes & Saks, 1983; Muraji, Miura & Nakasuji, 1989; Spence & Andersen, 1994). With the exception of the omnivorous Corixidae, all aquatic true bugs are predators, feeding on any organism that can be subdued by injection of a venom cocktail consisting of various toxins and proteolytic enzymes (Polhemus & Polhemus, 2007). On the other hand they serve as important prey for fish and other organisms at higher trophic levels (e.g. McCafferty, 1981; Zimmermann & Spence, 1989; Hutchinson, 1993; Klecka 2014; Boda et al., 2015).

Due to their general high abundance in many freshwater systems, their great value as bioindicators of water quality and their unique morphological and ecological specializations for exploiting specialized microhabitats, these groups has been in the focus of entomological and ecological research for a long time (e.g. Hufnagel, Bakonyi & Vásárhelyi, 1999; Polhemus & Polhemus, 2007; Whiteman & Sites, 2008; Skern, Zweimüller & Schiemer, 2010).) However, as a result of their highly similar morphology, the determination of various species is quite difficult and requires the help of experienced taxonomists. Furthermore, it is very difficult or even impossible to identify nymphal stages or females of some species, e.g. some species of the genus *Sigara* Fabricius, 1775. In term of males of the Corixidae, typical diagnostic traits include the shape and size of the tarsus of the first leg (pala), the arrangement of pala pegs, and the morphology of the genitalia (e.g. Jansson, 1986; Savage 1989).

Because aquatic Heteroptera are of high importance for ecological and conservational studies, however, the correct species identification is essential (e.g. Hufnagel, Bakonyi & Vásárhelyi, 1999; Whiteman & Sites, 2008; Skern, Zweimüller & Schiemer, 2010). This is

96 especially true for juveniles and females which can, depending on the life history of a species,
97 dominate within a population over a given period of a year (e.g. Barahona, Millan & Velasco,
98 2005; Pfenning & Poethke, 2006; Wachmann, Melber & Deckert, 2006).

99 Since a few years, new molecular and genomic approaches become more and more
100 popular to overcome possible drawbacks of this traditional way of assessment. Given the recent
101 technological advancement of DNA-based methods, in particular in the field of modern high-
102 throughput technologies (e.g. Heather & Chain, 2016), it is expected that such techniques will
103 gradually replace traditional field and lab procedures in bioassessment studies over the coming
104 ten to fifteen years (Leese et al., 2016). For example, the EU COST Action CA15219 on
105 "Developing new genetic tools for bioassessment of aquatic ecosystems in Europe" – or
106 DNAqua-Net (http://dnaqua.net/) – aims to accelerate the use of DNA-based approaches for the
107 monitoring and assessment of aquatic habitats (Leese et al., 2016). Following these
108 considerations, the analysis of single specimens, bulk samples or environmental DNA will be
109 performed routinely as part of modern species diversity assessment studies (e.g. Yu et al. 2012,
110 Scheffers et al. 2012, Cristescu 2014, Kress et al. 2015, Creer et al. 2016). However, the
111 effectiveness of all these approaches highly relies on comprehensive sequence libraries that act as
112 valid references (e.g. Brandon-Mong et al. 2015, Creer et al. 2016). In this context, DNA
113 barcoding represents undoubtedly the most prominent and popular approach using sequence data
114 for valid species identification (e.g. Hajibabaei et al., 2007; Miller et al., 2016). The concept of
115 DNA barcoding relies on the postulate that the interspecific genetic variation between species is
116 higher than the intraspecific variation (Hebert, Ratnasingham & deWaard, 2003; Hebert et al.,
117 2003). As a consequence, every species is characterized by unique DNA barcode cluster. For
118 animals, an approximately 650 base-pair fragment of the mitochondrial cytochrome *c* oxidase
119 subunit I (COI) gene was proposed as global standard for the identification of unknown
120 specimens in terms of a given classification (*sensu* Hebert, Ratnasingham & deWaard, 2003;

Hebert et al. 2003). However, it should be noted that various problems may affect the use of mitochondrial DNA, e.g., recent speciation events (e.g. Balvín et al. 2012, Raupach et al., 2014), heteroplasmy (e.g. Boyce, Zwick & Aquadro, 1989; Kavar et al., 2006; Kmiec, Woloszynska & Janska, 2006), incomplete lineage sorting (e.g. Petit & Excoffier, 2009), (introgressive) hybridization (e.g. Jansson, 1979a; 1979b; Calabrese, 1982; Spence & Wilcox, 1986; Wilcox & Spence, 1986; Savage & Parkin, 1998; Raupach et al., 2014), the presence of alpha-proteobacteria as *Wolbachia* within terrestrial arthropods (Werren, Zhang & Guo, 1995; Xiao et al., 2011; Werren, Baldo & Clark, 2008), and the existence of mitochondrial pseudogenes (e.g. Leite 2012; Song, Moulton & Whiting, 2014). Nevertheless, a vast number of studies across a broad range of different animals demonstrate the efficiency of DNA barcoding (e.g. Spelda et al., 2011; Hausmann et al., 2013; Hendrich et al., 2015; Lin, Stur & Ekrem 2015; Raupach et al., 2015; Barco et al., 2016; Coddington et al., 2016; Morinière et al., 2017).

Despite the fact that more than 45,000 species of true bugs have been described worldwide until now (Henry, 2017), the number of studies analyzing the usefulness of DNA barcodes to discriminate species of this highly diverse insect taxon is still low. Some studies focus on selected species (Rebijith et al., 2012; Zhou et al., 2012; Lis, Lis & Ziaja, 2013), other on specific families (Grebennikov & Heiss, 2014; Kaur & Sharma, 2017), whereas four publications provide a greater representation of various families (Park et al., 2011; Jung, Duwal & Lee, 2011; Raupach et al., 2014; Tembe, Shouche & Ghate, 2014). However, all these studies focused primarily on terrestrial species, analyzing just small number of species belonging to the Gerromorpha and/or Nepomorpha (Park et al., 2011; Jung, Duwal & Lee, 2011; Raupach et al., 2014). To our knowledge, only two publications analyzed aquatic true bugs specifically until now: Castanhole et al. (2013) investigated the variability of 17 barcode sequences of a few species from Brazil, whereas Ebong et al. (2016) successfully tested the usefulness of DNA barcodes to discriminate various species from Cameroon.

The aim of this study was to build-up a baseline for a comprehensive library of DNA barcodes for aquatic Heteroptera (Gerromorpha, Nepomorpha) of Central Europe with a focus on the German fauna and to test the efficiency of DNA barcodes to discriminate the analyzed species. Moreover, our study provides the first thorough molecular study of the aquatic Heteroptera of Germany. In doing so, we analyzed more than 700 DNA barcodes representing more than 60 species. Our library included various morphological similar taxa, e.g. species of the genera *Sigara* Fabricius, 1775 and *Notonecta* Linnaeus, 1758 as well as water striders of the genus *Gerris* Fabricius, 1794 from different localities in Germany. In addition to this we added various specimens from other European countries for comparison, e.g. specimens of the expansive small-bodied backswimmer *Anisops sardeus* Herrich-Schaeffer, 1849 (Berchi, 2011; Klementová & Svitok, 2014).

Material & Methods

Species collection and identification

All analyzed Gerromorpha and Nepomorpha were collected between the years 2003 and 2017. Most specimens were adults (n = 584; 96.8%). Specimens were stored in ethanol (96%) immediately after collection and identified by some of the authors (NH, MMG, MJR, PS, RN) using various keys (Nieser 1982; Jansson, 1986; Savage, 1989; Stoffelen et al., 2013; Strauss & Niedringhaus, 2014) based on the most recent taxonomic classification (Aukema & Rieger, 1995). All specimens were carefully checked multiple times by some of the authors in order to prevent a misidentification. For our analysis we also included 109 DNA barcodes of aquatic bugs that were part of a previous barcoding study of true bugs of Central Europe and in which species identification was verified by the authors for comparison (Raupach et al., 2014). Most of the analyzed bug specimens were collected in Germany (n = 616: 86.5%), but various studied individuals were sampled in Austria (37; 5.2%), Greece (20; 2.8%), Spain (16; 2.3%),

Switzerland (8; 1.1%), Italy (7; 1.0%), Poland (6; 0.8%), and Portugal (2; 0.3%) for comparison (Fig. 2). In this context we also included specimens from four species that are not recorded for Germany: I. *Anisops sardeus* Herrich-Schaeffer, 1849, (n = 5) from Greece, II. *Mesovelia vittigera* Horváth, 1895 (n = 4) from Greece, III. *Sigara dorsalis* (Leach, 1817) (n = 1) from Switzerland, and IV.*Velia currens* (Fabricius, 1794) (n = 3) from Switzerland. The total data set consisted of 712 DNA barcodes with 63 species that are documented for Germany. The number of analyzed specimens per species ranged from one (8 species) to a maximum of 41 for *Notonecta glauca* Linnaeus, 1758.

DNA barcode amplification, sequencing and data depository

The DNA barcode amplification was either performed at the German Centre of Biodiversity Research (Senckenberg am Meer) in Wilhelmshaven, the Carl von Ossietzky University of Oldenburg, or the Bavarian State Collection of Zoology in Munich (SNSB-ZSM). Following the guidelines of DNA barcoding studies (Ratnasingham & Hebert, 2007), all species were documented by photographs before molecular work started. In the majority of the studied animals, all legs of one side of the body were dissected and used for DNA extraction. In case of larger specimens of the genera *Notonecta* Linneaus, 1758, *Ilyocoris* Stål, 1861, *Ranatra* Fabricius, 1790, *Nepa* Linnaeus, 1758, and *Aphelocheirus* Westwood, 1833, however, only one leg was used. For some very small specimens with a body length <3 mm, e.g. species of the genus *Microvelia* Westwood, 1834, complete specimens were used for DNA extraction. All voucher specimens as well as DNA extracts are stored in a local collection at the Carl von Ossietzky University of Oldenburg.

