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An integrative revision of the subgenus *Liophloeodes* (Coleoptera: Curculionidae: Entiminae: Polydrusini): taxonomic, systematic, biogeographic and evolutionary insights

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Abstract

The subgenus *Liophloeus* Weise, 1894 of *Liophloeus* Germar, 1817 (Coleoptera: Curculionidae: Entiminae: Polydrusini) consists of five morphologically similar species traditionally diagnosed based on the shape of the aedeagus. However, traits of the genital apparatus exhibit substantial and overlapping inter- and intraspecific variation. All five species have the same ecological requirements and occur in central and eastern Europe, mostly in montane areas. The focus of this work was to verify the taxonomic status and validity of *Liophloeodes* species using a combination of molecular and morphometric techniques. Specimens were collected from the entire distribution range and initially assigned to a species according to the aedeagal shape. Genetic diversity and phylogeny of the subgenus were studied using three molecular markers (two ribosomal, 28S-D2 and ITS2, and one mitochondrial, COI). Moreover, several morphological characters were used for multivariate morphometric analyses. Finally, presence and prevalence of bacterial endosymbionts among species were investigated. Phylogenies based on ribosomal markers suggest that traditional species are correctly delimited, whereas COI phylogeny suggests hybridization and introgression occurring between *Liophloeodes* species. Morphometric analyses confirmed low interspecific diversity. Two major bacterial endosymbionts, *Rickettsia* and *Wolbachia*, were detected in many populations. We argue that *Liophloeodes* consists of young lineages whose evolution and diversification was possibly mediated by cyclic climate change events.

Key words

molecular markers, morphometry, phylogeny, taxonomy, weevils.

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Introduction

Integrative taxonomy is a relatively new approach based on the idea that results obtained using different methods should be integrated to increase robustness of taxonomic hypotheses (Dayrat 2005; Schlick-Steiner et al. 2010; Yeates et al. 2011), to which a degree of expected stability may be associated (Padial et al. 2010). Over the last 15 years, this integrative approach has become the most popular (and useful) taxonomic method, giving robust, reliable and often unexpected results for many groups of organisms (Miralles et al. 2011; Schutze et al. 2017; Vitecek et al. 2017, Stec et al. 2020a; Stec et al. 2020b). Integrated data include not only those obtained by "traditional" morphological and "modern" molecular methods, but also rigorous statistical testing of detailed morphometric measurements as well as ecological and biogeographical data, and even infection by microorganisms that may affect the organisms' biology (Gebiola et al. 2012).

Weevils (Coleoptera: Curculionoidea) are one of the most diverse groups of living organisms (McKenna et al. 2009) with more than 60,000 known species (of which over 50,000 belong to the family Curculionidae), and many ecological forms that evolved over millions of years of coevolution with (mostly angiosperm) plants (Oberprieler et al. 2007). Due to this huge morphological diversity, weevils represent a big challenge for taxonomists. Traditional taxonomy has been verified by molecular markers and by phylogenomic data at the level of subfamilies (Marvaldi et al. 2002; Shin et al. 2018). However, phylogenies and taxonomies of many tribes and genera are still poorly known and mostly unresolved. Integrative taxonomy as a tool that allows combining morphological knowledge with molecular data has proven helpful for studies that focus on weevils (Grobler et al. 2006; Toševski et al., 2014; Brown 2017).

The genus *Liophloeus* Germar, 1817 (Coleoptera: Curculionidae) includes two subgenera: *Liophloeus sensu stricto* and *Liophloeodes* Weise, 1894 (Fig. 1). *Liophloeus s.s.* consists of three species comprising both bisexual and parthenogenetic populations, and its range is much wider than *Liophloeodes*, as it covers the most of Europe (including Scandinavia and British Isles) and has wider ecological requirements (it can occur both in the cold and wet biotopes and in the warmer habitats in the lowlands). All *Liophloeodes* species are exclusively bisexual, and their geographic range overlaps with only one species of *Liophloeus s.s.*, *L. tessulatus.* Here we examined the morphological diversity and phylogenetic systematics of the subgenus *Liophloeodes.* Systematics of *Liophloeodes* has undergone many changes over time because of the extreme morphological similarity that is noted between its taxa, whereas the distinction between this subgenus and *Liophloeus s. s.* is straightforward, even in the field. The basis for current taxonomy of *Liophloeodes* is the work by Weise (1894), who established a subgenus that included the following group of species: *Liophloeus* (*Liophloeodes*) *schmidti* Boheman, 1842, *Liophloeus* (*Liophloeodes*) *lentus* Germar, 1824, *Liophloeus* (*Liophloeodes*) *chrysopterus* Boheman, 1842, *Liophloeus* (*Liophloeodes*) *gibbus* Boheman, 1842 and *Liophloeus* (*Liophloeodes*) *liptoviensis* Weise, 1894, which can be distinguished only by the shape of the aedeagus. Later, this nomenclature has been modified a few times (Apfelbeck 1928; Petri 1912; Reitter 1916). Smreczyński (1958) differentiated two species: *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *pupillatus* Apfelbeck, 1928, and at the same time split the first taxon into several distinct subspecies [*Liophloeus* (*Liophloeodes*) *lentus lentus*, *Liophloeus* (*Liophloeodes*) *lentus gibbus*, *Liophloeus* (*Liophloeodes*) *lentus liptoviensis*, *Liophloeus* (*Liophloeodes*) *lentus herbstii and Liophloeus* (*Liophloeodes*) *lentus ovipennis* with uncertain status]. More than 20 years later, Dieckmann (1980) elevated *Liophloeus* (*Liophloeodes*) *lentus* subspecies to the species level [except for uncertain *Liophloeus* (*Liophloeodes*) *lentus ovipennis*]. Currently, the subgenus *Liophloeodes* comprises five nominal species [it includes four Smreczynski's subspecies – except for *Liophloeus* (*Liophloeodes*) *lentus ovipennis* – and *Liophloeus* (*Liophloeodes*) *pupillatus*] whose taxonomy is rather poorly understood, and identification, based mainly on shape of male genitalia (Fig. 2) is considered extremely challenging. Furthermore, many formerly described taxa, despite being later synonymized, can still be found in faunistic surveys and species checklists which underlines even more the need for a taxonomic revision.

All *Liophloeodes* species prefer wet and cold biotopes and their host plants are species from the families Apiaceae (*Aegopodium* spp., *Chaerophyllum* spp., *Heracleum* spp.), Asteraceae (*Petasites* spp., *Tussilago* spp.) and Urticaceae (*Urtica* spp.). They can be found near streams and rivers in the mountains or sub-mountainous areas in south-eastern Europe (Fig. 3), across the whole Carpathians, eastern Alps, Dinaric Alps, Balkan Mountains, the Sudetes, and small montane chains in Pannonian Basin. Species belonging to this subgenus are partially sympatric. Their ecological and geographical similarity matches the low level of morphological differentiation among species. A general problem with the morphology of *Liophloeodes* is the lack of reliable diagnostic characters that would allow for confident species identification. The only diagnostic trait is the shape of the aedeagus; hence, females can only be distinguished by association with males from the same population. However, even identifying males can be problematic, due to the high intraspecific and low interspecific phenotypic plasticity of aedeagus. The diversity of *Liophloeodes* could be described more as a gradient of differences between species (Smreczyński 1958). Another major problem with *Liophloeodes* taxonomy is the limited knowledge about populations living south of the Pannonian Basin and, more generally, about their biology. The only exception is a study on Microsporidia infecting some Polish populations of *Liophloeus* (*Liophloeodes*) *lentus* (Ovcharenko et al. 2013).

