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Morphology, genetics and *Wolbachia* endosymbionts support distinctiveness of *Monochamus sartor sartor* and *M. s. urussovii* (Coleoptera: Cerambycidae)

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Abstract. *Monochamus sartor sartor* from Central European mountain ranges and *M. s. urussovii* from the Eurasian boreal zone are subspecies whose taxonomic statuses have been questioned. This sawyer beetle is a natural element of spruce forests but is considered to be a timber pest in spruce plantations. In this study, different sets of data (morphology, genetics and ecology) were used to verify the taxonomic status of *M. sartor* across its entire range. Morphologically, not only both subspecies but also European and Asian populations of *M. s. urussovii* were found to be distinct. Genetic data also showed that both subspecies have distinct mitochondrial haplogroups; however, divergence between them is very weak (of ca. 1%), suggesting they split very recently, possibly at the end of the Pleistocene glaciations. Species delimitation methods gave discordant results, either rejecting the species status of *M. s. sartor* and *M. s. urussovii* (Poisson tree processes) or confirming them as distinct taxa (the multispecies coalescent model for species validation). Host plant preferences also partially differentiate the subspecies, as *M. s. urussovii* has a broader diet than the generally monophagous, spruce-dependent *M. s. sartor.* Moreover, each subspecies is infected by different strains of the intracellular bacterium *Wolbachia*, which could be one of the factors causing their genetic isolation, regardless of geographic isolation. Aside from broadening the basic knowledge on the taxonomy and genetics of *Monochamus sartor*, this study shows that any research on these sawyers needs to consider their separate phylogenetic lineages, as do any plans for population management or conservation.

Key words. Cerambycidae, longhorn beetles, dead wood, phylogeny, species delimitation, integrative taxonomy.

1. Introduction

Subspecies is the only recognized rank below species level that can receive a name in the zoological code (International Code of Zoological Nomenclature 2000). Recognising subspecies is difficult. According to the biological species concept, organisms belonging to different species can be verified on the basis of their ability to interbreed and produce fertile offspring (Wilson & Brown 1953; Ehrlich 1957; Mayr 1982); however, there is no strict concept for the subspecies level. Biologists can identify subspecies on the basis of whether geographically separate populations of a species exhibit recognizable phenotypic differences (Wilson & Brown 1953;

Ehrlich 1957; Mayr 1982). These differences should also be visible in their genotypes, e.g. by the occurrence of distinct phylogenetic lineages. However, the border between intraspecific (interpopulation) diversity and divergence between subspecies is not clearly demarcated. Moreover, distinct phylogenetic lineages can be assigned as Evolutionary Significant Units (Ryder 1986; Moritz 1994), which are utilized in conservation genetics but which can also be valuable in taxonomic/phylogenetic studies. In nature, subspecies are mainly unable to interbreed due to geographic isolation of distant populations (Barrowclough 1982; Cracraft 1983). Moreover, it is expected that differences between subspecies should usually be less distinct than differences between species, but this assumption is rarely studied with respect to different characters. Most subspecies have been recognized on the basis of slight but significant differences in their morphological features (Nei 1972; Ball & Avise 1992), which are identified arbitrarily by observers/taxonomists. Relatively rarely is their taxonomic distinctiveness later verified with other data, e.g. on their genetics or ecologies (Phillimore & Owens 2006). This especially concerns insects, which are the most diverse group of organisms on Earth (May 1992; Mora et al. 2011), and particularly beetles (Coleoptera), which form the most species rich order of insects (Farrell 1998; Grove & Stork 2000). Detailed, integrative studies that use morphometrics, molecular markers and/or ecological features often find intraspecific variation. The taxonomic status of such polytypic species should be verified to broaden basic taxonomic knowledge, understand phylogenetic relations among units below species level and properly organize studies (to be sure which and how many units or taxa are investigated), as well to conserve/manage rare or economically important taxa.

Here, we focus on the sawyer beetle *Monochamus sartor* (Fabricius, 1787), which belongs to the longhorn beetles (Cerambycidae: Lamiinae). There are about 140 species and 25 subspecies of *Monochamus* worldwide, 50 of which inhabit the Palaearctic – mainly the boreal zone and mountain areas (Danilevsky 2017; http://insecta.pro/search?search=Monochamus). These species are highly dependent on the dead wood of mainly conifer trees (pines *Pinus* spp., spruces *Picea* spp., firs *Abies* spp., larches *Larix* spp. and cedars *Cedrus* spp.) (Hellrigl 1970; Isaev et al. 1988; Wallin et al. 2013). Larvae bore holes inside the wood of thicker branches and trunks, and thus are considered timber pests. Furthermore, species in the genus *Monochamus* are the main vectors of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Linit et al. 1983), a quarantine species that causes pine wilt disease (PWD), mainly in East Asia and recently also in Portugal (Kondo et al. 1986; Miller et al. 2013). For this reason, they are considered serious pests of conifer tree plantations (Hellrigl 1970; Evans et al. 2004; Wang 2017). On the other hand, they are one of the species responsible for the decay of dead wood in mature conifer forests in the boreal zone and on mountain ranges and are an important food source for numerous bird species (e.g. woodpeckers; Winkler et al. 1995).

Monochamus sawyers have been the objects of numerous taxonomic, systematic and phylogenetic studies (Hellrigl 1970; Tomminen & Leppänen 1991; Cesari et al. 2004; Koutroumpa et al. 2013; Wallin et al. 2013; Rossa et al. 2016). Much less is understood on the phylogeography of particular taxa as almost all such studies concern Asian *Monochamus alternatus* Hope (Kawai et al. 2006; Shoda-Kagaya et al. 2008; Hu et al. 2013); just recently a single study on the European *Monochamus galloprovincialis* (Olivier) was published (Haran et al. 2017). On the other hand, such knowledge on the *Monochamus sartor*-complex is insufficient. Until relatively recently, the two currently recognized subspecies, i.e. *M. sartor sartor* and *M. sartor urussovii* (Fischer von Waldheim, 1806), were considered distinct species, i.e. *M. sartor* and *M. urussovii*, respectively (Bense 1995; Sama 2002; Löbl & Smetana 2010). Sláma (2006) used the subspecies rank for both taxa; however, his justification for this splitting is lacking. Some other literature also supports this division (e.g. Wallin et al. 2013), based on the detailed characteristics of adults and the genital morphology of males and females. However, there are still many uncertainties, especially related to the distribution of both taxa. There is no certainty if the subspecific rank of these taxa is appropriate, and if it is, the question arises whether there should be another subspecies distinguished for the populations of north-east Europe.