The DNA extraction was performed using the NucleoSpin Tissue Kit by Macherey and Nagel (Düren, Germany), following the extraction protocol. Polymerase chain reaction (PCR) has been used for amplifying the COI barcode fragment by using the established primer pairs

LCO1480/HCO2198 (Folmer et al., 1994), LCO1480/NANCY (Simon et al., 1994), jgLCO1490/jgHCO2198 (Geller et al., 2013), or LepF1/LepR1 (Hebert et al., 2004) for most specimens. For various specimens of the Gerromorpha, however, a new specific forward primer HETF1 (5´-ATG AAT TAT TCG AAT TGA AAT AGG-3´) was designed and used in combination with HCO2198 for amplification, resulting in a somewhat smaller fragment with a length of 579 base pairs (bp) of the barcode region. All primers were modified with M13 forward and reverse tails to provide defined base sequences for sequencing (see Ivanova et al., 2007; Khalaji-Pirbalouty & Raupach, 2014).

Barcode amplicons were amplified using illustraTM puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) in a total volume of 20 µl, containing 17.5 µl sterile molecular grade H_2O, 2 µl DNA template with an DNA amount between 2 to 150 ng/µl and 0.25 µl of each primer (20 pmol/µl). The PCR thermal conditions included an initial denaturation at 94 °C (5 min), followed by 38 cycles at 94 °C (denaturation, 45 s), 48 °C (annealing, 45 s), 72 °C (extension, 80 s), and a final extension step at 72 °C (7 min). All PCR amplification reactions were conducted using an Eppendorf Mastercycler Pro system (Eppendorf, Hamburg, Germany). Negative and positive controls were included with each round of reactions. Two µl of the amplified products were verified for size conformity by electrophoresis in a 1% agarose gel with GelRed or SYBR Green using commercial DNA size standards, whereas the remaining PCR product was purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Purified PCR products were cycle-sequenced and sequenced in both directions at a contract sequencing facility (GATC, Konstanz, Germany) using the given M13 tail sequences. Double stranded sequences became assembled and checked for mitochondrial pseudogenes (numts) analyzing the presence of stop codons, frameshifts as well as double peaks in chromatograms with the Geneious program package version 7.0.4 (Biomatters, Auckland, New Zealand) (Kearse et al., 2012). Ambiguous parts at the 5´-end or 3´-end of the sequences were

removed. For verification, BLAST (nBLAST, search set: others, program selection: megablast) and/or BOLD (identification engine; species level barcode records) searches were performed to confirm the identity of all new sequences as bug sequences based on already published sequences.

Detailed voucher information, taxonomic classifications, photos, DNA barcode sequences, used primer pairs and trace files (including their quality) are publicly accessible through the public data set "DS-BAHCE Barcoding Aquatic Heteroptera of Central Europe" (Dataset ID: dx.doi.org/10.5883/DS-BAHCE) on the Barcode of Life Data Systems workbench (BOLD; www.boldsystems.org) (Ratnasingham & Hebert, 2007). All new barcode data were also deposited in GenBank (MG665389-MG665993).

DNA barcode analysis

We analyzed intra- and interspecific distances of the studied aquatic Heteroptera using the provided analytical tools of the BOLD workbench (align sequences: BOLD aligner; ambiguous base/gap handling: pairwise deletion) based on the Kimura 2-parameter model of sequence evolution (K2P; Kimura, 1980). Furthermore, all analyzed COI sequences became subject to the Barcode Index Number (BIN) system implemented in BOLD which clusters DNA barcodes in order to generate operational taxonomic units that closely correspond to species (Ratnasingham & Hebert, 2013). We used a recommended threshold of 2.2% for a rough differentiation of intraspecific as well as interspecific K2P distances (Ratnasingham & Hebert, 2013).

A Neighbor Joining cluster analysis (NJ; Saitou & Nei, 1987) was performed for all studied species for a graphical representation of the genetic differences between sequences and clusters of sequences using MEGA v7.0.18 (Kumar, Stecher & Tamura, 2016). The K2P model was chosen as the model for sequence evolution for comparison purposes. For validation, non-parametric bootstrap support values were obtained by resampling and analyzing 1,000 replicates

(Felsenstein, 1985). All analysis were based on an alignment that was generated using MUSCLE (Edgar, 2004) implemented in MEGA v7.0.18 for all studied barcode sequences. Additionally, statistical maximum parsimony networks were constructed exemplarily for species with interspecific distances ranging from zero to 1% (see Table 1) by using TCS networks (Clement, Posada & Crandall, 2002) as part of the software package of PopArt v.1.7 (Leigh & Bryant, 2015). Such networks allow the identification of haplotype sharing between species as a consequence of recent speciation or on-going hybridization processes (e.g. Raupach et al., 2010; Raupach et al., 2014).

Results

Our analyzed DNA barcode library comprised 63 species that are documented for Germany, representing 91% of the known aquatic bug species diversity of this country (Nepomorpha: $n = 43$ (92%); Gerromorpha: $n = 20$ (91%)), and additional four species that were collected in other countries and not recorded for Germany. In total, we generated 603 new barcodes of 64 species. The complete alignment of all analyzed sequences ($n = 712$) had a length of 658 bp, with fragments lengths ranging from a minimum of 366 bp to the full barcode fragment size of 658 bp. For some studied specimens of *Cymatia coleoptrata* (Fabricius, 1777) ($n = 22$), our analysis revealed two characteristic deletions of 39 (alignment position: 110 – 148) and nine nucleotides (629 – 637) for all studied specimens (Fig. S1). Average base frequencies were A = 32%, C = 17%, G = 16%, and T = 35%. Intraspecific distances ranged from zero to maximum values of 8.3% (*Plea minutissima* Leach, 1817) and 9.44% (*Cymatia coleoptrata*) (Table 1). Maximum intraspecific pairwise distances with values >2.2% were found for 11 species (Table 1). In terms of interspecific divergence, values ranged from zero to 18.58%, with 18 species pairs having values <2.2% (Table 1). We found interspecific distances below 1% for 9 species. For eight of these species, only one barcode sequence was generated (Table 1). Unique

BINs were recorded for 55 species, whereas two BINs were identified for 10 species (Table 1). For two species that were represented only by one specimen, namely *Arctocorisa germari* (Fieber, 1848) and *Corixa dentipes* Thompson, 1869, our sequences did not have the required fragment length of at least 400 bp to fulfill the criteria for BIN assignment. As consequence, no BINs were available for these two species.

Our NJ analysis based on K2P distances revealed two large and distinct clusters, separating all analyzed Gerromorpha and all Nepomorpha specimens from each other (Fig. S2). For a better presentation, the topology has been split on this basis and shown in two figures (Gerromorpha: Fig. 3, Nepomorpha: Fig. 4). We found non-overlapping clusters with bootstrap values >90% for 57 species (85%) (Fig. 3; 4). Of the analyzed 59 species with more than one specimen, 52 (88%) were monophyletic, 3 (5%) paraphyletic, and 4 (7%) polyphyletic (Table 1, Fig S2).

The statistical maximum parsimony network analysis of species with interspecific distances below 1% revealed a close relationship between *Gerris asper* (Fieber, 1860) (n = 1) and *Gerris lateralis* Schummel, 1832 (n = 2) (Fig. 5). We found three haplotypes with a frequency of 1 (singletons) that were separated by only one or two mutational steps, with haplotype h1 (*Gerris asper*) connected with h2 (*Gerris lateralis*), which was in turn connected with haplotype h3 (*Gerris lateralis*). A similar situation was observed for *Sigara limitata* (Fieber, 1848) (n = 2) and *Sigara semistriata* (Fieber, 1848) (n = 5) (Fig. 5). Three different haplotypes were identified, with h1 representing all studied specimens of *Sigara semistriata*. Both unique haplotypes of *Sigara limitata* (h2, h3) were directly connected to this haplotype by two or three mutational steps. In the case of *Callicorixa praeusta* (Fieber, 1848) (n = 23) and *Callicorixa producta* (Reuter, 1880) (n = 1) we found five different haplotypes (Fig. 5), with h1 representing the dominant haplotype which includes 19 specimens of *Callicorixa praeusta* and the only specimen of *Callicorixa producta*. All other four haplotypes (h2-h5) were only scored in one specimen and connected

with h1 by one or two mutational steps. A much more complex network was revealed for *Sigara distincta* (Fieber, 1848) (*n* = 7), *Sigara falleni* (Fieber, 1848) (*n* = 12), and *Sigara iactans* Jansson, 1983 (*n* = 12) (Fig. 6). We identified 16 different haplotypes in total, with six haplotypes (h1-h6) shared by more than one specimen. Three of these haplotypes (h2, h3, h5) were shared by specimens of *Sigara falleni* and *Sigara iactans*. Furthermore, haplotypes of both previously mentioned species were randomly distributed within the network. In many cases, haplotypes of *Sigara falleni* were separated merely by two mutational steps from haplotypes of *Sigara iactans* (e.g. h6 and h13) and vice versa. We found four singletons for *Sigara falleni* and five for *Sigara iactans*. In contrast to these two species, we identified only two haplotypes (h1, h8) for the seven analyzed specimens of *Sigara distincta*. Moreover, most specimens (*n* = 6) were identical (h1) and located at the periphery of the network. The other haplotype (h8), a singleton collected among others at Apen (Lower Saxony), was separated by more than 25 mutational steps from the network and represents the most isolated haplotype in this network by far. Therefore, *Sigara distincta* shared no haplotypes with other species.