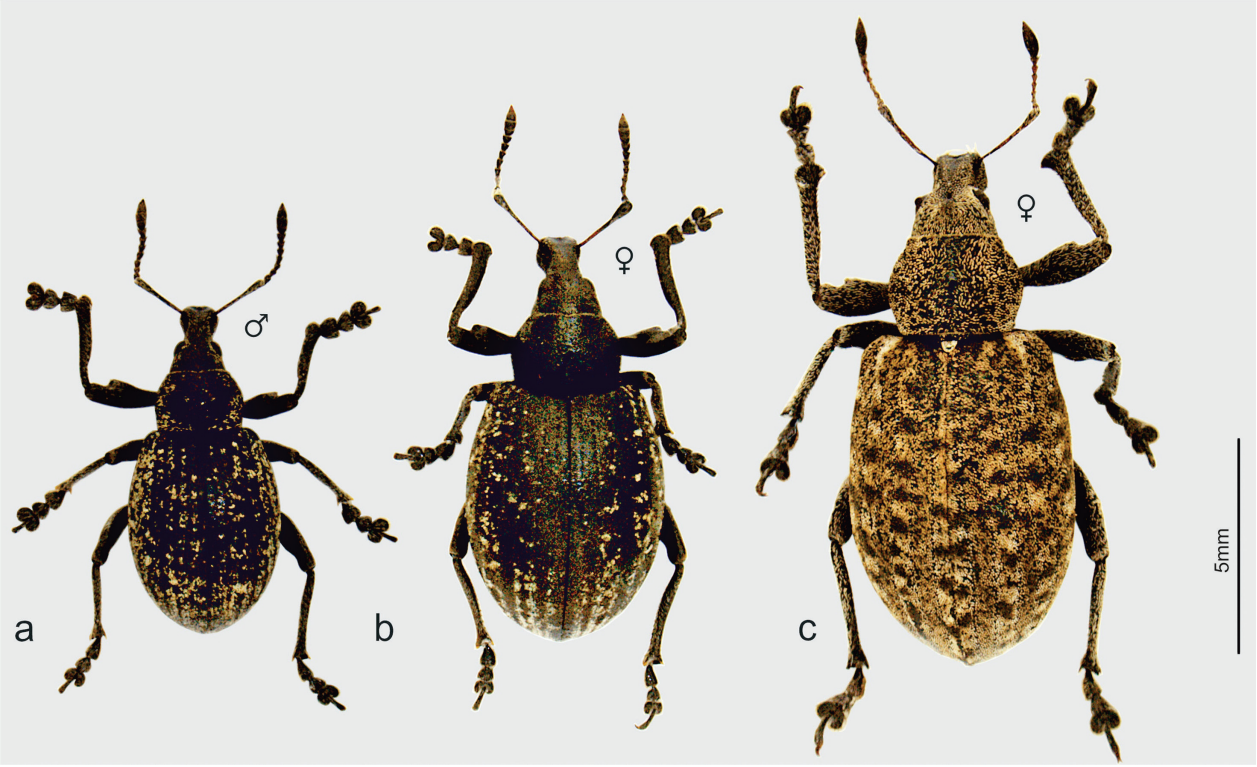

Figure 1. Habitus of *Liophloeus* (*Liophloeodes*) *lentus* male (a) and female (b) and *Liophloeus tessulatus* female (c). Scale bar: 5 mm.

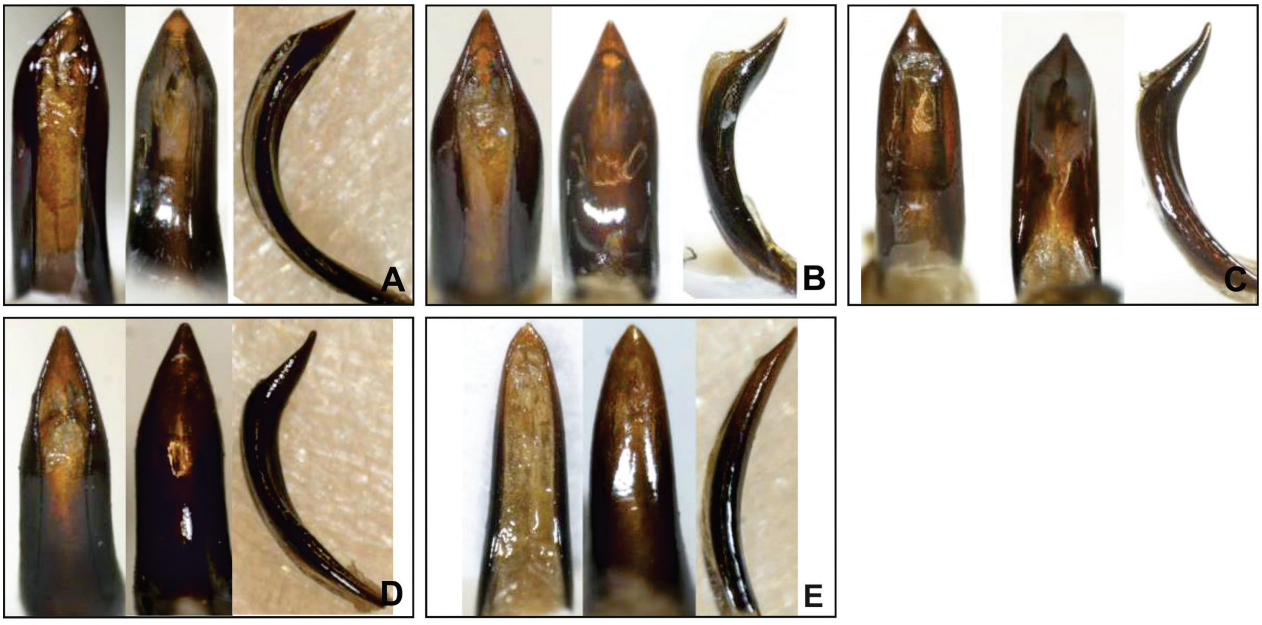

Figure 2. Aedeagi of *Liophloeus* (*Liophloeodes*) species in dorsal, ventral, and lateral views. **A**- *Liophloeus* (*Liophloeodes*) *herbstii,* **B** – *Liophloeus* (*Liophloeodes*) *gibbus*, **C** – *Liophloeus* (*Liophloeodes*) *lentus*, **D** – *Liophloeus* (*Liophloeodes*) *liptoviensis*, **E** – *Liophloeus* (*Liophloeodes*) *pupillatus*.

Taking into consideration all mentioned issues concerning these weevils, their systematics can be considered as highly uncertain and should be verified by integrative taxonomy, using morphological, molecular and morphometric data. Using traditional aedeagus shape-based species identification as a starting hypothesis, the diversity of *Liophloeodes* species from the entire known distribution range was iteratively assessed by a combination of morphological and molecular examination. Three molecular (two ribosomal, one mitochondrial) markers were used as distinct lines of evidence. The morphometric measurements were also taken and analysed as another independent method. Additionally, endosymbionts occurrence and phylogeny has been shown to be another potentially important line of evidence to support differences between species (Gebiola et al. 2012). Endosymbiont research