Both, *M. sartor sartor* and *M. s. urussovii*, occur throughout the natural range of Norway spruce, *Picea abies* (L.) H. Karst, while also rarely utilizing pines and firs. Only in eastern Siberia, Korea, and Japan they are also reported to develop on other spruce species as well as on cedars and birches *Betula* L. sp. (Cherepanov 1983). However, data on the distribution of these taxa in Europe is often insufficient and sometimes contradictory. For example, Danilevsky (2012) stated that both *M. sartor sartor* and *M. s. urussovii* co-occur in several European countries (e.g. Belarus, Estonia, Latvia, Lithuania and Ukraine). Nevertheless, he questioned the occurrence of the latter subspecies in north-eastern Poland, and further stated that the populations of *M. sartor* from this area are identical to those found in the Carpathians (i.e. to *M. s. sartor*). As a consequence, the western parts of Belarus (i.e. the Białowieża Primeval Forest) would supposedly be populated by *M. s. sartor*, while the eastern parts by *M. s. urussovii* (Danilevsky 2012). Meanwhile, a different distribution pattern for both taxa was proposed by Löbl & Smetana (2010) and later Wallin et al. (2013), who suggested the two taxa co-occur in six European countries, namely in Belarus, Czechia, Latvia, Lithuania, Estonia and Poland. These authors, however, did not specify the areas of distribution of *M. s. urussovii* in these countries.

The distributional pattern is less obscure for *M. s. sartor*, which is characterized by a European type of distribution – its range is less extensive than *M. s. urussovii* and covers the Alps, Carpathians, Dinaric Alps and Bul-

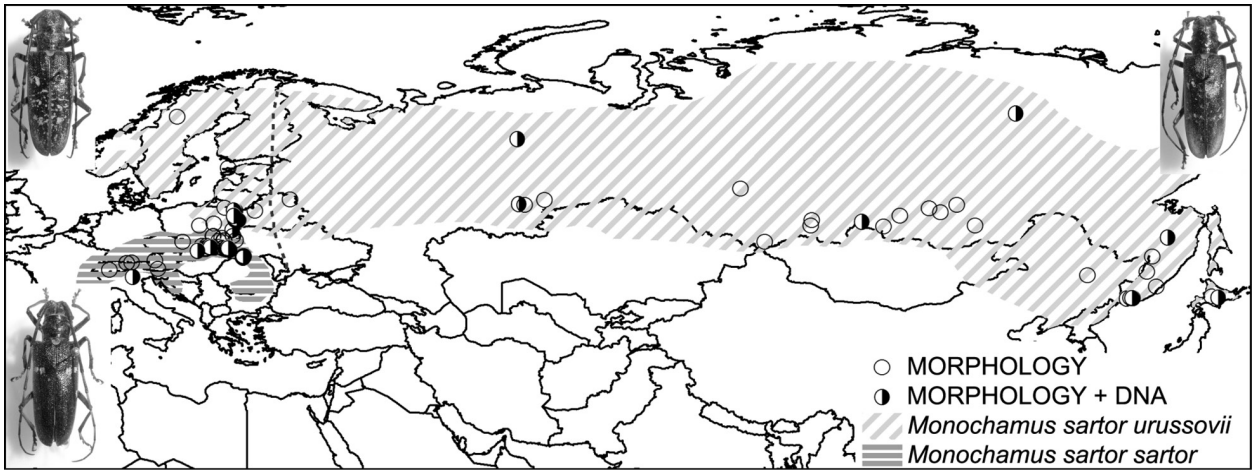


Fig. 1. Distribution of *Monochamus sartor sartor* and *M. s. urussovii* in Eurasia with localization of sampling sites for morphological and molecular studies. Broken line – approximate border between eastern and western populations of *M. s. urussovii*.

garian mountains, and their foothills (Heyrovský 1955; Mikšič & Korpič 1985; Dominik & Starzyk 2004; orig. inf.).

Morphology and morphometric information complements molecular data. Moreover, these types of data are often supplemented with ecological features (e.g. habitat requirements and interactions with other organisms, such as host plants for herbivores), and together such a comprehensive elaboration of species distinctiveness and relations is called "integrative taxonomy" (Dayrat 2005; Schlick-Steiner et al. 2010). Among ecological features, relations with symbionts or parasites are often utilized (Valentini et al. 2009), with great emphasis on microbiota (Steinert et al. 2000; Hosokawa et al. 2006). Intracellular bacteria can be especially important for arthropods, as some endoparasites or endosymbionts can directly influence host fitness, development and diversity, which may in turn have implications on host speciation (Hurst & Jiggins 2000; Engelstädter & Hurst 2009). Notable examples of such endosymbionts/parasites are the maternally inherited bacteria *Wolbachia* (Bourtzis & O'Neill 1998; Stouthamer et al. 1999; Zchori-Fein & Perlman 2004; Kikuchi 2009; White et al. 2009, 2011). Finally, phylogenetic and systematic studies are especially challenging in cases where the examined taxa are presumed to be of subspecies status (Avise & Wollenberg 1997; Presgraves 2010). This is because often it is hard to decide to which (intraspecific, taxonomic) level these taxa should be assigned.

Here, we use a combination of morphological and molecular (including *Wolbachia* endosymbiont diversity) features to identify phylogenetic lineages within populations of *Monochamus sartor* sawyers and particularly to examine the taxonomic statuses of presumed subspecies. We aimed to verify the following hypotheses: that i) *Monochamus sartor sartor* from mountainous areas of Europe (i.e. the Carpathians and Alps) and *M. s. urussovii* from the boreal zone of Eurasia are distinct subspecies, which evolved in distinct Pleistocene refugia; ii) *M. s. urussovii* from its westernmost range in central-eastern and northern Europe is taxonomically distinct from *M. s. urussovii* in northern and eastern Asia; iii) *Wolbachia* infection differs between *M. s. sartor* and *M. s. urussovii*, which suggests it played a role in the divergence of these sawyers.

2. Material and methods

2.1. Sampling of specimens

All specimens of *Monochamus sartor sartor* and *M. s. urussovii* used in our study were collected between 1902–2016 by various entomologists (Fig. 1). Thus, most of them were dried specimens that we borrowed from various institutions and private entomological collections. Furthermore, adult specimens were collected by us in 2014–2017 in NE (Białowieża Forest, Augustów Forest and Knyszyn Forest) and SE (Bieszczady, Beskid Niski, and Pieniny Mts.) Poland (Fig. 1). In total, 531 specimens of both subspecies of *M. sartor* were collected (see Supplementary Tables S1 and S2 for detailed characteristics of the gathered material).