Discussion

Our comprehensive DNA barcode library represents an important step for the molecular characterization of the freshwater fauna in Central Europe and adjacent regions. As COI sequences are used routinely in phylogeographic, phylogenetic and evolutionary studies as well, our data can be also implemented in projects analyzing the genetic variation of species in relation to historical, geographical and ecological factors (Galactos, Cognato & Sperling, 2002; Damgaard, 2005; Damgaard, 2008b; Gagnon & Turgeon 2010; Ye et al., 2016). Unique BINs were found for 55 species, allowing a valid identification of 82% of the analyzed 67 species. Distinct and monophyletic lineages, however, were revealed for 52 species (78%). Our study also indicates the need of further detailed taxonomic revisions, using state-of-the-art methods for a

316 fine-scaled characterization (Raupach et al. 2016). This is especially true for the species-rich
317 family Corixidae. In the following we will discuss noticeable species with high intraspecific
318 and/or low interspecific distances more in detail.

319 **Interspecific K2P distances with values below 2.2%**

320 The efficiency of DNA barcoding highly depends on distinct mitochondrial lineages,
321 ideally coupled with moderate to high genetic interspecific distances. If sister species, however,
322 have low interspecific distances and haplotype sharing as a result of a recent ancestry and/or
323 ongoing gene flow, DNA barcoding will fail (e.g. Tautz et al., 2003; Frezal & Leblois, 2008;
324 Raupach & Radulovici, 2015). For the analyzed species of the Gerromorpha and Nepomorpha,
325 minimum interspecific K2P distances with values below 2.2% were found for 18 species (Table
326 1). Distance values ranged from 0% (four species: *Callicorixa praeusta* (Fieber, 1848),
327 *Callicorixa producta* (Reuter, 1880), *Sigara falleni* (Fieber, 1848), *Sigara iactans* Jansson, 1983)
328 to 2.11% (*Sigara venusta* (Douglas & Scott, 1869)). Distinct monophyletic clusters were revealed
329 for *Notonecta obliqua* Thunberg, 1787 and *Notonecta glauca* Linnaeus, 1758 (1.08%), *Notonecta*
330 *lutea* Müller, 1776 and *Notonecta reuteri* Hungerford, 1928 (1.24%), *Sigara dorsalis* (Leach,
331 1817) and *Sigara striata* (Linnaeus, 1758) (1.71%) (but see Savage and Parkin, 1998), and
332 *Sigara venusta* (Douglas & Scott, 1869) and *Sigara limitata* (Fieber, 1848)/*Sigara semistriata*
333 (Fieber, 1848) (2.11%), indicating a close relationship of these species pairs with distinct lineages
334 (Table 1). Furthermore, the analyzed specimen of *Arctocorisa germari* (Fieber, 1848) was nested
335 in the paraphyletic cluster of *Arctocorisa carinata* (C. R. Sahlberg, 1819) (1.03%) (Fig. S1). In
336 this context it should be noted that experimental crosses gave viable hybrids between both
337 *Arctocorisa* species with intermediate characters (Jansson, 1979). These examples show that
338 recent speciation events as well as hybridization may represent important processes in these
339 groups. Future studies including more specimens and other genetic markers should be conducted

to resolve the eco-evolutionary events leading to the low interspecific variation. Species pairs with interspecific K2P distances <1% will be discussed more in detail below.

Species pairs with interspecific distances below 1%

I. *Gerris asper* (Fieber, 1860) and *Gerris lateralis* Schummel, 1832

From a morphological perspective, both species are very similar (e.g. Wagner & Zimmermann, 1955; Wachmann, Melber & Deckert, 2006). Not surprisingly, *Gerris asper* is suggested as a south-eastern vicariant of its boreo-montane sister species *Gerris lateralis* (Jeziorski et al., 2012). Whereas *Gerris lateralis* has a distribution ranging from Europe to the Far East of Russia, *Gerris asper* is found in Southern and Central Europe, extending to Afghanistan (Jeziorski et al., 2012). In spite of the fact that our sample sizes were very small (*Gerris asper*: $n = 1$, *Gerris lateralis*: $n = 2$), our molecular data set clearly support the proposed close relationship of both water striders species (Fig. 5, Table 1). Future studies including more specimens covering a larger geographic range are needed to test whether both taxa represent distinct lineages or hybridization still takes place as it is known from other species of this genus (e.g. Calabrese, 1982).

II. *Sigara limitata* (Fieber, 1848) and *Sigara semistriata* (Fieber, 1848)

Both species belong to the subgenus *Retrocorixa* Walton, 1940 and have a similar distribution, ranging from Europe eastwards to Siberia (Jansson, 1986; Wachmann, Melber & Deckert, 2006; Coulianos, Økland & Økland, 2008). A close relationship as it has been indicated by our data has not been proposed yet. In contrast to our results, morphological characters suggest *Sigara venusta* (Douglas & Scott, 1869) as sister species of *Sigara semistriata* (see Jansson, 1986). As part of our study, *Sigara venusta* represents the sister species of *Sigara limitata* and *Sigara semistriata* with a distance of 2.11% (Fig. 5, Table 1). Due to the fact that

neither *Sigara limitata* nor *Sigara semistriata* were monophyletic and the observed interspecific distances were very low (0.15%) (Table 1), we suggest a recent ancestry of both species. Hybrids are currently not known. Future studies are needed to verify this hypothesis.

III. *Callicorixa praeusta* (Fieber, 1848) and *Callicorixa producta* (Reuter, 1880)

The genus *Callicorixa* White, 1873 includes five medium sized species (6 – 8 mm) that are recorded for Europe, with two species documented in Central Europe. Specimens of *Callicorixa praeusta* can be found throughout most Europe expect the Mediterranean region reaching to the Far East of Russia, whereas the distribution of *Callicorixa producta* ranges from the Northern parts of Central Europe to Fennoscandia, Northern Russia, Kazakhstan, Mongolia, and Siberia (Jansson, 1986; Wachmann, Melber & Deckert, 2006; Coulianos, Økland & Økland, 2008). Most identification keys for this genus rely largely on the shape and intensity of dark areas of the hind tarsus 1 (Jansson, 1986; Savage 1989; Strauss & Niedringhaus, 2014). While this morphological trait is fairly good for the determination of most typical specimens, existing variation is rather wide, making it unreliable in many cases (Jansson, 1986). Similar to other species, our DNA barcode data give evidence for a recent ancestry or ongoing gene flow between *Callicorixa praeusta* and *Callicorixa producta* (Fig. 5). However, only one (female) specimen of *Callicorixa producta* was available, demonstrating the need for more detailed studies to clarify the underlying processes.

IV. *Sigara distincta* (Fieber, 1848), *Sigara falleni* (Fieber, 1848), and *Sigara iactans* Jansson, 1983

Some decades ago, a comprehensive revision revealed that the well-known species *Sigara falleni* of the subgenus *Subsigara* Stichel, 1935 was actually a mixture of four closely related and highly similar species, including *Sigara iactans* (see Jansson, 1983). Whereas the identification

386 of females is not always reliable, males of both species can be recognized by the shape of their
387 pala: specimens of *Sigara falleni* are characterized by triangular pala, whereas trapezoidal pala
388 are found for *Sigara iactans* (Jansson, 1983; 1986). Intermediate specimens, however, have been
389 also documented and indicate on-going hybridization between both species (Jansson, 1983;
390 1986). Water bugs of *Sigara distincta* are found from the British Isles through North and Central
391 Europe to Asia as far as East Siberia and Mongolia (Jansson, 1986; Savage, 1989; Coulianos,
392 Økland & Økland, 2008). A similar distribution is known for *Sigara falleni*, ranging throughout
393 most of Europe eastwards to Siberia and China (Jansson, 1986; Savage, 1989; Coulianos, Økland
394 & Økland, 2008). In contrast to both previous species, *Sigara iactans* is found in two disjunct
395 areas, one in North and Central Europe, and the other in Southeastern Europe (Jansson, 1986,
396 Wachmann, Melber & Deckert, 2006). Our DNA barcode data revealed multiple haplotype
397 sharing between *Sigara falleni* and *Sigara iactans*, supporting the close relationship and on-going
398 hybridization between both species (Fig. 6). Beside this, our results revealed a close relationship
399 of *Sigara (Subsigara) distincta* with *Sigara falleni* and *Sigara iactans*, as it has been discussed in
400 the past also (Jansson, 1986). However, we found no shared haplotypes yet. Additional studies
401 involving more specimens of a larger geographic region are needed to validate the species status
402 within this subgenus.

403 **Intraspecific K2P distances with values >2.2%**

404 Various phenomena can generate distinct lineages within DNA barcode data, e.g.
405 phylogeographic processes (e.g. Andersen et al., 2000; Damgaard 2005; 2008b; Ye et al., 2016),
406 the presence of maternally inherited endosymbionts as *Wolbachia* (e.g. Lis, Maryańska-
407 Nadachowska & Kajtoch, 2015), or the existence of cryptic species (e.g. Paterson et al., 2016; Jiu
408 et al., 2017). In this context, we found 11 species with intraspecific K2P distances >2.2%, ranging
409 from 2.32% (*Mesovelia vittigera* Horváth, 1895) to a maximum of 9.44 (*Cymatia coleoptrata*

(Fabricius, 1777)). For most species, excluding *Sigara iactans* (2.67%), *Sigara falleni* (3.37%), and *Sigara distincta* (5.77%) (see discussion above), we are currently unable to clarify the background of the observed high nucleotide distances and distinct lineages based on the given data set. However, exceptionally high intraspecific distances with values >8% were found within the pygmy backswimmer *Plea minutissima* Leach, 1817 (8.3%) and *Cymatia coleoptrata* (Fabricius, 1777) (9.44%) (Table 1). Both will be discussed more in detail.