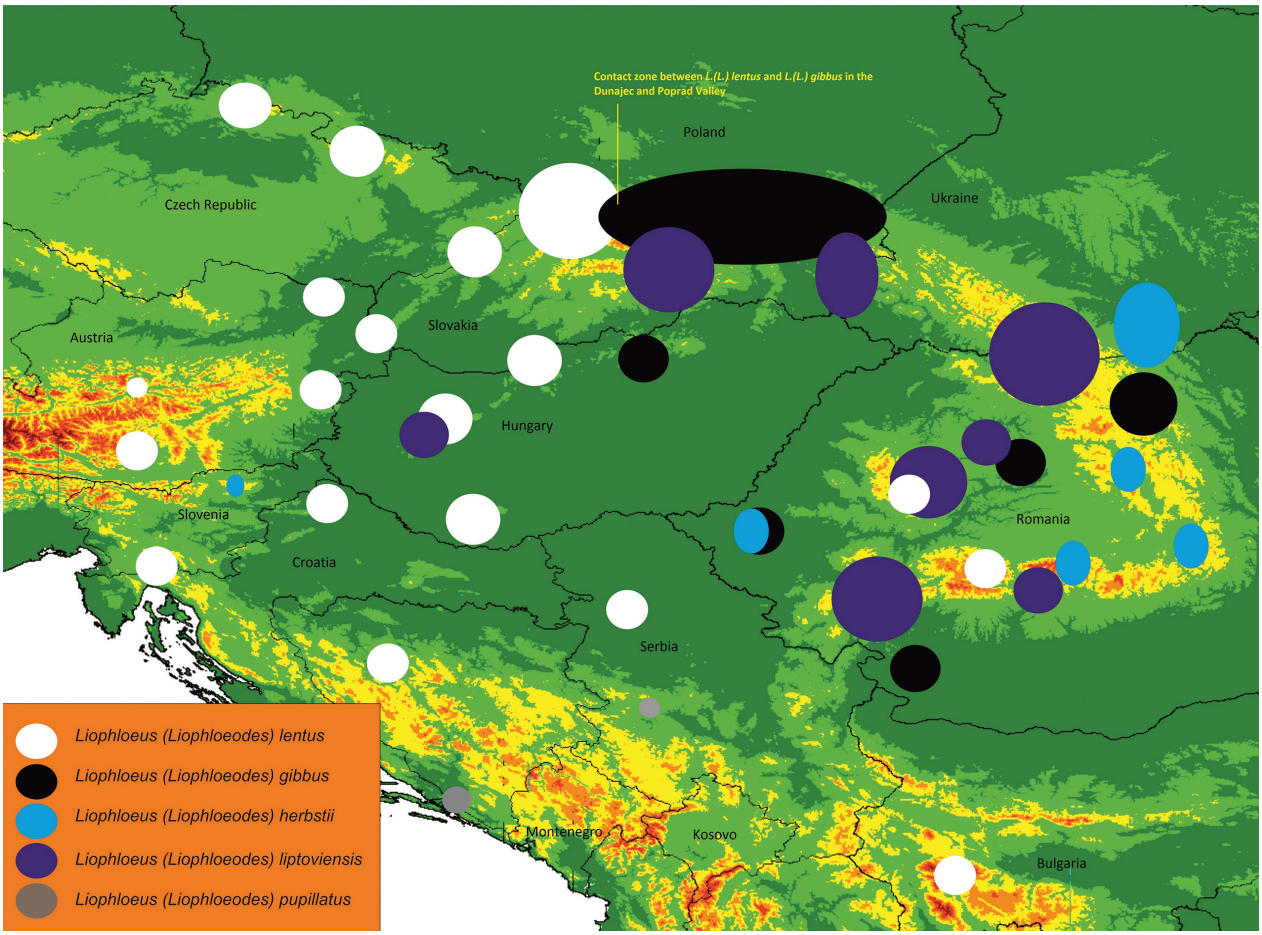

Figure 3. Geographical distribution of the species belonging to the genus *Liophloeus* (*Liophloeodes*) and contact zone between *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus* in Dunajec and Poprad valley.

could also deliver additional information about possible paths of evolution, diversification and speciation within the taxon, as symbionts may have an impact on reproduction isolation by causing reproductive manipulations (Shropshire et al. 2020).

Materials and Methods

Material collection

Specimens were collected in 2009–2010 in Poland and Slovakia, and in 2013–2017 in Poland, Slovakia, the Czech Republic, Austria, Slovenia, Croatia, Bosnia and Herzegovina, Serbia, Hungary, Romania, Bulgaria, and Ukraine (Table S1, Fig. 3). Specimens (481 individuals, now deposited in the Department of Entomology, Jagiellonian University) were collected by hand or using a sweeping net, immediately put in 95% alcohol, transferred to the laboratory and stored at -20°C until use. The collection sites were determined using faunistic literature [mainly Smreczyński (1958), but also local faunistic papers] and knowledge about preferred landscape and environment. Most specimens were collected in the valleys, near streams and rivers in wet and cold biotopes, in plant communities, mainly consisting of Apiaceae, but also *Petasites* spp. and *Urtica* spp.

Liophloeus (*Liophloeodes*) *ovipennis*, which was described based on a single specimen collected in the French Alps, is probably a misidentified weevil belonging to *Liophloeus s.s.*, because there are no other data about the occurrence of *Liophloeodes* in this part of Europe. Specimens of *Liophloeus tessulatus* occurring in the sampling areas were also collected to be included in phylogenetic analyses, to help understand interspecific phylogenetic relationship in the genus.

Molecular techniques

DNA was isolated from whole insect bodies. Before the extraction, the abdomen of every specimen was poked laterally with a sterile needle to facilitate DNA extraction. Isolation was made using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's instructions. Three molecular markers were amplified for *Liophloeodes*: two ribosomal: 28S-D2 (GenBank accession: MN190722-MN191039) and ITS2 (GenBank accession: MN191040-MN191233) and one mitochondrial: the standard COI barcoding region (GenBank accession: MT858362-MT858668), using primers as in Table 1. A nested PCR was used to amplify bacterial DNA, by per-

Table 1. Primers used in this study.

DNA marker	Primers	References
COI	LCO1490 / HC02198	(Folmer et al. 1994)
28S-D2	D2F / D2R	(Campbell et al. 1994)
ITS2	LC1 / HC2	(Navajas et al. 1992)
16S	27F / 1513R	(Weisburg et al. 1991)
16S *Spiroplasma*	27F / TKSSsp	(Fukatsu and Nikoh 2000)
16S *Cardinium*	CLOF / CLOR	(Weeks et al. 2003)
16S *Arsenophonus*	27F / ARS16SR	(Tsuchida et al.2002)
16S *Rickettsia*	Rb-F / Rb-R	(Gottlieb et al., 2006)
wsp *Wolbachia*	wsp_F1 / wsp_R1	(Baldo et al., 2006)
ftsZ *Wolbachia*	ftsZ_F1 / ftsZ_R1	(Baldo et al., 2006)
16S *Microsporidia*	V1 / 1492	(Vossbrinck and Friedman 1989; Zhu et al. 1993)
16S *Nardonella*	16SA1F / Nard733R	(White et al., 2015)

forming the first PCR using generic primers targeting the 16S gene (GenBank accession MN621120–MN621139), followed by a second PCR with primers specific to *Wolbachia*, *Arsenophonus*, *Rickettsia*, *Spiroplasma*, *Cardinium*, *Nardonella* and Microsporidia. For *Wolbachia*, ftsZ (GenBank accession MT500574–MT500577) and *wsp* (GenBank accession MT611140–MT611153) genes were amplified with primer pairs listed in Table 1. The PCR reaction was conducted using the following mix: 11.5 µl of ddH_2O, 2 µl of 10X DreamTaq buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 uM for each primer, 0.4 µl of 10 mM dNTPs, 0.1 µl of 5 U/µl Taq polymerase and 2 µl DNA. PCR products were sent for sequencing to the companies HongKe XiLin Biotechnology Co (China) and Macrogen (Netherlands). Obtained sequences were visually analysed and edited using SeqMan (Swindell and Plasterer 1997) or BioEdit (Hall 1999). COI sequences were translated into amino acids using the ExPASy translate tool (Gasteiger et al. 2003). Sequences were aligned by MAFFT (Katoh 2002) using the G-INS-1 algorithm. Due to many insertions and deletions, the ITS2 dataset was aligned using Fastgap (Borchsenius 2009), which allows for coding indels as traits for Bayesian analysis. *Pseudomeira obscura* Solari & Solari, 1907 (GenBank accessions HE818408 and HE818407) and *Eusomus ovulum* Germar, 1824 (KU341552 and MH746366), both from the tribe Entiminae were selected as outgroups for phylogenetic analyses based on nuclear markers, because both ITS2 and 28S-D2 were available for these species. For COI, *E. ovulum* (KU341536) was used along with *Graptus triguttatus* (Fabricius, 1775) (KY110616) and *Prothrombosternus tarsalis* Voss (1965) (KU748541), from the tribes Entiminae and Molytinae, respectively. For the phylogenetic reconstruction of *Rickettsia* symbionts, homologous sequences of several *Rickettsia* strains available in GenBank used are reported in Table S5. Evolutionary models for each alignment and the best partitioning scheme were chosen using PartitionFinder (Lanfear et al. 2017). Phylogenetic reconstructions were obtained by Bayesian inference using MrBayes v 3.2 with 1 cold and 3 heated Markov chains for 10,000,000 generations, and trees sampled every 1000th generation. (Huelsenbeck and Ronquist 2001) – there have been built tree for every marker and also tree from concatenated nuclear markers. Obtained trees were visualized using FigTree 1.4.3 (Rambaut 2009) and graphically edited using CorelDraw Graphic Suite X7. Genetic distances within and between lineages were calculated using the *p*-distance method in MEGA 6 (Tamura et al. 2013). Phylogeography of *Liophloeodes* populations based on COI was inferred by statistical parsimony using the software TCS (Clement et al. 2000). The network was graphically edited using the software PopArt (Leigh and Bryant 2015) to better visualize taxonomic and geographic signals.