2.2. Morphological analyses

The morphological study was based on selected body characteristics of 523 adult sawyers (247 females, 276 males). Namely, the maximal width of thorax (i.e. the mesothorax section) and the length of the right elytra of each specimen were measured. The measurements were taken using a Zeiss Stemi 2000-C stereomicroscope, within a 0.1 mm accuracy.

The normality of the distribution of data was checked using a Shapiro-Wilk test. A Kruskal-Wallis test for independent groups, i.e. beetles from: mountainous areas of Europe (group I), boreal Europe (group II), and Central and East Asia (group III), was used to compare the

Table 1. Genetic diversity of mitochondrial DNA in examined populations of *Monochamus sartor* sawyers. — ***Abbreviations***: N – sample size, H – number of haplotypes, S – number of segregating sites, Hdiv – haplotype diversity, πdiv – nucleotide diversity, SD – standard deviation.

Subspecies	Area	N	H	S	Hdiv ± SD	πdiv ± SD
Monochamus sartor urussovii	Asia eastern	8	7	11	1.00 ± 0.08	0.003 ± 0.002
	Asia central	5	5	10	1.00 ± 0.13	0.004 ± 0.002
	Asia - total	13	13	21	1.00 ± 0.03	0.005 ± 0.002
	Poland north-eastern	16	13	17	0.98 ± 0.03	0.004 ± 0.002
	Eurasia	29	25	28	0.99 ± 0.01	0.004 ± 0.002
Monochamus sartor sartor	Alps	4	3	2	0.83 ± 0.22	0.001 ± 0.001
	Carpathians	26	10	9	0.70 ± 0.09	0.001 ± 0.001
	Alps & Carpathians	30	12	11	0.71 ± 0.09	0.001 ± 0.001

studied morphological features. Due to expected sexual dimorphism both sexes of sawyer beetles were treated separately. Statistical analyses were carried out in Statistica 10 (STATSOFT 2011).

2.3. Molecular analyses

Molecular analyses were conducted on 59 specimens representing all parts of both subspecies' ranges in Europe and Asia (details listed in Table 1). Most specimens were directly preserved in 99% ethanol and kept in –20°C freezer until use – this concerns beetles collected in SE (*M. s. sartor*) and NE Poland (*M. s. urussovii*). Other specimens (mostly *M. s. urussovii* from Russia and Japan) were preserved as dry samples.

2.3.1. Laboratory procedures

DNA was extracted from internal tissues of abdomens (specimens were retained for morphological measurements and collection) using Nucleospin Tissue kits (Macherey-Nagel) following the manufacturer's instructions. Two different beetle genes were amplified, sequenced and used for the following analyses. Partial sequences of mitochondrial cytochrome oxidase subunit I (*coxI*) and nuclear elongation factor 1-alpha (*ef-1α*) were amplified using primers C1-J-1751 and L2-N-3014 (SIMON et al. 1994), and EFs149 and EFa1R (NORMARK et al. 1999; SANZ MUÑOZ 2010), respectively. The details of amplification, purification and sequencing procedures were reported in KUBISZ et al. (2012). *CoxI* was amplified for all examined beetles, whereas *ef-1α* could only be amplified from fresh-preserved specimens (for *M. s. sartor* these were collected from the Carpathians and for *M. s. urussovii* from North-eastern Poland). Because the initial sequencing of *ef-1α* revealed no polymorphism in *M. s. sartor* and *M. s. urussovii*, we did not analyse this gene further. Moreover, we downloaded all available *coxI* sequences of *M. s. sartor*, which originated from Italian (Alpine) specimens (GenBank accession numbers: AY260838 – AY2608340).

Wolbachia infection was initially screened in all individuals with ftsZ_F1 / ftsZ_R1 and hcpA_F1 / hcpA_R1 primers for two *Wolbachia*-specific genes (BALDO et al. 2005). Next, all positively infected individuals were genotyped with respect to all five genes included in the Multilocus Sequence Typing system accepted for *Wolbachia* (details available at http://pubmlst.org/wolbachia/). We excluded from the analysis all dry-preserved beetle specimens as we could not rule out whether lack of amplification of bacterial genes really indicated a lack of infection or DNA from these specimens was just too degraded.

The sequences of presumed *Monochamus* and *Wolbachia* genes were compared with the online NCBI databank using the Basic Local Alignment Search Tool (BLAST) (ALTSCHUL et al. 1990) to check if the primers had specifically amplified the targeted sequences of sawyers and α-proteobacteria.

The obtained electropherograms, after correction using BioEdit v.7.0.5.2 (HALL 1999), were deposited in GenBank (accession numbers: MF327393 – MF327421 and MF371175 – MF371201 for *coxI*; MF405509 – MF405514 for *ef-1α;* MF405515 – MF405520 for *gatB*, MF405521 – MF405526 for *coxA*, MF405527 – MF405532 for *hcpA*, MF405533 – MF405539 for *ftsZ* and MF405540 – MF405545 for *fbpA*).

Protein-coding DNA sequences (*cox1* and *ef-1α*) were aligned using MAFFT (KATOH et al. 2005). Pairwise nucleotide divergences for both sawyer markers were calculated using MEGA v6 (TAMURA et al. 2013).

2.3.2. Genetic diversity

Haplotypes were identified and standard genetic indices such as haplotype diversity (Hdiv), nucleotide diversity (πdiv), number of private haplotypes (Hpriv) and number of segregating sites (S) for populations were computed using the program DnaSP v.5 (LIBRADO & ROZAS 2009). Population samples were grouped according to their geographical locations (Table 1). F_{ST} indices were calculated using ARLEQUIN v.3.5 (EXCOFFIER & LISCHER 2010). A Mantel test (MANTEL 1967) was performed in the program ARLEQUIN to check if the genetic structure of the sampled localities (five) fits an isolation by distance model (IBD) (SLATKIN 1993), using pairwise F_{ST} values and straight-line geographic distances in kilometers. To test for the presence of barriers between populations, an analysis of molecular variance (AMOVA) was conducted in ARLEQUIN. Moreover, a minimum-spanning (MS)

haplotype network (Bandelt et al. 1999) was reconstructed for *coxI* in PopArt (http://popart.otago.ac.nz/).

2.3.3. Phylogenetic analyses and species delimitation

Nucleotide substitution models were estimated for the datasets using the Smart Model Selection tool implemented in PhyML 3.0 software (Guindon et al. 2010) and the best nucleotide evolution model was selected according to the Bayesian information criterion. For *coxI*, GTR was selected as the best model of nucleotide evolution both for ingroup and outgroup taxa.