Small and cryptic: two highly distinct DNA barcode clusters within *Plea minutissima* Leach, 1817

Pygmy backswimmers are small bugs, usually less than 3.5 mm in length and confine themselves to the vegetation in which they hide and where they prey on mosquito larvae and other small arthropods (Schuh & Slater, 1995). For Europe, only one species of the Pleidae is recorded, namely *Plea minutissima*. As part of our study we found two distinct lineages within the sixteen analyzed specimens with high distances ranging from 8.1 to 8.3%. Both lineages were supported by high bootstrap values (99%) (Fig. 7). Most specimens of lineage A (n = 8) were found in Brandenburg and Bavaria, but also two specimens were collected in Lower Saxony (Jaderberg). In contrast to this, all specimens of lineage B (n = 8) were collected in Lower Saxony (Jaderberg, Lingen, Norderney). Whether this surprisingly high molecular diversity is a result of effects as incomplete lineage sorting (e.g. Damgaard, 2008) or whether we found evidence for the existence of two sibling species (e.g. Damgaard, 2005), is not within the scope of this study but clearly needs further investigation.

A currently unknown species of the genus *Cymatia* Flor, 1860?

For the genus *Cymatia*, three European species are documented so far: *Cymatia coleoptrata* (Fabricius, 1777), *Cymatia bonsdorffii* (C. R. Sahlberg, 1819), and *Cymatia*

433 *rogenhoferi* (Fieber, 1864). In terms of a morphological identification, all species can be
434 identified according to their size and hemelytral patterns without doubt (Janssen 1986, Stoffelen
435 et al., 2013). Our study revealed two distinct lineages within the analyzed specimens of *Cymatia*
436 *coleoptrata* (lineage A and B), with a K2P distances ranging from 9.13 – 9.42% and bootstrap
437 support values of 99% (Fig. 8). Whereas lineage A includes 22 specimens from Lower Saxony (*n*
438 = 21, Lingen) and Baden-Württemberg (*n* = 1, Wolperstwende), lineage B contains two
439 specimens that were collected in Brandenburg (Voßberg). Both specimens of lineage B were
440 small adult males, with a body size between 4.3 and 4.5 mm, and were identified using
441 morphological traits as *Cymatia coleoptrata* at first sight. Interestingly, their barcode sequences
442 did not have the characteristic nucleotide deletions of this species (Fig. S1). Furthermore, we
443 found no other similar sequences using the BOLD identification engine (Best ID: *Cymatia*
444 *coleoptrata*) (date of request: 2017-11-20). Unfortunately, both *Cymatia* vouchers got lost,
445 preventing a closer reanalysis of the specimens. Nevertheless, our results should motivate
446 heteropterologists to study more specimens of this genus, in particular from the Eastern parts of
447 Germany, in order to verify the presence of this putative new species.

448 **Conclusion**

449 In this study we lay the foundations for a comprehensive DNA barcode data set for the
450 aquatic Heteroptera in Central Europe and adjacent regions, which will act as useful reference
451 library for freshwater bioassessment studies using modern high-throughput sequencing
452 technologies. Unique BINs were revealed for 55 species determined species, representing of 82%
453 of the analyzed 67 species. Furthermore, monophyletic lineages were found for 52 species (78%).
454 Nevertheless, our molecular data highlights discordance between the generally accepted but
455 exclusively morphologically-based taxonomy and observed molecular diversity within some
456 species of the Gerromorpha and Nepomorpha. The analysis of additional specimens from other

localities and of other molecular markers, for example microsatellites or SNPs, will give us more insights into the taxonomic status of these species as well as in the eco-evolutionary processes underlying the observed genetic patterns. However, it should be kept in mind that the traditional aims of taxonomy are unchanged and include various aspects, e.g. detailed high-quality descriptions and delimitation of species, a classification that reflects evolution, a dynamic nomenclature, and fast and reliable identification tools. Therefore, our DNA barcode library may be considered as a promoter for such studies.

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Figure 1(on next page)

Representative images of analyzed aquatic bug species.

A: *Nepa cinerea* Linnaeus, 1758 (Nepidae), **B:** *Corixa affinis* Leach, 1817 (Corixidae), **C:** *Sigara (Subsigara) scotti* (Douglas & Scott, 1868) (Corixidae), **D:** *Ilyocoris cimicoides* (Linnaeus, 1758) (Naucoridae), **E:** *Aphelocheirus aestivalis* (Fabricius, 1794) (Aphelocheiridae), **F:** *Notonecta viridis* Delcourt, 1909 (Notonectidae), **G:** *Plea minutissima* Leach, 1817 (Pleidae), **H:** *Mesovelia furcata* Mulsant & Rey, 1852 (Mesovelidae), **I:** *Hydrometra gracilenta* Horváth, 1899 (Hydrometridae), **J:** *Hebrus ruficeps* Thompson, 1871 (Hebridae), **K:** *Velia caprai* Tamanini, 1947 (Velidae), **L:** *Gerris costae* (Herrich-Schaeffer, 1850) (Gerridae). Scale bars = 1 mm. All images were obtained from www.corisa.de

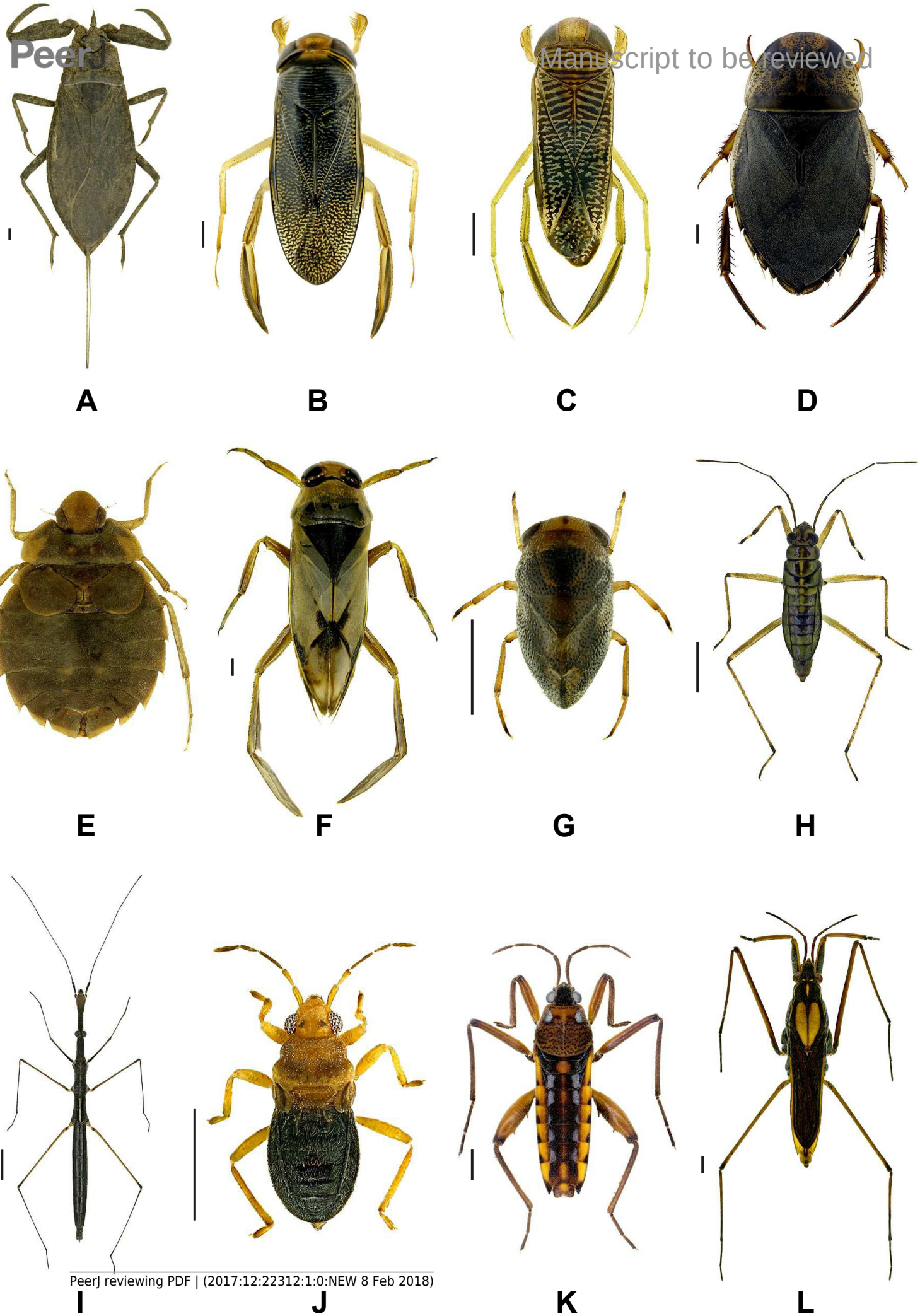

Figure 2

Sampling sites of the studied aquatic true bugs (Gerromorpha, Nepomorpha) across Europe.