Morphological and morphometric study

After identifying the species based on the male aedeagus morphology (Fig. 2) every female *Liophloeodes* specimen from the same area was initially assigned to the same species, except for areas where males of two species were collected (those females were classified only as *Liophloeodes*). All collected specimens of *Liophloeus sensu stricto* were identified as *Liophloeus tessulatus*, based on the morphology and the geographical ranges of the species and were female (there are no bisexual populations in the sampling areas, see Smreczynski 1958).The specimens were dissected and their elytra, heads, antennae, legs, pronota, abdomens and spermathecae (females) were glued on cardboard and measured using a Nikon SMZ1500 binocular microscope and the NIS Elements BR 2.30 software (Fig. S1). Due to the destruction of some structures while collecting or mounting, and consequent lack of some measurements, missing data were replaced by the mean of measurements from a particular trait and species (Arbour and Broun 2014). Results of the measurements were presented separately for both sexes using Principal

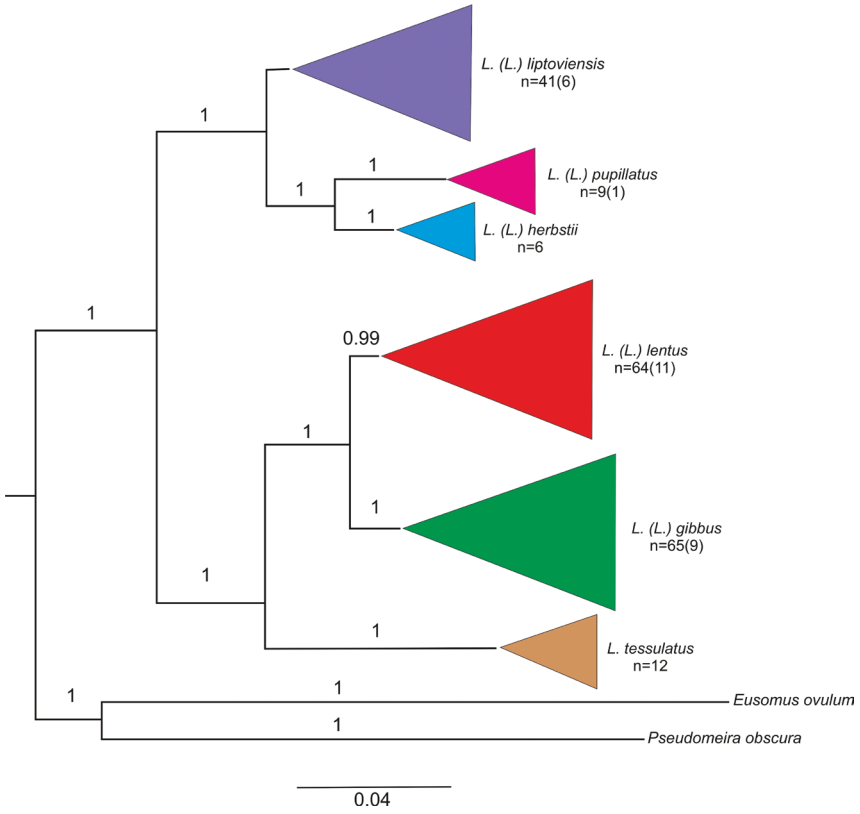

Figure 4. Bayesian phylogenetic tree based on the ITS2 marker. (GenBank accessions: MN191040–MN191233). The first number in each collapsed clade is the total number of specimens sequenced, the number in parenthesis indicates unidentified specimens (from populations where there were no males). Numbers above branches represent posterior probabilities. *Eusomus ovulum* and *Pseudomeira obscura* were used as outgroups.

Component Analysis (PCA) in the R environment using a modified script (Baur and Leuenberger 2011). Results of PCA were analysed using the Generalized Linear Model (GLM) method with factor scores as dependent variables and species as the predictor (Kuszewska and Woyciechowski 2015). Principal components that explained most of the variance were used in the analysis. When differences were statistically significant, the Tukey post-hoc test was performed to assess differences between species.

Results

Phylogenetic trees and networks

Phylogenies based on 28S-D2 (581 bp) and ITS2 (750 bp – length of full alignment) were consistent with morphological identification based on the aedeagal shape (Fig. 2), with each clade including male specimens assigned to only one particular species (along with the females from the same collection area, where available) (Figs 4–6). However, topologies of trees and networks differed by markers. ITS2 tree (Fig. 4) was divided into two main sister clades: [*Liophloeus tessulatus* + [*Liophloeus* (*Liophloeodes*) *lentus* +*Liophloeus* (*Liophloeodes*) *gibbus*)]] and [*Liophloeus* (*Liophloeodes*) *liptoviensis* + [*Liophloeus* (*Liophloeodes*) *pupillatus* + *Liophloeus* (*Liophloeodes*) *herbstii*]]. However, 28S tree topology (Fig. 5) was different with three clades: [*Liophloeus* (*Liophloeodes*) *lentus*], [*Liophloeus* (*Liophloeodes*) *gibbus*], [*Liophloeus* (*Liophloeodes*) *liptoviensis* + *Liophloeus* (*Liophloeodes*) *pupillatus* + *Liophloeus tessulatus*] and unresolved *Liophloeus* (*Liophloeodes*) *herbstii.* Heterozygosity (double peaks in the chromatograms) was detected at diagnostic polymorphic sites of 28S and ITS2 sequences of specimens occupying contact zones (Fig. S4). Combined tree from both nuclear markers was, similarly to ITS2 tree divided into two main clades: [*Liophloeus tessulatus* + [*Liophloeus* (*Liophloeodes*) *lentus* + *Liophloeus* (*Liophloeodes*) *gibbus*]] and [*Liophloeus* (*Liophloeodes*) *liptoviensis* + [*Liophloeus* (*Liophloeodes*) *pupillatus* + *Liophloeus* (*Liophloeodes*) *herbstii*]] (Fig.6).

Phylogeny based on the 650-bp COI alignment was incongruent with traditional taxonomy and nuclear phylogenies (Fig. S2, Figs 7–11). *Liophloeus tessulatus* was the only monophyletic clade (Fig. S2, Fig. 7, clade A). Three clades corresponding to three morphological species, [*Liophloeus* (*Liophloeodes*) *liptoviensis*, *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus*)] could be distinguished (Fig. S2), yet each of those clades also included specimens from the other species (at least specimens from two species in one clade). The *Liophloeus* (*Liophloeodes*) *liptoviensis* clade includes specimens from this species along with specimens from *Liophloeus* (*Liophloeodes*) *gibbus*, *Liophloeus* (*Liophloeodes*) *herbstii* and *Liophloeus* (*Liophloeodes*) *pupillatus*

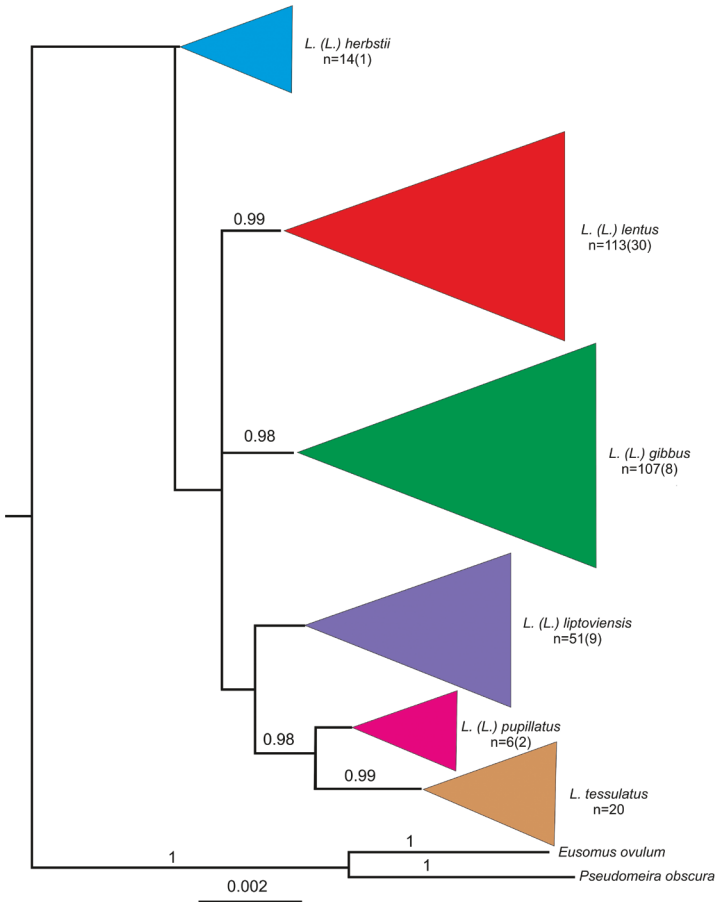

Figure 5. Bayesian phylogenetic tree based on the 28S-D2 marker (GenBank accessions: MN190722–MN191039). The first number is the number of specimens in the clade, the number in parenthesis is the number of unidentified specimens (from populations where there were no males). Numbers above branches represent posterior probabilities. *Eusomus ovulum* and *Pseudomeira obscura* were used as outgroups.