For *cox1* phylogenetic reconstruction, single sequences were randomly selected from each sample site and the following outgroup taxa were added from GenBank: *Monochamus galloprovincialis* (GenBank accession number: AY260835), *M. saltuarius* (Gebler) (AY260842), *M. alternatus* (KF737828), *M. sutor* (L.) (AY264403) and *Anoplophora glabripennis* (Motschulsky) (EU914688). Phylogenetic trees were reconstructed adopting the Bayesian inference (BI) and maximum likelihood (ML) approaches. BI was performed using MrBayes 3.2.2 (Ronquist et al. 2012) in two independent runs, each with one cold and five heated Markov chains ($\lambda = 0.1$) run each, for 2×10^7 generations that were sampled every 100 generations. Stationarity was considered to be reached when the average standard deviation of the split frequencies was less than 0.01; however, the convergences of each run were also visually inspected using TRACER (Drummond et al. 2012). An appropriate number of sampled trees were discarded as burn-in, and a majority-rule consensus tree was obtained. The ML analyses were performed using PhyML 3.0 software (Guindon et al. 2010) using the command line version. Branch support was obtained by the Approximate Likelihood-Ratio Test (aLRT) (Anisimova & Gascuel 2006).

Molecular species delimitation analyses were performed on *coxI* of all *Monochamus* species included in this study, adopting the tree-based method the Bayesian Poisson tree process model (bPTP; Zhang et al. 2013), and the Bayesian Markov chain Monte Carlo program for Phylogenetic and Phylogeographic analyses under the multispecies coalescent model (BPP; Yang 2015). bPTP and BPP methods have been extensively used to recognize and delimit species (e.g. Hambäck et al. 2013; Cranston & Krosh 2015; Lecocq et al. 2015), as well as to support the description of new insect taxa (e.g. Leaché & Fujita 2010; Montagna et al. 2016a). bPTP analysis, performed on the BI tree, was carried out with the bPTP web server (http://species.h-its.org/ptp/) with the following parameters: 500,000 MCMC generations, thinning every 200 generations, and 0.2 % of generation discarded as burn-in. The BPP guide tree was drawn on the basis of the BI tree topology. We performed A01 and A11 analyses four times, each with different combinations of prior gamma distributions: i) Θ : G(2,200), τ : G(2,400); ii) Θ : G(2,200), τ : G(2,200); iii) Θ : G(2,200), τ : G(2,2000); and iv) Θ : G(2,2000), τ : G(2,200). Each analysis consisted of 100,000 MCMC generations sampled every 20 generations and discarding the first 20% of the samples as burn-in. Moreover, mean genetic distances among sites were calculated using MEGA5 (Tamura et al. 2011) under the Kimura 2-parameter model (K2P).

2.3.4. *Wolbachia* infection

Allelic profiles of MLST genes were generated for each infected individual. Next, we utilized an approach similar to that of Montagna et al. (2014) to compare allelic profiles generated from *Monochamus* beetles with some representative sequence types from other species that harbored bacteria belonging to supergroups A (ST-1 from *Drosophila melanogaster* Meigen), B (ST-15 from *Drosophila simulans* Sturtevan), D (ST-35 from unspecified nematode), F (ST-8 from *Cimex lectularius* L.), and H (ST-90 from *Zootermes angusticollis* (Hagen)). Moreover, the allelic profiles found for the only European beetles with full allelic profiles in the MLST database were added to this set of MLST sequences: *Eusomus ovulum* Germar (Mazur et al. 2016), *Oreina cacaliae* (Schrank) (Montagna et al. 2014), and *Crioceris quinquepunctata* Scopoli (Kubisz et al. 2012). We then used the generated alignment of MLST genes for the construction of a phylogenetic network in SplitsTree4 (Huson & Bryant 2006) by using neighbor-net algorithm distance estimates. In contrast to traditional phylogenetic trees, this allows for visualization of multiple connections among examined sequences, which can represent recombination events. The PHI test implemented in SPLITSTREE v. 4 (Huson & Bryant 2006) has been shown to identify the presence/absence of recombination within a range of sequence samples (both insect and bacterial markers) with a low false-positive rate (Bruen et al. 2006). The PHI test rejected the hypothesis assuming recombination among MLST genes ($p = 1.000$). Additionally, the most similar hits to all MLTS (gene) sequences generated from *Monochamus* sawyers were identified with the BLAST search tool (Altschul et al. 1990) against NCBI GenBank resources.

3. Results

3.1. Morphological differentiation

Statistical analyses revealed significant differences between the studied size features of *M. sartor* populations from the three areas of distribution (female thoracic width: $H = 53.78$, $df = 2$, $P < 0.001$; female elytral length: $H = 42.13$, $df = 2$, $P < 0.001$; male thoracic width: $H = 24.03$, $df = 2$, $P < 0.001$; male elytral length: $H = 16.74$, $df = 2$, $P < 0.001$). In general, females from boreal Europe (group II) had a smaller body size, as reflected by mean thoracic width and elytral length, than females from both mountainous areas of Europe and Central and East Asia (groups I and III, respectively). A similar pat-