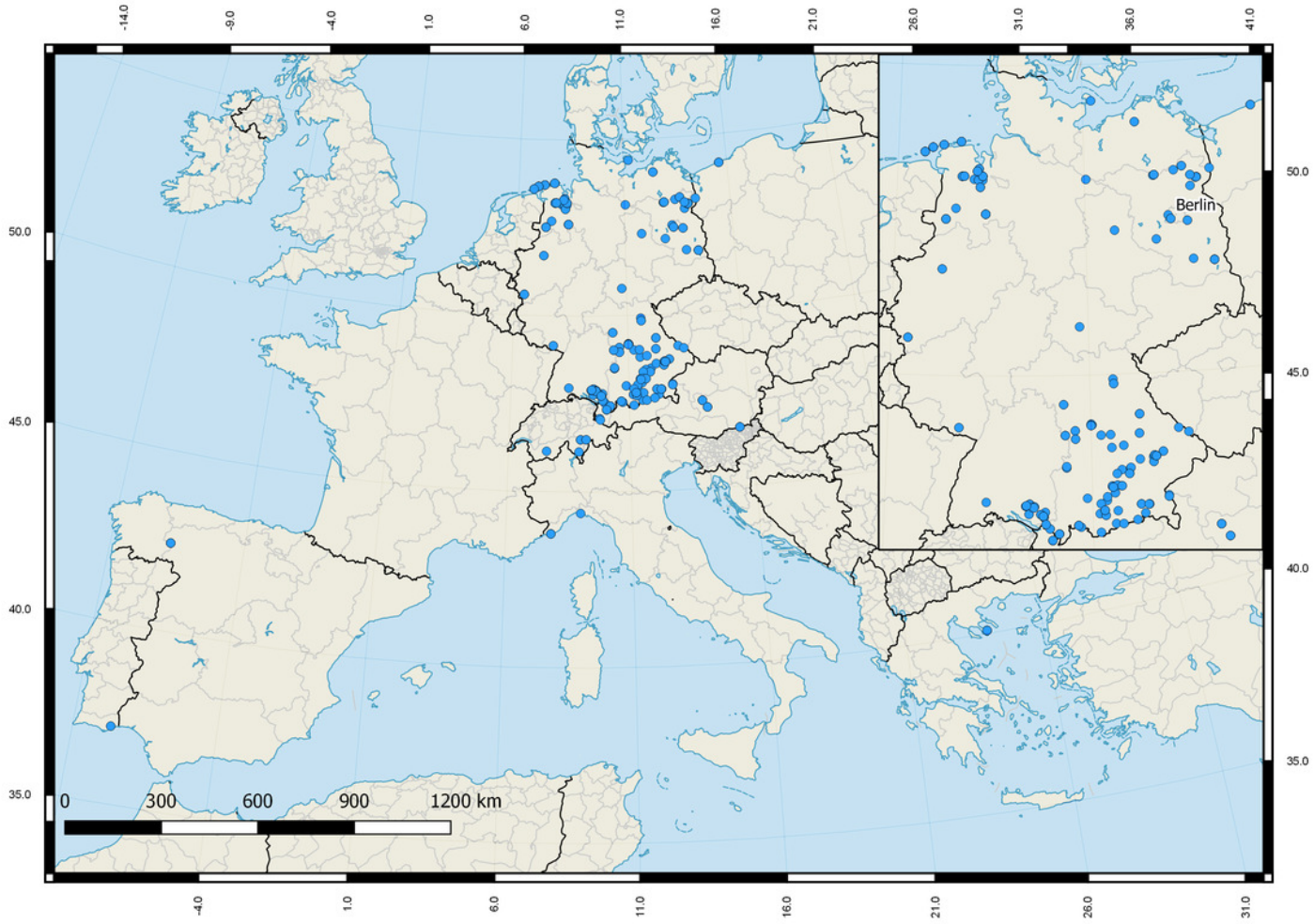

Figure 3(on next page)

Neighbor Joining (NJ) topology of the analyzed species of the Gerromorpha based on Kimura 2-parameter distance.

Triangles indicate the relative number of individual's sampled (height) and sequence divergence (width). Blue triangles indicate species with intraspecific maximum pairwise distances >2.2%, red triangles species pairs with interspecific distances <2.2%. Numbers next to nodes represent non-parametric bootstrap values >80% (1,000 replicates). Asterisks indicate species not recorded in Germany. All images were obtained from www.corisa.de

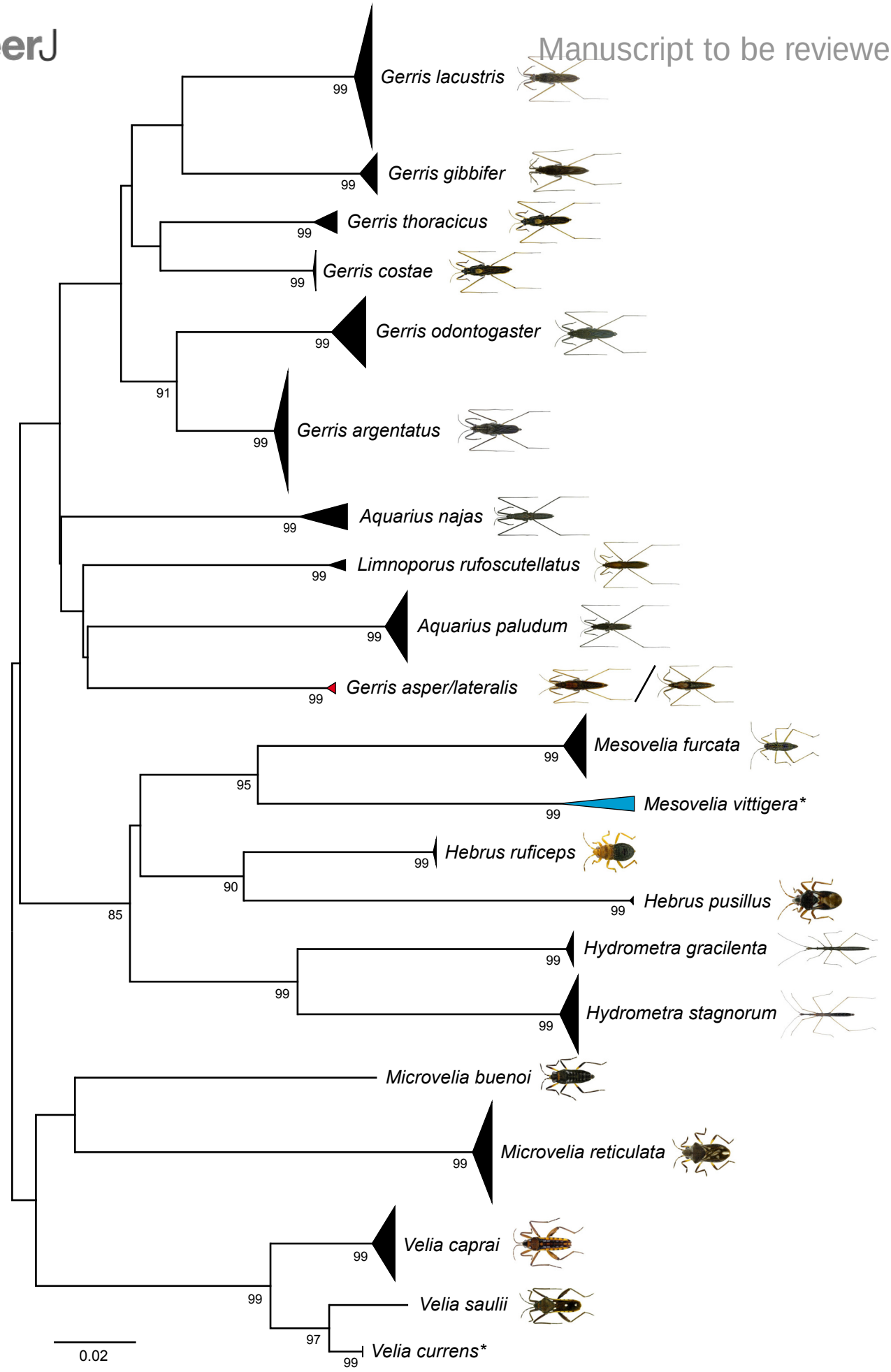
Gerris lacustris
Gerris gibbifer
Gerris thoracicus
Gerris costae
Gerris odontogaster
Gerris argentatus
Aquarius najas
Limnoporus rufoscutellatus
Aquarius paludum
Gerris asper/lateralis
Mesovelia furcata
Mesovelia vittigera*
Hebrus ruficeps
Hebrus pusillus
Hydrometra gracilenta
Hydrometra stagnorum
Microvelia buenoi
Microvelia reticulata
Velia caprai
Velia saulii
Velia currens*
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Figure 4(on next page)

Neighbor Joining (NJ) topology of the analyzed species of the Nepomorpha based on Kimura 2-parameter distance.