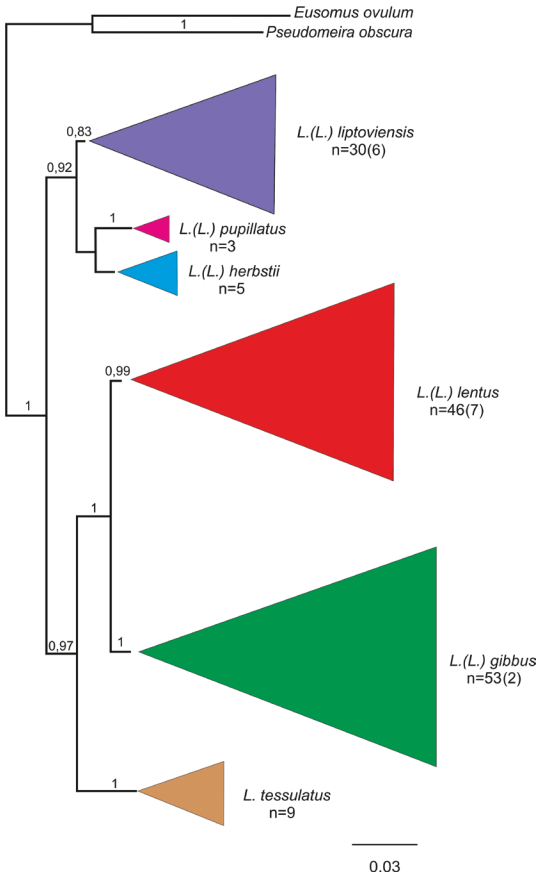

Figure 6. Bayesian phylogenetic tree based on the combined markers 28S-D2 and ITS (GenBank accessions: MN190722–MN191039 and MN191040–MN191233). The first number is the number of specimens in the clade, the number in parentheses is the number of unidentified specimens (from populations where there were no males). Numbers above branches represent posterior probabilities. *Eusomus ovulum* and *Pseudomeira obscura* were used as outgroups.

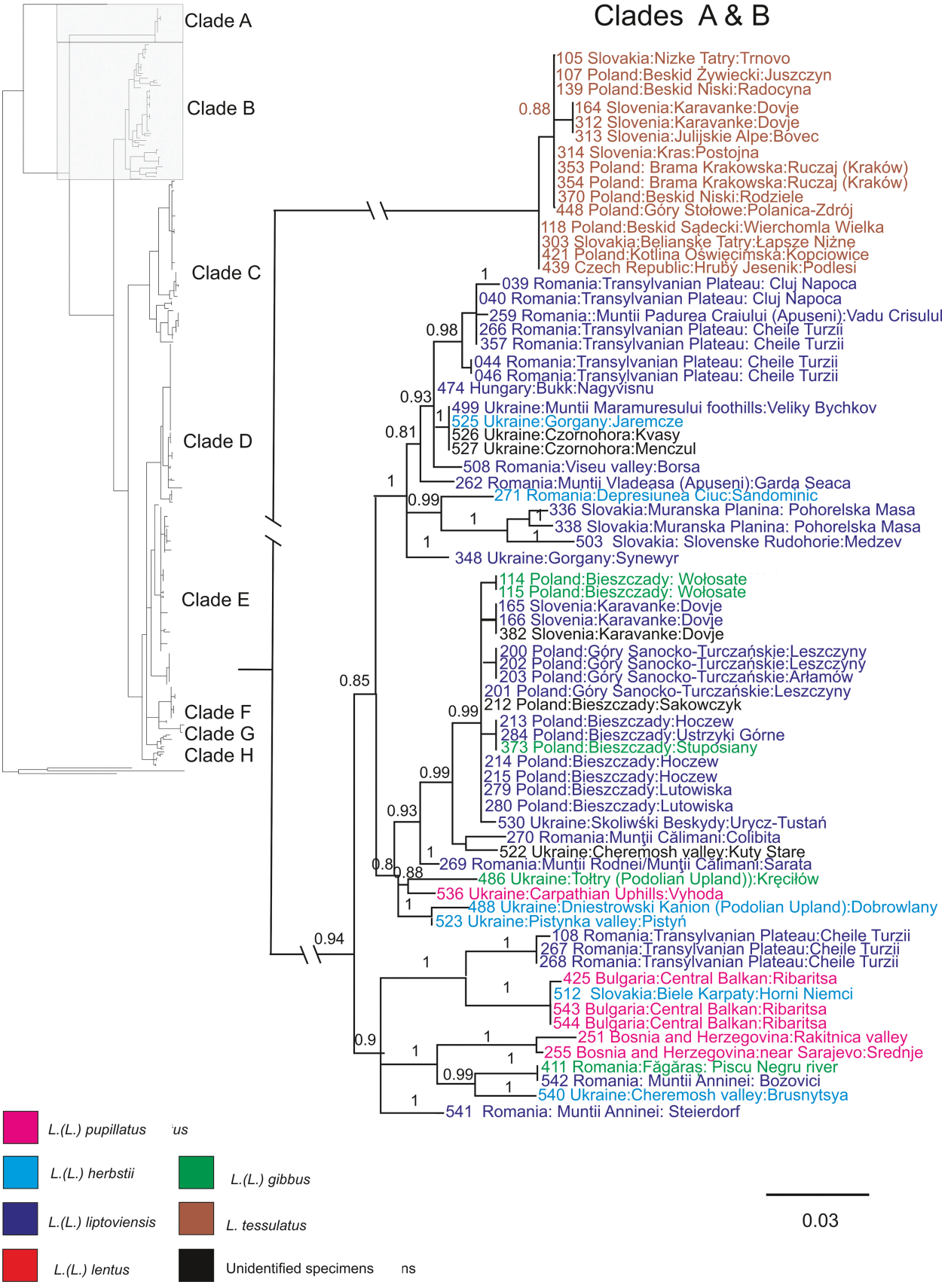

Figure 7. Bayesian phylogenetic tree of the subgenus *Liophloeodes* based on COI sequences (GenBank accession: MT858362-MT858668): *Liophloeus tessulatus* and *Liophloeus* (*Liophloeodes*) *liptoviensis*, clades A and B.

from neighbour populations (Fig. 7, clade B). Also, in the *Liophloeus* (*Liophloeodes*) *lentus* clade, we can find specimens from neighbouring populations of *Liophloeus* (*Liophloeodes*) *liptoviensis* (Fig. 8, clade C). *Liophloeus* (*Liophloeodes*) *gibbus* clade includes few subclades (Figs 9–11, clades D–H), one of them (Fig. 9, clade D) most-

Figure 8. Bayesian phylogenetic tree of the subgenus *Liophloeodes* based on COI sequences: *Liophloeus* (*Liophloeodes*) *lentus,* clade C.

ly contains *Liophloeus* (*Liophloeodes*) *lentus* specimens from Poland, localized western from the contact zone between *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus* (Dunajec and Poprad valley, Fig. 3). *Liophloeus* (*Liophloeodes*) *herbstii* and *Liophloeus* (*Liophloeodes*) *liptoviensis* can also be found in the *Lio-*