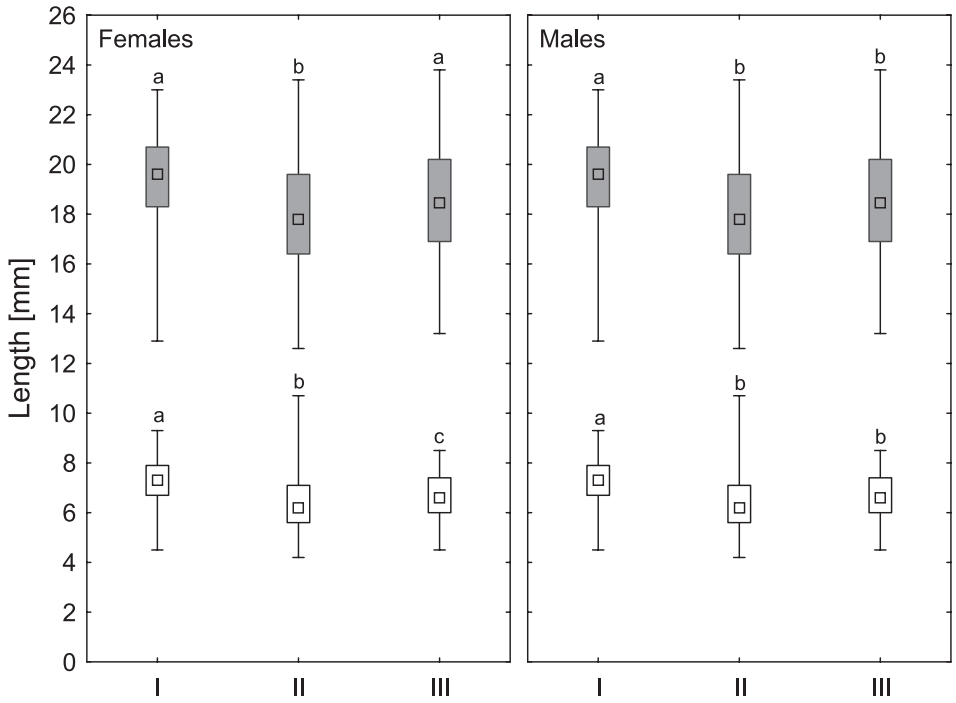


Fig. 2. Differences between the length of the right elytra (gray boxes) and the width of thorax (white boxes) of female and male *Monochamus sartor* sawyers from three distinguished areas of distribution: I – montane areas of Central Europe (Carpathians and Alps), II – boreal Europe, and III – Central and East Asia. Squares indicate medians, boxes indicate 25th and 75th percentiles and whiskers indicate minimum and maximum values; different letters indicate significant differences between studied populations; $p < 0.05$.

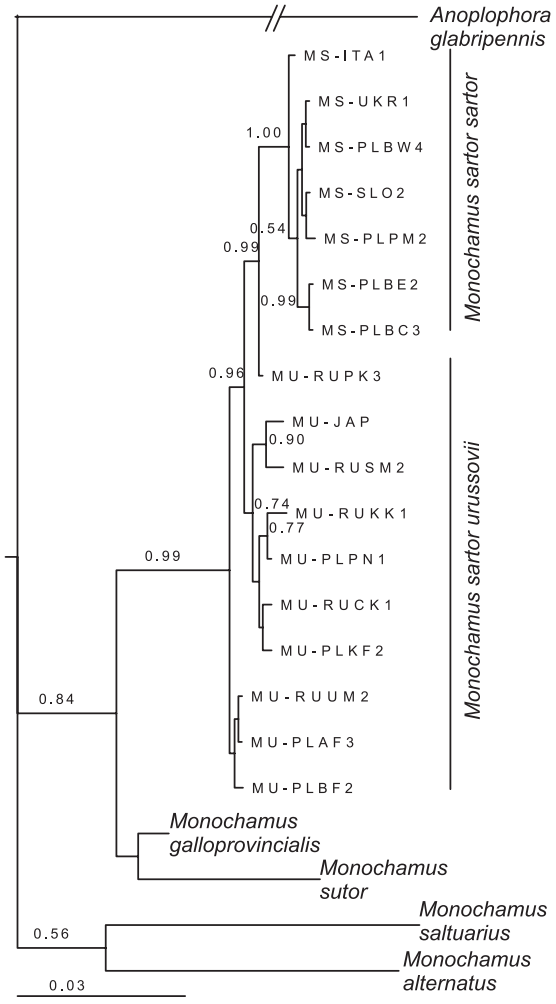
tern was observed for adult males, in which the studied body characteristics of specimens from boreal Europe differed significantly from those from mountainous areas of Europe, but were not different from specimens from Central and East Asia (Fig. 2).

3.2. Molecular differentiation

3.2.1. Genetic diversity

After trimming of ambiguous fragments of e*f-1α* sequences, the final alignment was 600 bp long. There were no stop codons, and only one indel (3 bp) differentiated *A. glabripennis* from *Monochamus* species. Due to lack of polymorphism in *ef-1α* in both *M. sartor* subspecies, all below-mentioned analyses were based only on the *cox1* dataset. The *coxI* alignment was 1187 bp long, and no stop codons or indels were detected. Genetic diversity was high in *M. s. urussovii* and was similarly high across all its geographic groups of population samples (Table 1). On the other hand, *M. s. sartor* had much lower genetic diversity (Table 1).

Monochamus sartor shows weak but significant isolation by distance (Mantel test: R = 0.245, P = 0.003).



→ **Fig. 3.** Bayesian phylogenetic tree reconstructed for examined *Monochamus sartor sartor* and *M. s. urussovii* sawyers on the basis of polymorphism of cytochrome oxidase I gene. Values indicate support of branches (posterior probabilities).