Triangles indicate the relative number of individual's sampled (height) and sequence divergence (width). Blue triangles indicate species with intraspecific maximum pairwise distances >2.2%, red triangles species with interspecific distances <2.2%. Numbers next to nodes represent non-parametric bootstrap values >=80% (1,000 replicates). Asterisks indicate species not recorded in Germany. All images were obtained from www.corisa.de

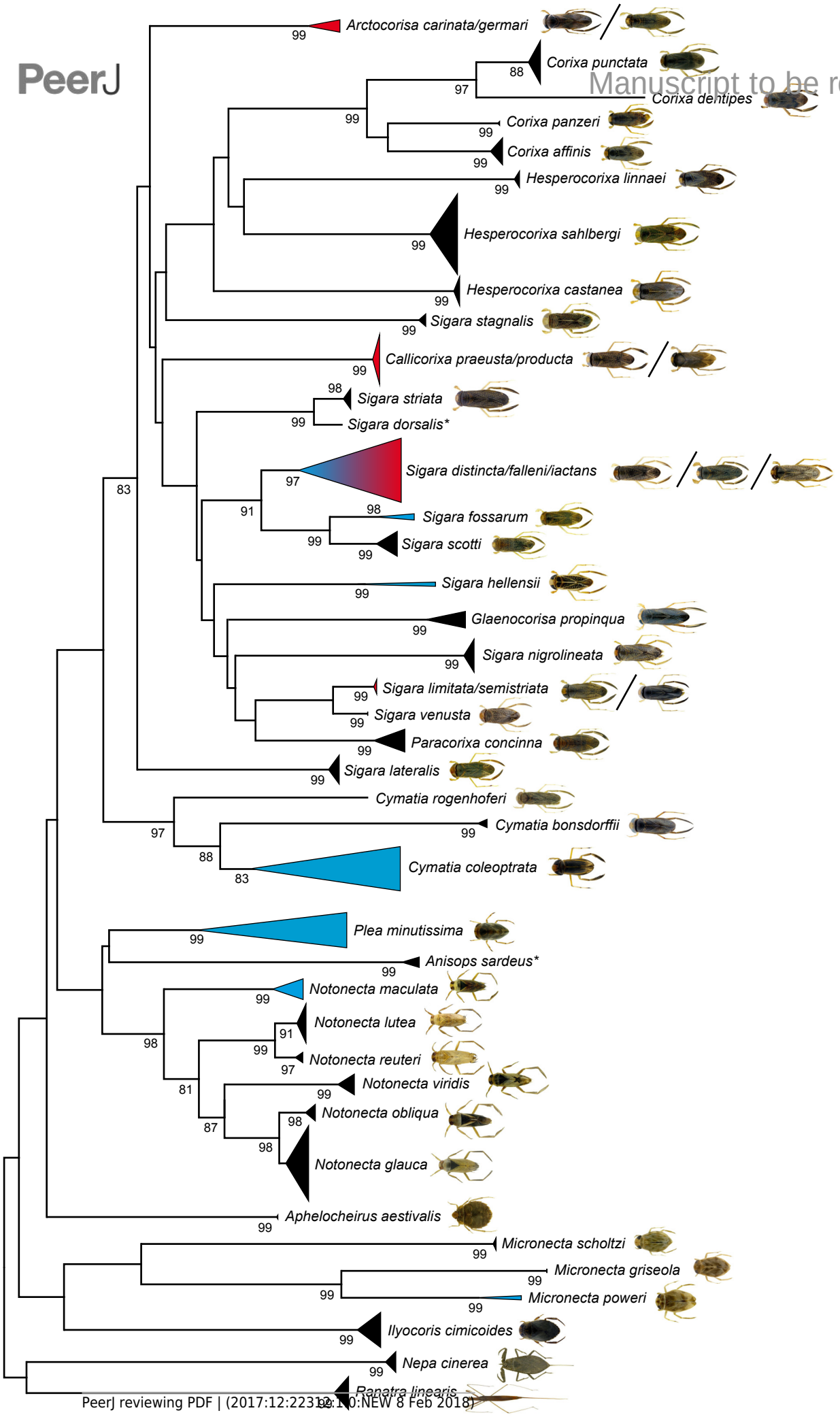

Figure 5(on next page)

Maximum statistical parsimony network of various species of the Gerromorpha and Nepomorpha with interspecific K2P-based distances of COI sequences <1%

A *Gerris asper* (Fieber, 1860) (*n* = 1) and *Gerris lateralis* Schummel, 1832 (*n* = 2); B *Sigara limitata* (Fieber, 1848) (*n* = 2) and *Sigara semistriata* (Fieber, 1848) (*n* = 5); C *Callicorixa praeusta* (Fieber, 1848) (*n* = 23) and *Callicorixa producta* (Reuter, 1880) (*n* = 1). Used settings included default settings for connection steps whereas gaps were treated as fifth state. Each line represents a single mutational change whereas small black dots and small black lines indicate missing haplotypes. The diameter of the circles is proportional to the number of haplotypes sampled (see open half circles with numbers). Color codes were given for each species. Scale bars = 1 mm. Aquatic bug images were obtained from www.corisa.de

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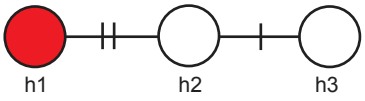
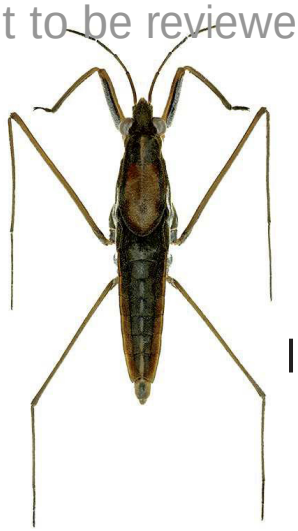
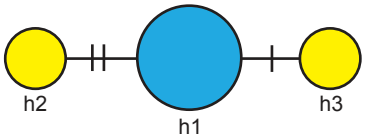


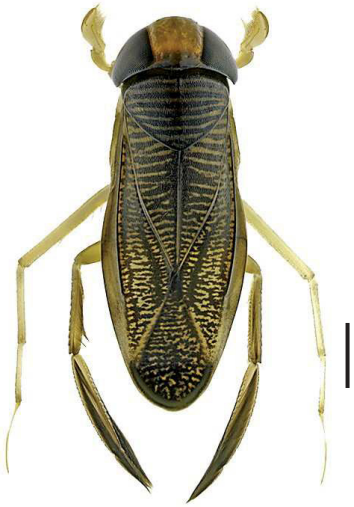


Gerris asper (Fieber, 1860)
(*n* = 1)

Gerris lateralis Schummel, 1832
(*n* = 2)

B

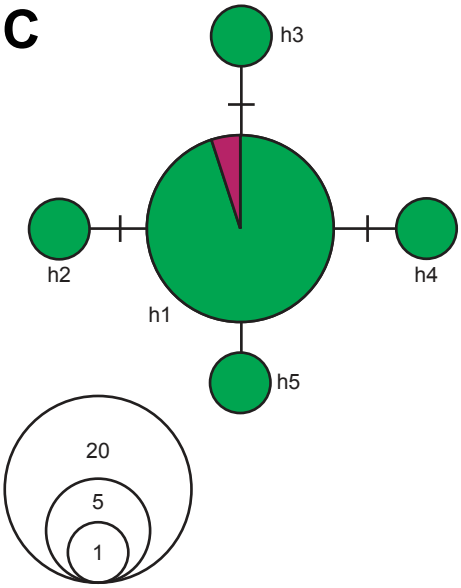




Sigara limitata (Fieber, 1848)
(*n* = 2)

Sigara semistriata (Fieber, 1848)
(*n* = 5)

C



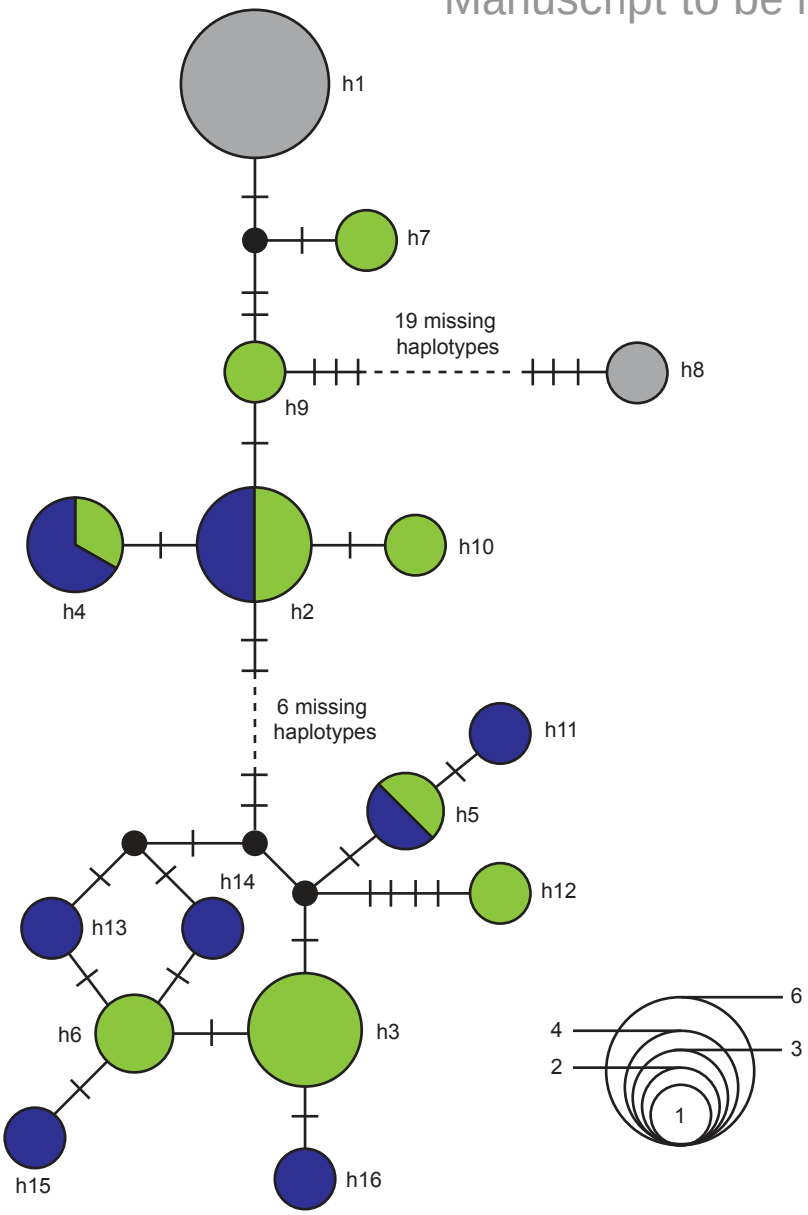
Callicorixa praeusta (Fieber, 1848)
(*n* = 23)