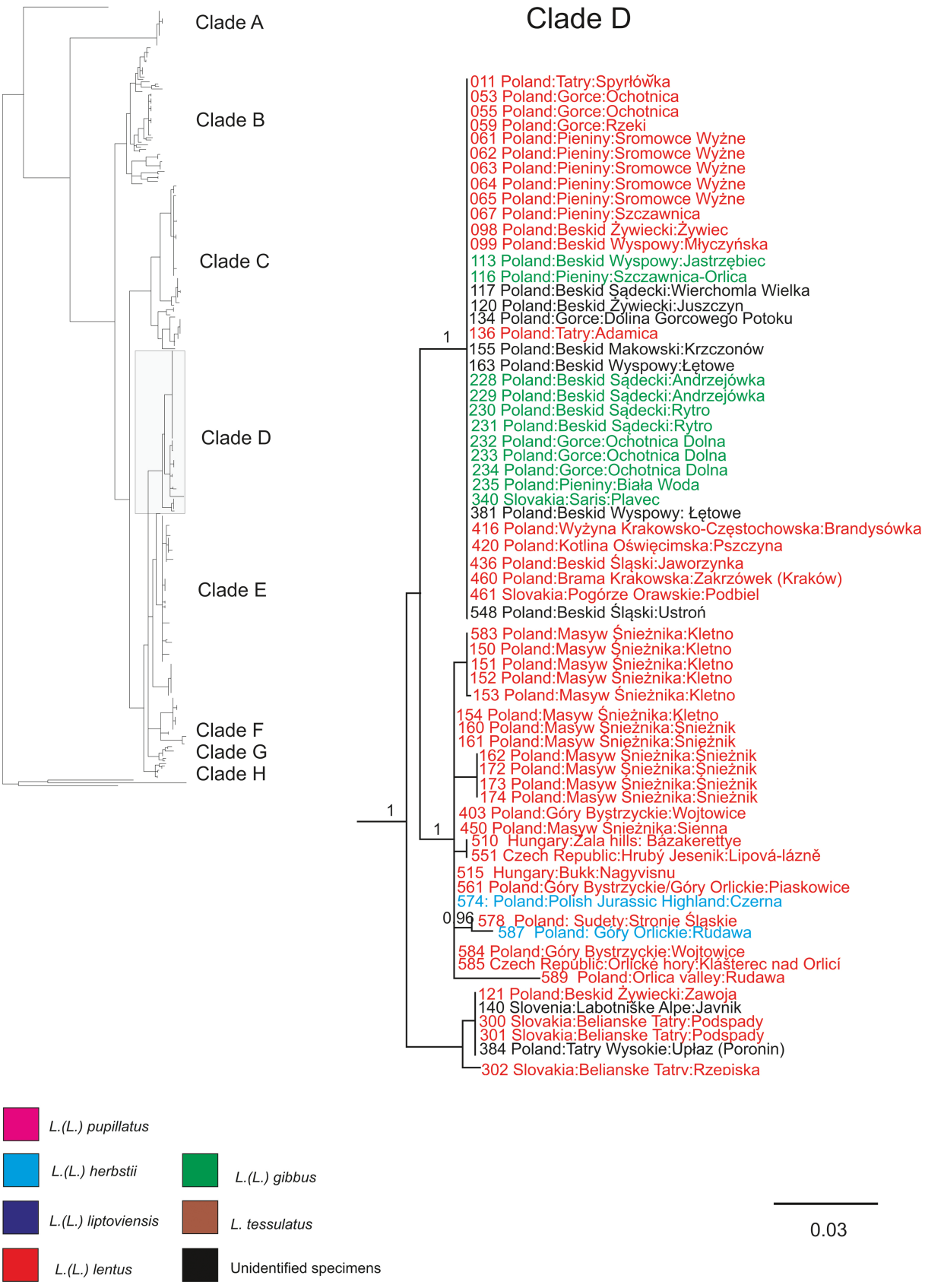

Figure 9. Bayesian phylogenetic tree of the subgenus *Liophloeodes* based on COI sequences: *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus,* clade D.

phloeus (*Liophloeodes*) *gibbus* clade. In some clades, a geographical structure could be seen. For example, there were subclades of *Liophloeus* (*Liophloeodes*) *liptoviensis* clade that included *Liophloeus* (*Liophloeodes*) *liptoviensis* specimens from Romania, Ukraine, or Polish Western Carpathians, along with specimens from different

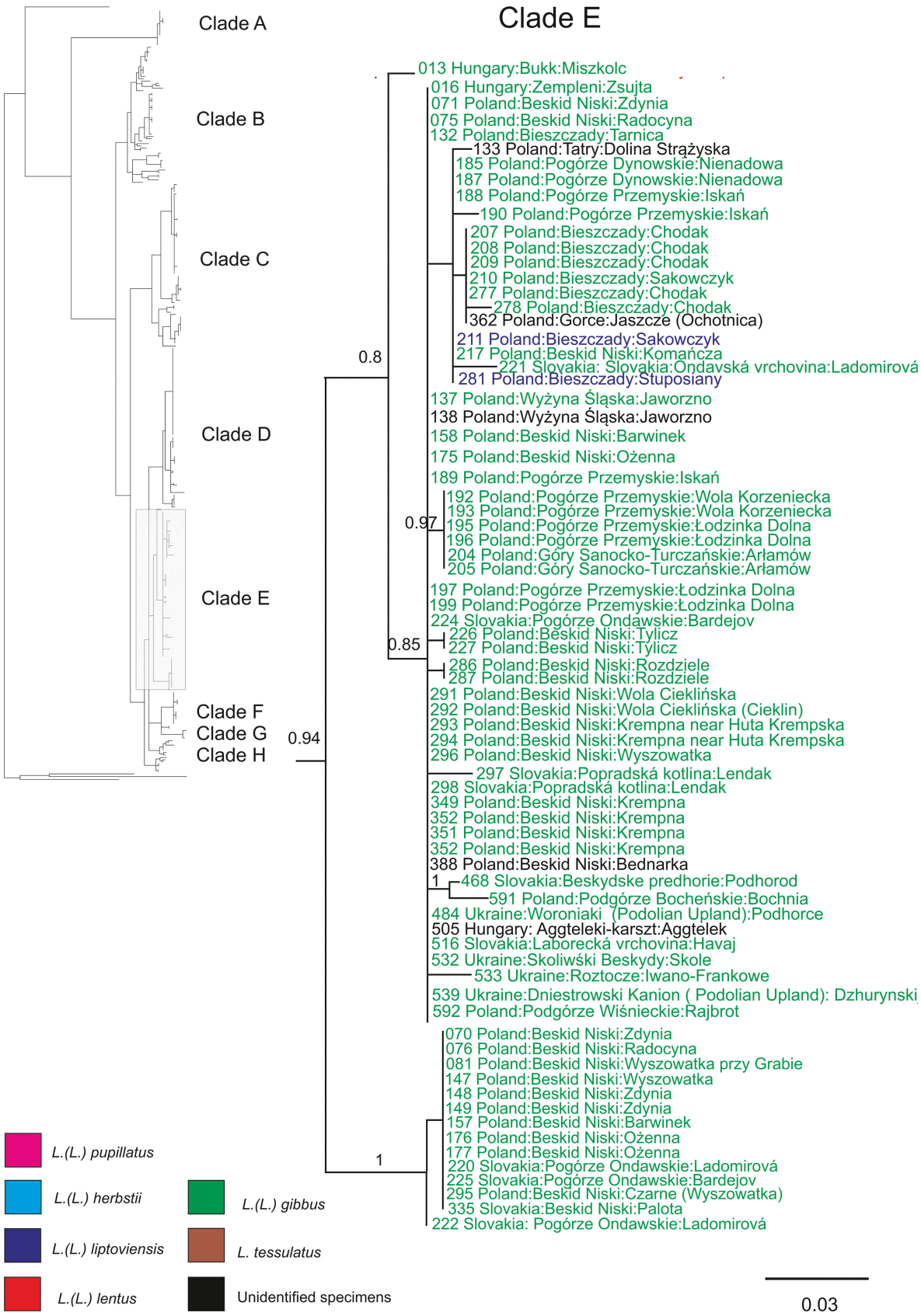

Figure 10. Bayesian phylogenetic tree of the subgenus *Liophloeodes* based on COI sequences: *Liophloeus* (*Liophloeodes*) *gibbus,* clade E.

species from the same areas (Fig. 7, clade B). Similarly, all *Liophloeus* (*Liophloeodes*) *lentus* from Slovakia formed one big subclade, which also included specimens of *Liophloeus* (*Liophloeodes*) *liptoviensis* from Slovakia (Fig. 8, clade C). All Polish populations of *Liophloeus* (*Liophloeodes*) *lentus* clustered in one of the *Liophloeus*