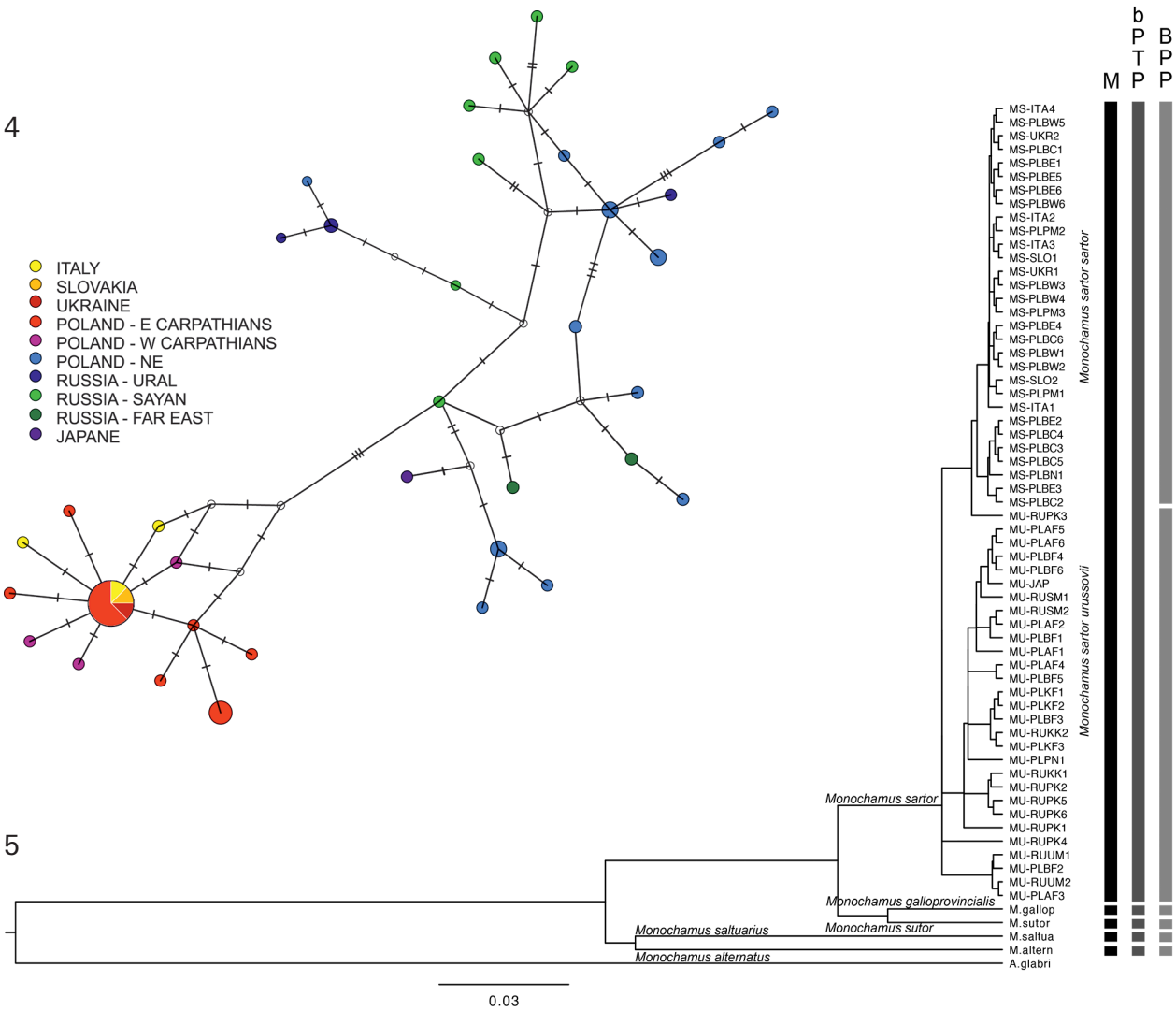


Fig. 4. Minimum-spanning haplotype network of cytochrome oxidase I (*cox1*) gene sequenced for *Monochamus sartor sartor* and *M. s. urussovii* sawyers.

Fig. 5. Ultrametric Bayesian phylogenetic tree reporting the results of the species delimitation analyses. Vertical bars correspond to morphology (M; black) and to the species delimitation results obtained by bPTP and BPP methods, respectively in dark and light grey.

AMOVA showed that 66.69% of the molecular variance could be attributed among groups of population samples, 24.80% within populations and only 8.51% among population samples within groups (F_{SC} = 0.255, F_{ST} = 0.751, F_{CT} = 0.666, all P < 0.001). F_{ST} values were low between Asian and Polish populations of *M. s. urussovii* (0.056) and very high between *M. s. urussovii* and *M. s. sartor* (0.770 and 0.783, respectively).

3.2.2. Phylogenetic analyses and species delimitation

Both BI and ML methods resulted in trees of congruent topologies and therefore only BI trees were presented. Phylogenetic reconstruction, calculated for both markers supported the monophyly of *M. sartor*. *Ef-1α* failed to distinguish *M. s. urussovii* from *M. s. sartor*, as both taxa shared a haplotype of this gene. On the other hand, *coxI* suggested the presence of two clusters: *M. s. urussovii* and *M. s. sartor*; however, they were not monophyletic, as the second was nested within the first (Fig. 3).

The haplotype network showed that M. s. urussovii and M. s. sartor form distinct clusters, whose closest haplotypes are divided by only seven substitutions (Fig. 4). Within *M. s. urussovii* K2P nucleotide divergence was in the range of 0.1–0.7%, within *M. s. sartor* 0.0–0.2%, and between both subspecies 0.4–1.2%.

Species delimitation with the bPTP method recognized five entities (Fig. 5; 95% CI 5–7 entities) with a Bayesian posterior probability ranging from 0.94 to 1. The method supported the species distinctiveness of all *Monochamus* species including *M. sartor*, but rejected the distinctiveness of two subspecies of the latter. Whereas, BPP analyses adopting different priors were in close agreement on the best tree topology (i.e. (*M. alternatus*, (*M. saltuarius*, ((*M. galloprovincialis*, *M. sutor*), (*M. sartor urussovii*, *M. sartor sartor*))))) and in accordance in recognizing the presence of 6 entities, with the

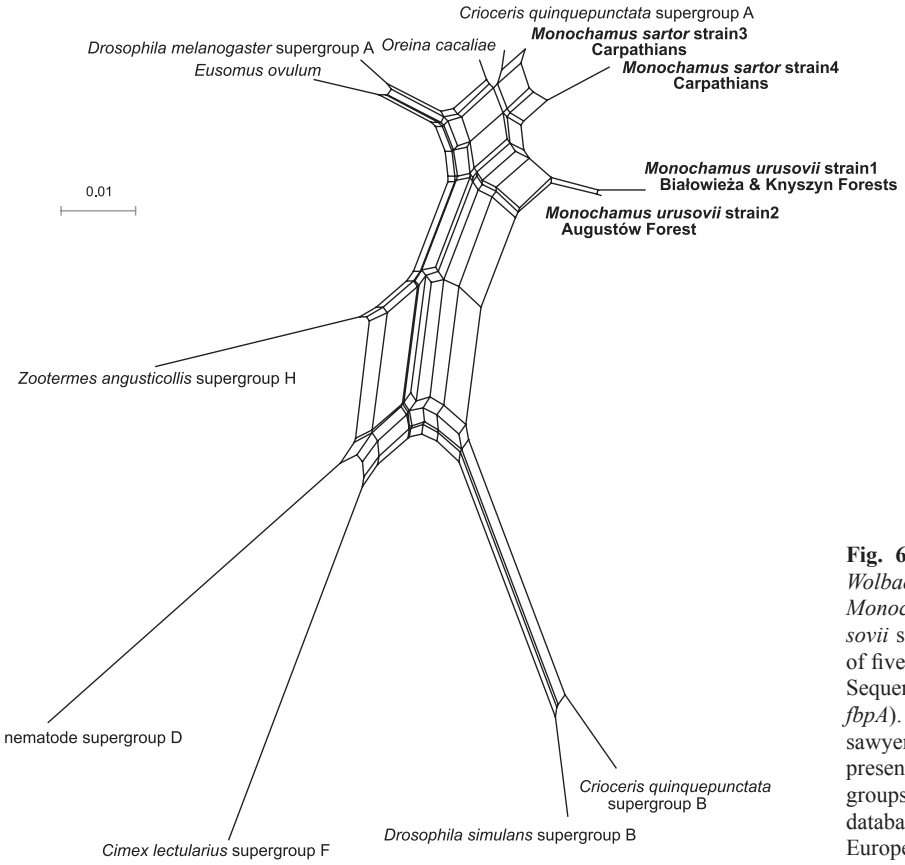


Fig. 6. Median network reconstructed for *Wolbachia* strains generated from examined *Monochamus sartor sartor* and *M. s. urussovii* sawyers on the basis of polymorphism of five *Wolbachia* genes used for Multilocus Sequence Typing (*gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA*). *Wolbachia* strains from examined sawyers are shown with relations to the representative strains of all *Wolbachia* supergroups found in Multilocus Sequence Typing databases and some strains found in other European beetles.

two *M. sartor* subspecies delimited as separate species (delimitation posterior probability ranging from 0.84 to 0.99) (Fig. 5).