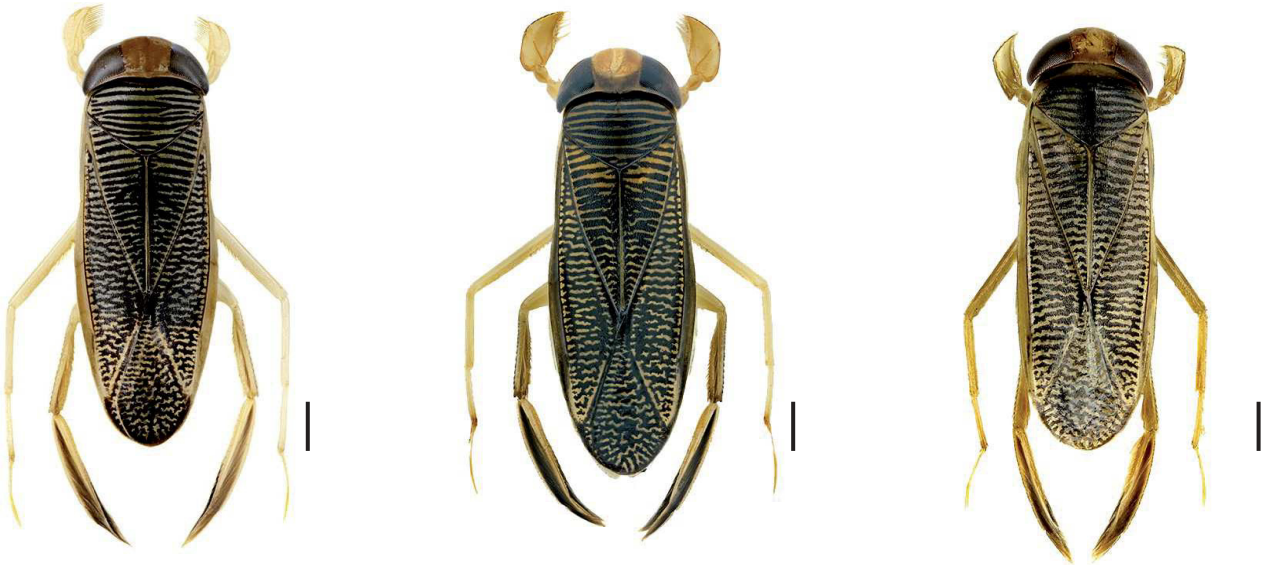
Callicorixa producta (Reuter, 1880)
(*n* = 1)

Figure 6(on next page)

Maximum statistical parsimony network of three *Sigara* species with interspecific K2P-based distances of COI sequences <1%

Used settings included default settings for connection steps whereas gaps were treated as fifth state. Each line represents a single mutational change whereas small black dots and small black lines indicate missing haplotypes. The diameter of the circles is proportional to the number of haplotypes sampled (see open half circles with numbers). Color codes were given for each species. Scale bars = 1 mm. Aquatic bug images were obtained from www.corisa.de





Sigara distincta (Fieber, 1848) (*n* = 7)

Sigara falleni (Fieber, 1848) (*n* = 12)

Sigara iactans Jansson, 1983 (*n* = 12)

Figure 7 (on next page)

Subtree of the Neighbour Joining topology of the analyzed specimens of *Plea minutissima* Leach, 1817

Branches with specimen ID-Number from BOLD and species names. Numbers next to internal branches are non-parametric bootstrap values (in %). Scale bar = 1 mm. Image obtained from www.corisa.de

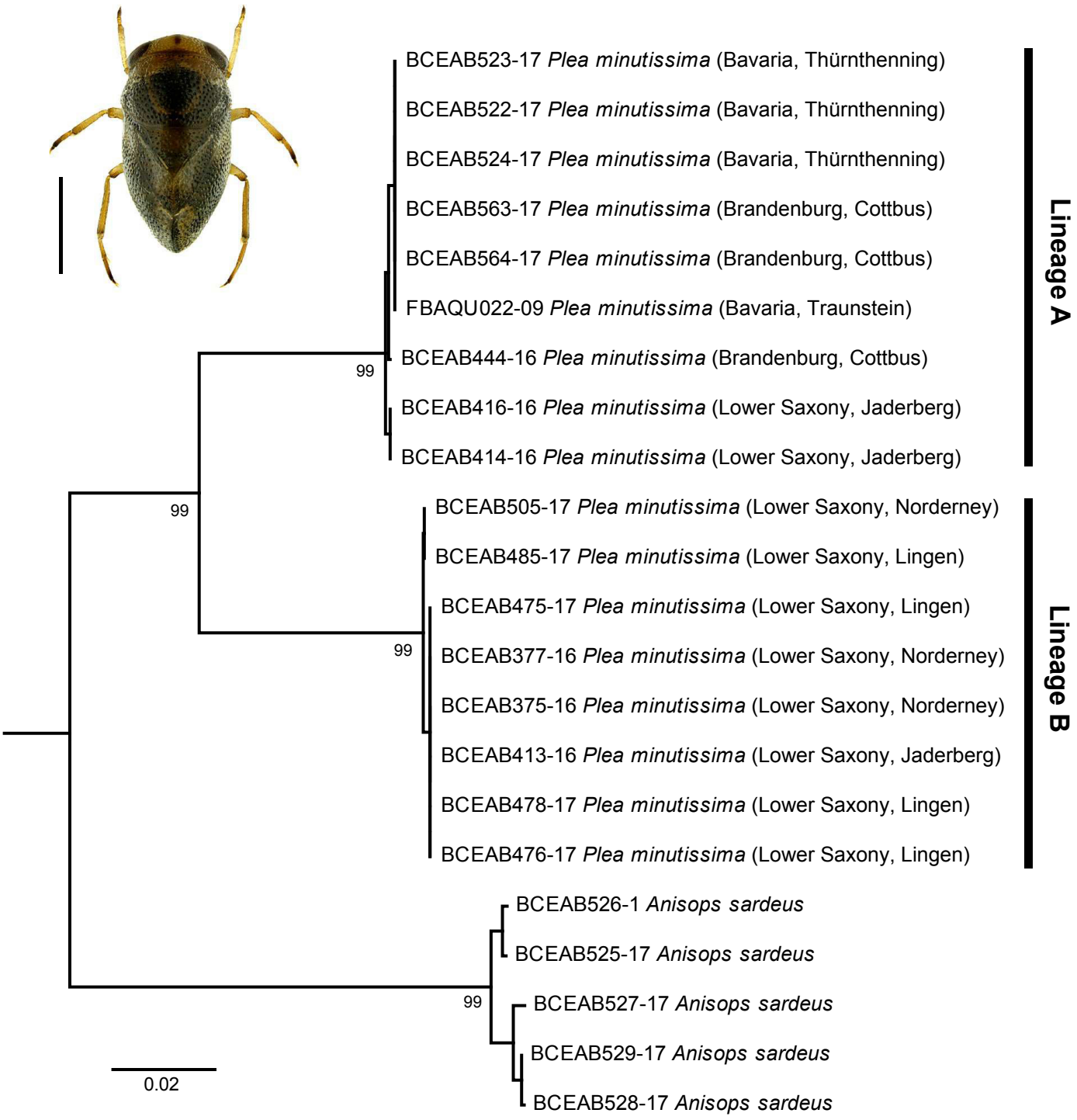

BCEAB523-17 Plea minutissima (Bavaria, Thürnthenning)
BCEAB522-17 Plea minutissima (Bavaria, Thürnthenning)
BCEAB524-17 Plea minutissima (Bavaria, Thürnthenning)
BCEAB563-17 Plea minutissima (Brandenburg, Cottbus)
BCEAB564-17 Plea minutissima (Brandenburg, Cottbus)
FBAQU022-09 Plea minutissima (Bavaria, Traunstein)
BCEAB444-16 Plea minutissima (Brandenburg, Cottbus)
BCEAB416-16 Plea minutissima (Lower Saxony, Jaderberg)
BCEAB414-16 Plea minutissima (Lower Saxony, Jaderberg)
BCEAB505-17 Plea minutissima (Lower Saxony, Norderney)
BCEAB485-17 Plea minutissima (Lower Saxony, Lingen)
BCEAB475-17 Plea minutissima (Lower Saxony, Lingen)
BCEAB377-16 Plea minutissima (Lower Saxony, Norderney)
BCEAB375-16 Plea minutissima (Lower Saxony, Norderney)
BCEAB413-16 Plea minutissima (Lower Saxony, Jaderberg)
BCEAB478-17 Plea minutissima (Lower Saxony, Lingen)
BCEAB476-17 Plea minutissima (Lower Saxony, Lingen)
BCEAB526-1 Anisops sardeus
BCEAB525-17 Anisops sardeus
BCEAB527-17 Anisops sardeus
BCEAB529-17 Anisops sardeus
BCEAB528-17 Anisops sardeus
99
99
99
Lineage A
Lineage B
0.02

Figure 8(on next page)

Subtree of the Neighbour Joining topology of the analyzed species of the genus *Cymatia* Flor, 1860.