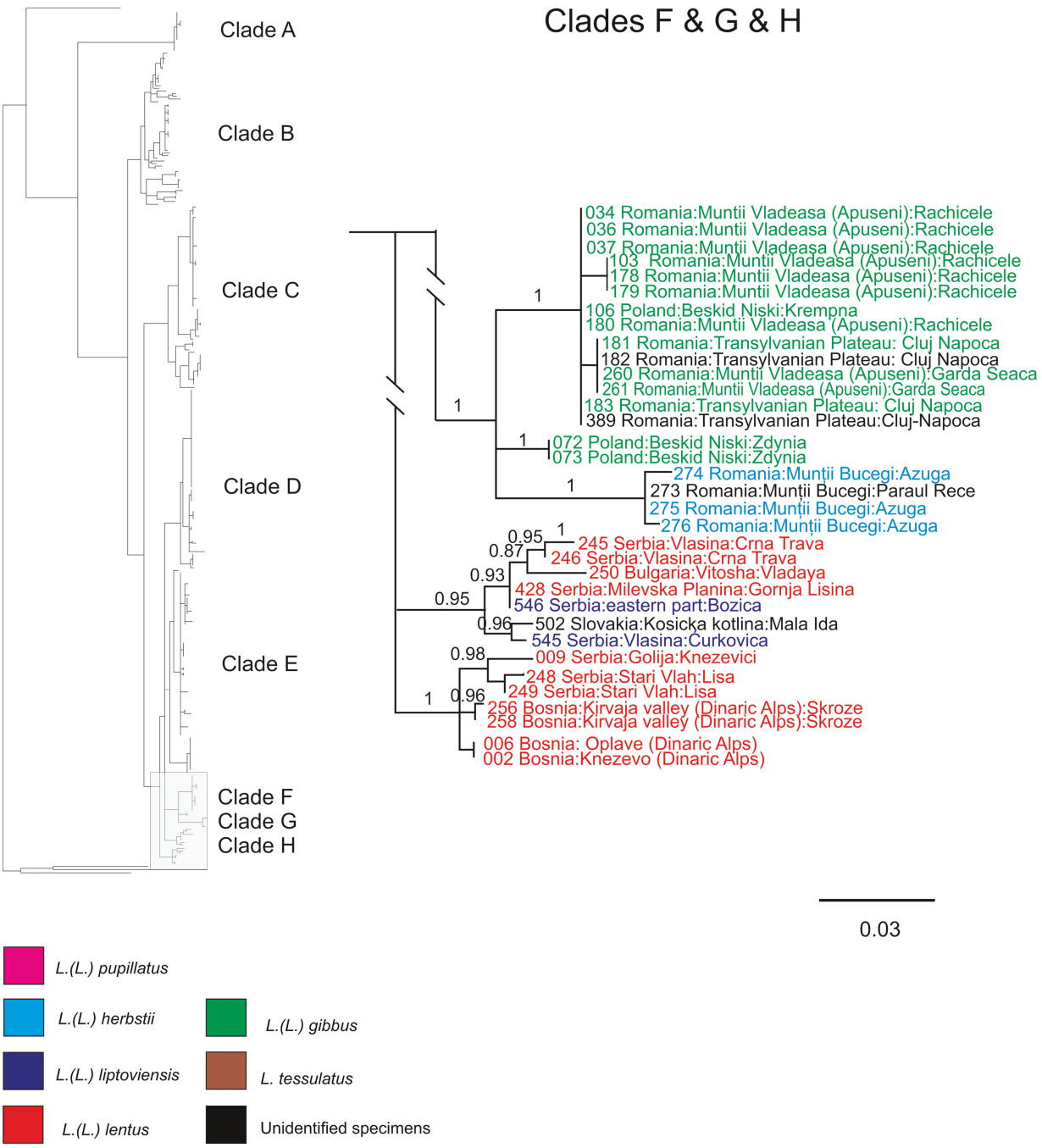

Figure 11. Bayesian phylogenetic tree of the subgenus *Liophloeodes* based on COI sequences *Liophloeus* (*Liophloeodes*) *gibbus* and *Liophloeus* (*Liophloeodes*) *lentus,* clades F&G&H.

(*Liophloeodes*) *gibbus* subclades, along with some Polish *Liophloeus* (*Liophloeodes*) *gibbus* specimens (Fig. 9, clade D). All and *Liophloeus* (*Liophloeodes*) *pupillatus* specimens gathered in the *Liophloeus* (*Liophloeodes*) *liptoviensis* clade (Fig. 7, clade B). The COI statistical parsimony networks (Figs. 12a, 12b) also confirmed the presence of a strong geographic signal, with different species connected by reticulation events.

Genetic distances

For all three markers and all *Liophloeodes* species, the highest proportions of differing nucleotides were found when they were paired with *Liophloeus tessulatus*. The most differing species among *Liophloeodes* species was *Liophloeus* (*Liophloeodes*) *liptoviensis*, however the differences within this subgenus were minor. For COI: minimum *Liophloeus* (*Liophloeodes*). *liptoviensis-Liophloeus* (*Liophloeodes*) *pupillatus* 0.9%, maximum *Liophloeus* (*Liophloeodes*) *herbstii-Liophloeus* (*Liophloeodes*) *lentus* 8.6%; for 28S-D2: minimum: *Liophloeus* (*Liophloeodes*) *herbstii-Liophloeus* (*Liophloeodes*) *gibbus* 0.4%, maximum *Liophloeus* (*Liophloeodes*) *pupillatus-Liophloeus* (*Liophloeodes*) *lentus* 2.1%, *Liophloeus* (*Liophloeodes*) *lentus-Liophloeus* (*Liophloeodes*). *liptoviensis* 2.1%; for ITS minimum: *Liophloeus* (*Liophloeodes*) *liptoviensis-Liophloeus* (*Liophloeodes*) *lentus* 3.6%, maximum: *Lio-*

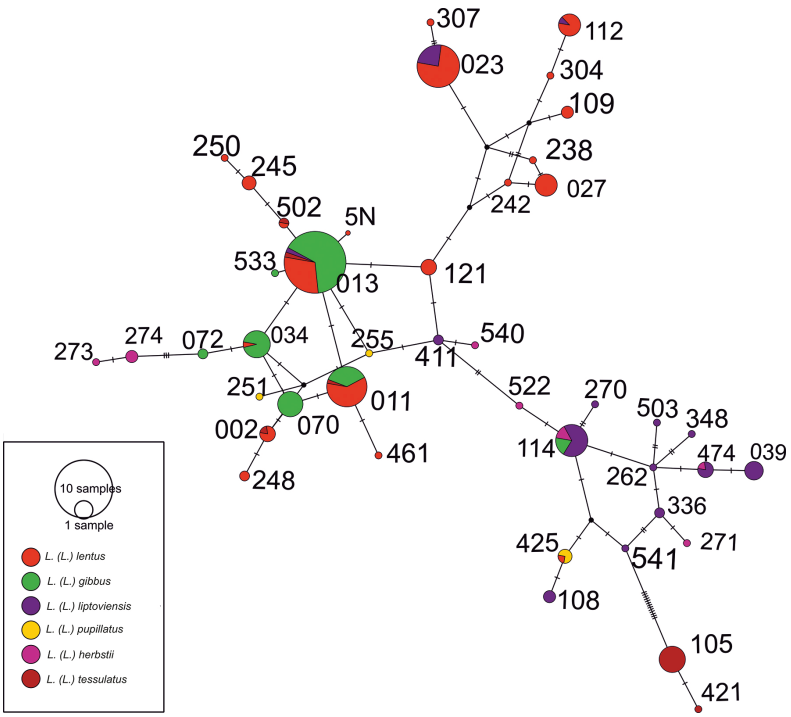

Figure 12a. TCS network inferred from mitochondrial sequences, with groups corresponding to species. The relative size of circles is proportional to the number of sequences of the same haplotype.

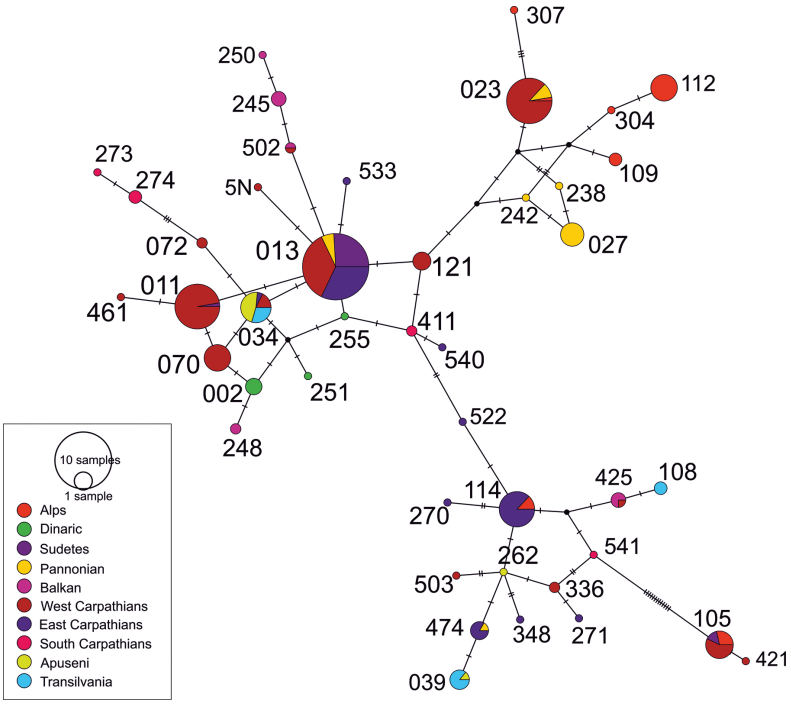

Figure 12b. TCS network with groups corresponding to geography.

phloeus (*Liophloeodes*) *gibbus-Liophloeus* (*Liophloeodes*) *pupillatus* 19.7%). The highest distances were detected for the ITS2 and the lowest for 28S-D2 (Tables S2–S4).