3.2.3. *Wolbachia* infection

Both of the examined subspecies of *Monochamus sartor* (from the Carpathians, n = 26 and NE Poland, n = 16) were found to be infected and all tested specimens harboured *Wolbachia*. Both subspecies were infected with two *Wolbachia* strains, but each harbored different strains, so overall the species was found to be infected by four strains – all belonging to the A supergroup (Fig. 5). *Monochamus sartor urussovii* was infected by strain 1 in Białowieża and Knyszyn Forests and by strain 2 in Augustów Forest (Fig. 6). *Monochamus sartor sartor* was infected by strains 3 and 4 (Fig. 5). According to a BLAST search against the MLST database, the most similar loci were found in the following species: *Evagetes parvus* (Cresson) wasp (3 genes similar), *Ceutorhynchus obstrictus* (Marsham) weevil (2 genes similar) and *Agelenopsis naevia* (Walckenaer) spider (3 genes similar). The BLAST comparison of *Wolbachia* genes against GenBank resources showed that similar variants were found in the following species: *Ceutorhynchus obstrictus* weevil, *Oreina liturata* (Scopoli), *Altica impressicollis* (Reiche) and *Hermaeophaga mercurialis* (Fabricius) leaf beetles, *Leptopilina clavipes* (Hartig) wasp, and *Lutzomyia stewarti* (Mangabeira & Galindo) fly.

4. Discussion

This study aimed to verify whether *Monochamus sartor sartor* and *M. s. urussovii* are valid species or just subspecies. This issue has been the topic of many studies that often show contradictory conclusions (HELLRIGL 1970; ISAEV et al. 1988; BENSE 1995). The preliminary phylogenetic study of CESARI et al. (2004) rejected the distinctiveness of these subspecies, however only on the basis of very limited sampling. On the other hand, ROSSA et al. (2016) showed that these two subspecies differ significantly with respect to wing venation. Moreover, the host plant preferences of both subspecies (*M. s. sartor* is associated almost exclusively with spruces, whereas *M. s. urussovii* develops on a larger variety of hosts, especially in its Asiatic range) show that there are some ecological and possibly adaptational differences between these taxa – however, these differences could also have arisen due to phenotypic plasticity. In this study, all collected types of data (morphology, genetics and ecology) support the distinctiveness of both taxa, while simultaneously showing that their differentiation is very shallow. These integrative data suggest that species status should not be supported. On the other hand, *Monochamus sartor* could be an example of recent divergence, with a split forming between the boreal *M. s. urussovii* and mountain *M. s. sartor*. The shallow divergence and *M. s. sartor* haplotypes nested within *M. s. urussovii* are arguments against treating these two subspecies as distinct species.

But, as sister species often do not form reciprocal monophyletic clades in molecular data (Knowles & Carstens 2007), we are not able to definitively rule out that these two subspecies are in fact separate species. This issue requires some further study, like experimental crossing of members of both groups to verify if they produce offspring. If yes, the genetics, reproduction, fitness, ecology and behaviour of progeny should be examined to check if there are any postzygotic barriers supporting species status.

There are several morphological characters that distinguish *M. s. sartor* from the Central-European mountains from *M. s. urussovii* from the Eurasian semiboreal and boreal zones. These characters include density of hairs in distal parts of elytra and their punctuation (e.g. Plavil'shhikov 1958; Wallin et al. 2013). The question is whether these differences are just due to phenotypic plasticity and environmental adaptations (Grenier et al. 2016), or in fact represent phenotypic proof for the existence of two separate species. The genetic data collected in this study confirm that mountain and boreal populations of *Monochamus sartor* are characterized by different mitochondrial haplogroups and that no haplotypes are shared between subspecies, but also that haplotypes of *M. s. sartor* are nested within *M. s. urussovii,* according to the phylogenetic tree reconstruction. On the other hand, the closest haplotypes belonging to these two groups are only distant by approximately 1%. This value is much below the threshold that is usually observed between sibling species, which for Cerambycidae is higher than 4% (Nakamine & Takeda 2008; Ohbayashi & Ogawa 2009), and similar or larger interspecific distances have been observed for other closely related beetles (e.g. Kubisz et al. 2012; Montagna et al. 2016b). Within *Monochamus* sawyers, interspecific distances between *M. galloprovincialis* and *M. sutor* reach 3.1% (Koutroumpa et al. 2013). The species delimitation methods gave discordant results concerning the status of *M. s. sartor* and *M. s. urussovii*. Bayesian PTP rejected the species distinctiveness of the two taxa (while simultaneously supporting species status of the other *Monochamus* species), while BPP supported their species status. For *M. s. sartor* and *M. s. urussovii*, it is hard to tell which method gave more reliable results, since the results obtained with bPTP can only be considered putative species that should be confirmed by other methods (Zhang et al. 2013). On the other hand, the coalescence adopted by BPP can only delimit population structure and not species boundaries (Sukumarana & Knowles 2017). In any case, these contrasting results may highlight limitations associated with the use of single locus data.

Other evidence, from the analyses performed on the available sequence data, suggest that there is no separation between *M. s. sartor* and *M. s. urussovii*. For example, the low genetic distance between them is similar to the distances of 1–2% that have been observed between presumed subspecies of *M. galloprovincialis* and *M. sutor*, whose subspecies statuses have also been questioned (Koutroumpa et al. 2013). A lack of genetic support for the distinctiveness of both taxa is also indicated by the presence of the same *ef-1α* haplotype (the only one) in both *M. s. sartor* and *M. s. urussovii*, but explanation of this low (or lack) of nuclear variation needs further studies with more variable markers like microsatellites. It is possible that *Monochamus sartor* sawyers just followed the recent expansion of its host plant – spruce (Taberlet et al. 1998; Latałowa & van der Knaap 2006), which is known to be of double (boreal and mountain) origin in some areas (e.g. Białowieża Forest) (Latałowa & van der Knaap 2006; Dering & Lewandowski 2009; Tollefsrud et al. 2015; Nowakowska et al. 2017). Moreover, timber harvesting and transportation could also have facilitated passive migrations of sawyers across large distances (Etxebeste et al. 2015).