Numbers next to internal branches are non-parametric bootstrap values (in %). Images obtained from www.corisa.de

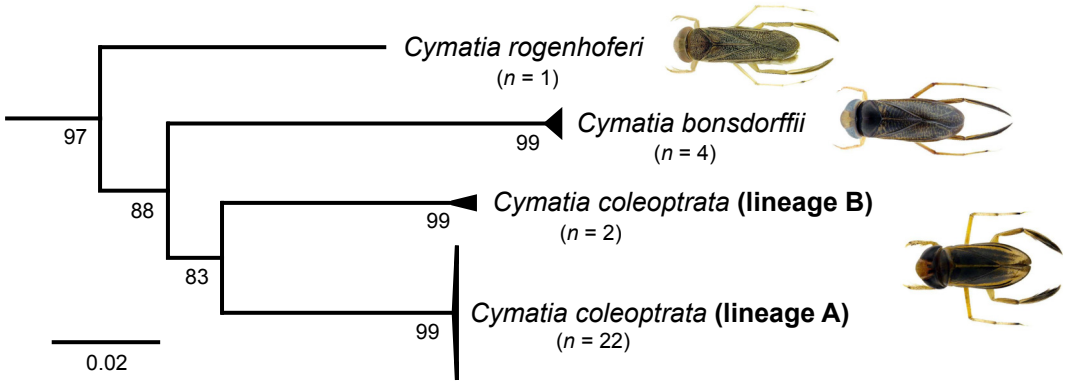

Cymatia rogenhoferi
(n = 1)
Cymatia bonsdorffii
(n = 4)
Cymatia coleoptrata (lineage B)
(n = 2)
Cymatia coleoptrata (lineage A)
(n = 22)
97
99
88
99
83
99
0.02

Table 1(on next page)

BOLD distance analysis of the studied Gerromorpha and Nepomorph.

With the number of analyzed specimens (*n*), phylogenetic categories, barcode index number (BIN), maximum intraspecific pairwise K2P distances (MID), minimum interspecific pairwise K2P distances to the nearest neighbor species (DNN), and the nearest neighbor species (NNS). Maximum intraspecific distances >2.2% and minimum interspecific distances <2.2% are marked in bold. At least one specimen of the compared species showed a distance value above or below this threshold in terms of a pairwise comparison. Asterisks (*) indicate species not recorded for Germany

Family	Species	*n*	Phylogenetic categories	BIN	MID	DNN	NNS
Aphelocheiridae	*Aphelocheirus aestivalis*	2	monophyletic	ABX0398	0	11.86	*Notonecta maculata*
Corixidae	*Arctocorisa carinata*	5	paraphyletic	AAJ7903, ACY1261	**2.36**	**1.03**	*Arctocorisa germari*
	Arctocorisa germari	1	n. a.	-	0	**1.03**	*Arctocorisa carinata*
	Callicorixa praeusta	23	paraphyletic	AAK1938	0.31	**0**	*Callicorixa producta*
	Callicorixa producta	1	n. a.	AAK1938	0	**0**	*Callicorixa praeusta*
	Corixa affinis	13	monophyletic	ACY0615	1.92	5.92	*Corixa panzeri*
	Corixa dentipes	1	n. a.	-	0	6.08	*Corixa punctata*
	Corixa panzeri	2	monophyletic	ACX9506	0	5.92	*Corixa affinis*
	Corixa punctata	21	monophyletic	ACB1799	0.77	6.08	*Corixa dentipes*
	Cymatia bonsdorffii	4	monophyletic	ABX0396	0.62	12.34	*Cymatia coleoptrata*
	Cymatia coleoptrata	24	monophyletic	ACB1796, ADD1561	**9.44**	12.4	*Cymatia bonsdorffii*
	Cymatia rogenhoferi	1	n. a.	ACB2132	0	12.7	*Cymatia coleoptrata*
	Glaenocorisa propinqua	8	monophyletic	ABX4248	1.55	9.96	*Sigara semistriata*
	Hesperocorixa castanea	14	monophyletic	ABX0447	0.32	13.22	*Paracorixa concinna*
	Hesperocorixa linnaei	8	monophyletic	ABX0448	0	11.89	*Sigara venusta*
	Hesperocorixa sahlbergi	39	monophyletic	AAN0795	1.7	11.34	*Corixa panzeri*
	Micronecta griseola	2	monophyletic	AAK6480	0	10.63	*Micronecta poweri*
	Micronecta poweri	2	monophyletic	ACB1970	**2.39**	10.63	*Micronecta griseola*
	Micronecta scholtzi	6	monophyletic	AAK6479	0.16	18.58	*Sigara semistriata*
	Paracorixa concinna	11	monophyletic	ABV3365, ADG5371	1.71	7.03	*Sigara semistriata*
	Sigara distincta	7	polyphyletic	ABY7152, ABV4484	**5.77**	**0.37**	*Sigara falleni*
	*Sigara dorsalis**	1	n. a.	AAJ6688	0	**1.71**	*Sigara striata*
	Sigara falleni	12	polyphyletic	AAH9524, ABY7152	**3.37**	**0**	*Sigara iactans*
	Sigara fossarum	3	monophyletic	AAJ6707, ADD1512	**2.82**	2.72	*Sigara scotti*
	Sigara hellensii	2	monophyletic	ADH9592, ACT7694	**4.41**	9.09	*Sigara distincta*
	Sigara iactans	12	polyphyletic	ABY7152, AAH9524	**2.67**	**0**	*Sigara falleni*
	Sigara lateralis	14	monophyletic	AAJ6697	0.81	9.84	*Sigara striata*
	Sigara limitata	2	paraphyletic	ACM1221	0.48	**0.15**	*Sigara semistriata*
	Sigara nigrolineata	16	monophyletic	ACB1978	0.46	10.12	*Sigara semistriata*
	Sigara scotti	12	monophyletic	ACY0807	1.08	2.72	*Sigara fossarum*
	Sigara semistriata	5	polyphyletic	ACM1221	0	**0.15**	*Sigara limitata*
	Sigara stagnalis	6	monophyletic	ACY0713	0.55	11.45	*Paracorixa concinna*
	Sigara striata	10	monophyletic	AAJ6688	0.93	**1.71**	*Sigara dorsalis*
	Sigara venusta	2	monophyletic	ABA5309	0	**2.11**	*Sigara semistriata*
Naucoridae	*Ilyocoris cimicoides*	17	monophyletic	AAF2590	1.03	15.06	*Hesperocorixa sahlbergi*
Nepidae	*Nepa cinerea*	10	monophyletic	AAK8359	0.34	17.06	*Notonecta maculata*
	Ranatra linearis	16	monophyletic	AAL1328	0.84	15.03	*Notonecta lutea*
Notonectidae	*Anisops sardeus**	5	monophyletic	ABV0079	1.24	12.84	*Notonecta maculata*
	Notonecta glauca	41	monophyletic	AAK4442	1.71	**1.08**	*Notonecta obliqua*
	Notonecta lutea	19	monophyletic	AAN1701	0.68	**1.24**	*Notonecta reuteri*
	Notonecta maculata	10	monophyletic	AAN1703	**2.43**	6.56	*Notonecta glauca*
	Notonecta obliqua	9	monophyletic	AAK4442	0.64	**1.08**	*Notonecta glauca*
	Notonecta reuteri	5	monophyletic	ACE8526	0.46	**1.24**	*Notonecta lutea*
	Notonecta viridis	10	monophyletic	ABV0133	1.18	5.03	*Notonecta glauca*
Pleidae	*Plea minutissima*	17	monophyletic	ACY0868, AAF3832	**8.3**	10.92	*Notonecta lutea*
Gerridae	*Aquarius najas*	7	monophyletic	AAN1521	2.14	11.75	*Gerris thoracicus*
	Aquarius paludum	19	monophyletic	AAI7450	1.24	12.61	*Gerris argentatus*
	Gerris argentatus	32	monophyletic	ADD1846	0.72	6.55	*Gerris odontogaster*
	Gerris asper	1	n. a.	ABA3327	0	**0.34**	*Gerris lateralis*
	Gerris costae	11	monophyletic	ACI6181	0	7.48	*Gerris thoracicus*
	Gerris gibbifer	11	monophyletic	ACB1756	0.88	8.91	*Gerris lacustris*
	Gerris lacustris	38	monophyletic	ACT3584	1.05	8.91	*Gerris gibbifer*
	Gerris lateralis	2	monophyletic	ABA3327	0.17	**0.34**	*Gerris asper*
	Gerris odontogaster	19	monophyletic	ABU6679, ADD1838	1.59	6.55	*Gerris argentatus*
	Gerris thoracicus	6	monophyletic	ACB1745	0.35	7.48	*Gerris costae*
	Limnoporus rufoscutellatus	3	monophyletic	AAV0261	0.88	11.86	*Gerris asper*
Hebridae	*Hebrus pusillus*	2	monophyletic	AAN0981	0.15	14.32	*Hebrus ruficeps*
	Hebrus ruficeps	7	monophyletic	AAI6967	0.15	14.32	*Hebrus pusillus*
Hydrometridae	*Hydrometra gracilenta*	9	monophyletic	AAN0857	0.46	13.06	*Hydrometra stagnorum*
	Hydrometra stagnorum	21	monophyletic	AAK5632	0.62	13.06	*Hydrometra gracilenta*
Mesoveliidae	*Mesovelia furcata*	17	monophyletic	AAN2451	1.39	16.24	*Mesovelia vittigera*
	*Mesovelia vittigera**	4	monophyletic	ACD4048	**2.32**	16.24	*Mesovelia furcata*
Veliidae	*Microvelia buenoi*	1	n. a.	ACY1789	0	15.06	*Gerris costae*
	Microvelia reticulata	27	monophyletic	AAG4341	0.77	15.04	*Gerris asper*
	Velia caprai	20	monophyletic	AAN0455	1.1	4.94	*Velia saulii*
	*Velia currens**	3	monophyletic	ADI1962	0	2.82	*Velia saulii*
	Velia saulii	1	n. a.	ABX0836	0	2.82	*Velia currens*