Another question in our study was to verify whether *M. s. urussovii* from Asia and from its westernmost range in Eastern Europe represent the same or distinct units. In this case, morphology and genetics show somewhat inconsistent patterns. Concerning morphology, there are important differences between these populations in females, but not males. Rossa et al. (2016) showed that wing venation of individuals from these two populations is distinct but only slightly if compared to *M. s. sartor*. They even suggested that *M. s. urussovii* from NE Poland could be hybrids between *M. s. sartor* and the Asian *M. s. urussovii*, but this could also be a result of ongoing gene flow (probably mediated by males if considering only mtDNA distinctiveness of these populations). In contrast to this, mitochondrial DNA did not indicate separation of *M. s. urussovii* populations, as haplotypes from Asia and NE Poland did not form distinct clusters and in some cases the same or highly related haplotypes were found in very distant localities. Such patterns are quite common for boreal species that have wide distributions across the Palaearctic boreal zone and which probably expanded from Asian refugia after the end of the Pleistocene glaciations following the expansion of coniferous forests. The phylogeography of some cambioxylophagous beetles follows this pattern (e.g. some bark beetles, Stauffer et al. 1999; darkling beetles, Painter et al. 2007). Moreover, the presented genetic data cannot solve the hybrid origin of *M. s. urussovii* from NE Poland, as the nuclear marker used in this study was found to be monomorphic across the entire species range. Further studies with microsatellites or single nucleotide polymorphism loci are needed to verify this hypothesis.

History of *Monochamus sartor* probably follows the history of boreal tree species, particularly spruce – its main host plant. The current range of *Monochamus sartor* is strictly associated with the range of *Picea* spp., and rarely has this species been found foraging on other conifers or birches. The genetic diversity of its two subspecies strongly indicates that they survived glacial periods in at least two refugia – in the Alps and/or in the Carpathians (*M. s. sartor*) and most probably somewhere in Asia or in Asia together with Eastern Europe (*M. s. urussovii*). Foothills of both the Alps and Carpathians (especially the Southern Carpathians) are known refugial areas for many

species, which survived there unfavorable glacial periods in so-called "cryptic" northern refugia (STEWARD & LISTER 2001; SCHMITT & VARGA 2012). Also, Eastern Europe (southern Russian Plains) and East Asia are known refugial areas for numerous continental and boreal species (STEWART et al. 2010). Low divergence between the two subspecies could suggest that their isolation occurred quite recently – probably during one of the last glacial periods. Worth noting is that during the Holocene gene flow between the two subspecies has probably not occurred or has been restricted to male-mediated dispersal.

Previous studies on the microbiota of some *Monochamus* species either found no *Wolbachia* infection like in *M. galloprovincialis* (VICENTE et al. 2013) or showed that although *M. alternatus* (Asian species) is currently not infected, it had to have been in the past as it carries some *Wolbachia* genes in its genome (AIKAWA et al. 2009, 2014). In this study, for the first time, we have confirmed the presence of *Wolbachia* in *Monochamus* species. Interestingly, we found that both subspecies of *Monochamus sartor* are infected (at least in their examined populations from the Carpathians and NE Poland). Moreover, both subspecies harbor different strains – two each, which all belong to supergroup A but are distinct from each other. The presence of different bacterial strains in the two subspecies could further indicate their distinctiveness. Discussing the role of this bacterium in subspecies formation via isolation (e.g. caused by cytoplasmic incompatibility) would be too speculative without further studies. *Wolbachia* can also be used as a biocontrol agent against some insect pests (LACEY & GOETTEL 1995; ZABALOU et al. 2004), so studies in this direction could also be interesting for controlling outbreaks of *Monochamus sartor* populations, especially with respect to its role as a vector for the pinewood nematode, *Bursaphelenchus xylophilus* (LINIT et al. 1983), a quarantine species that causes PWD (KONDO et al. 1986; MILLER et al. 2013).

5. Conclusions

Previous uncertainty on the taxonomic status of *Monochamus sartor sartor* and *M. s. urussovii*, and the Asian and European populations of the latter have been solved in this study. All the gathered types of data (morphology, genetics and ecology) indicate that these two subspecies should not be considered valid species, in contrast to what has been proposed in the past (BENSE 1995; SAMA 2002; LÖBL & SMETANA 2010). The question is whether these presumed subspecies should be considered subspecies. The data presented in this study provide several forms of evidence that despite weak divergence, the boreal and mountain populations differ with respect to their morphology, diversity of endosymbiotic bacteria and plasticity of host plant use. The evidence supports the hypothesis that they should be considered separate subspecies that split quite recently. Aside from broadening the basic knowledge on the taxonomy and genetics of *Monochamus sartor*, this study shows that any research on these sawyers needs to consider their separate subspecies status. Moreover, any plans for population management (if considering them to be forest pests) or population conservation (if considering them to be natural elements of mature forests with high shares of dead wood) of these longhorn beetles should take into account that there are two groups, which differ with respect to numerous characters and therefore could react in different ways to forest management or conservation practices.

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Electronic Supplement Files

at http://www.senckenberg.de/arthropod-systematics

File 1: plewa&kajtoch-monochamus-asp2018-electronicsupplement-1.pdf – **Table S1.** Specimens of *Monochamus sartor sartor* and *M. s. urussovii* sawyers used for morphological study. (specimen sources: DNF – Department of Natural Forest, Forest Research Institute in Białowieża; DFP – Department of Forest Protection, Forest Research Institute in Sękocin Stary; MIZ – Museum and Institute of Zoology Polish Academy of Sciences in Łomna; IFEP – Institute of Forest Ecosystem Protection, University of Agriculture in Kraków; DFPE – Department of Forest Protection and Ecology, Warsaw University of Life Sciences; priv. – private collection; codes for countries: AU – Austria, BY – Belarus, CZ – Czechia, ES – Estonia, GE – Germany, IT – Italy, JA – Japan, SK – Slovakia, SZ – Switzerland, PL – Poland, RU – Russia, SW – Sweden, UK – Ukraine).

File 2: plewa&kajtoch-monochamus-asp2018-electronicsupplement-2.pdf – **Table S2.** Characterization of *Monochamus sartor* samples used for molecular studies.