Endosymbiont survey

Of the seven groups of symbionts searched for in *Liophloeodes*, only two were found, *Wolbachia* and *Rickettsia*. (Table S6). *Wolbachia* and *Rickettsia* were recorded in 43% and 76% of tested specimens respectively. All obtained *Wolbachia* wsp sequences were identical and they belonged to the strain that can be also found in other beetles (*Otiorhynchus singularis* GU111688, *Byturus ochraceus* AJ585380). *Wolbachia* ftsZ sequences were identical, and this strain has also been found in many other arthropod groups (spiders-MN594716, wasps-MH742743, flies-CP042904, butterflies-KC959172). Differently, *Rickettsia* 16S rDNA sequences were more diverse than ftsZ and wsp, but they were all closely related to the ones that have been previously found in other weevil species (Fig. S3).

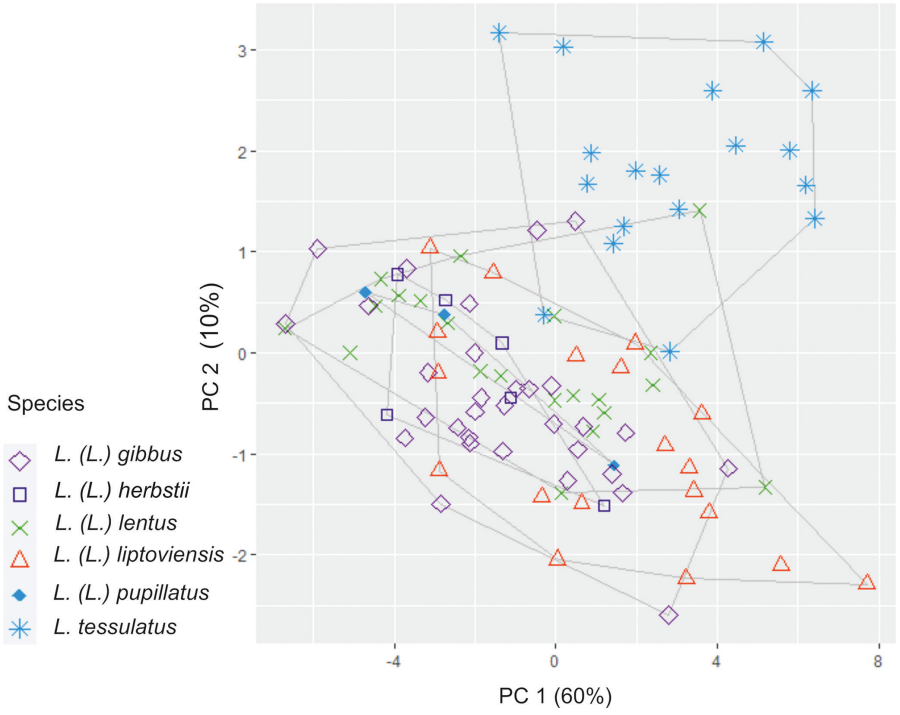

Figure 13a. Principal component analysis (PCA) of morphometric measurements of females. Scatterplot shows first against second shape PC. The variance explained by each principal component is given in parentheses.

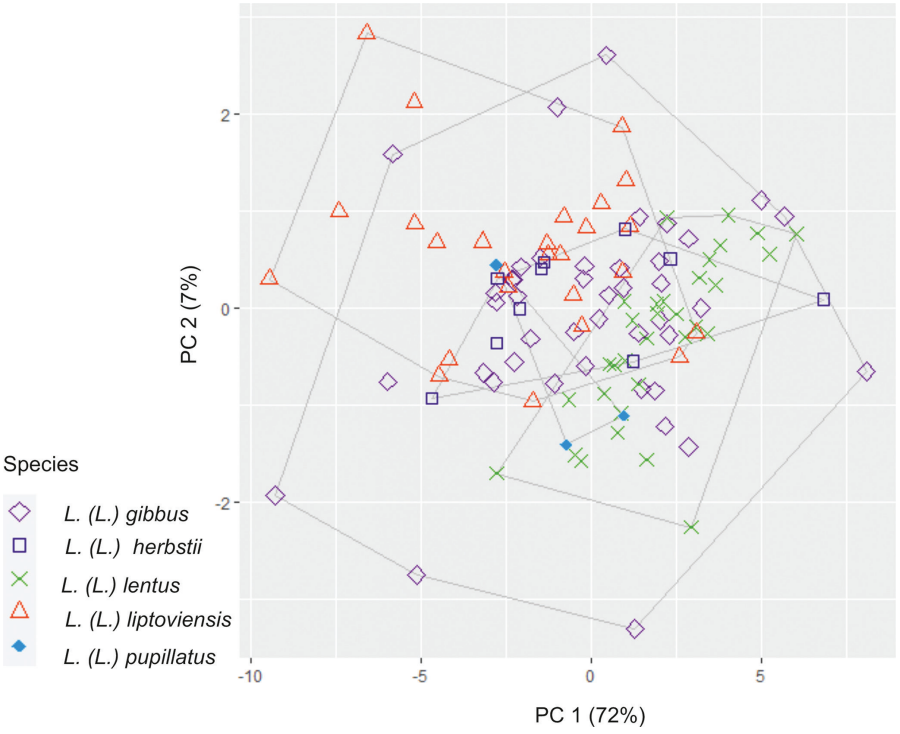

Figure 13b. Principal component analysis (PCA) of morphometric measurements of males. Scatterplot shows first against second shape PC. The variance explained by each principal component is given in parentheses.

Morphometrics

Females of all *Liophloeodes* species grouped together in the PCA scatter plot, however, in the plot of the first against the second shape PC, females of *Liophloeus tessulatus* formed a distinct group (Fig. 13a). In the PCA scatter plot of first against second shape PC for males, all species were overlapping (Fig. 13b). PCA for females showed a positive correlation of the elytra length (0.29) and width (0.24), abdomen length (0.25) and width (0.28), distance between eyes (0.25), femur (0.26), tibia (0.28), pronotum length (0.27) and width (0.26), scape length (0.24), rostrum width (0.26) and head width (0.29) with the first component, which integrated information about the body shape. The first principal component was used in the GLM as it explained 60% of the variance (with 10% of variance explained by the second principal component). Results of GLM showed that there is a statistically significant difference ($p<0.001$) in factor scores from the PCA between species. *Post-hoc* Tukey test for females confirmed the distinctiveness of *Liophloeus tessulatus* [statistically significant differences in all pairings,

except for pairing with *Liophloeus* (*Liophloeodes*) *liptoviensis* with *Liophloeus tessulatus* being significantly bigger] and showed a statistically significant difference between *Liophloeus* (*Liophloeodes*) *liptoviensis*, and *Liophloeus* (*Liophloeodes*) *gibbus*, with *Liophloeus* (*Liophloeodes*) *liptoviensis* being significantly bigger. PCA for males showed strong negative correlation of the elytra length (–0.29) and width (–0.27), abdomen length (–0.28) and width (–0.3), distance between eyes (–0.25), femur (–0.28), tibia (–0.29), pronotum length (–0.29) and width (–0.29) scape length (–0.28), rostrum width (–0.26) and head width (–0.3) with the first component, which integrated information about the body shape. The first principal component was used in the GLM, as it explained 72% of the variance (with 7% of the variance explained by the second principal component). Results of GLM showed that there is a statistically significant difference ($p<0.001$) in factor scores from the PCA between species. Post-hoc Tukey test for males showed significant difference between *Liophloeus* (*Liophloeodes*) *liptoviensis* and *Liophloeus* (*Liophloeodes*) *lentus*, with *Liophloeus* (*Liophloeodes*) *lentus* being significantly bigger.

Discussion

Diversity of *Liophloeodes*

Results of traditional species identification were usually consistent with data from faunistic papers, with some differences. In few regions where the subgenus was previously recorded no *Liophloeodes* were found (northern Slovenia, Croatia, a big part of south-western Romania). In northern Slovenia (Triglav) specimens of *Liophloeus* (*Liophloeodes*) *liptoviensis* were collected instead of *Liophloeus* (*Liophloeodes*) *herbstii*, which was the species known from this region. This last issue may be explained by the previous misidentification of these two species (the aedeagi of both species are often similar). Some new areas of distribution for *Liophloeodes* were also found (e.g., Balkan Mountains in Bulgaria). Nuclear markers showed full congruence with morphological identification of species (Figs 4–6), whereas the mitochondrial marker displayed a strong geographic pattern that suggests widespread hybridization and introgression events (Fig. S2, Figs 7–12a). This may be at least partly explained by the presence of two bacterial endosymbionts, *Wolbachia* and *Rickettsia*, which infect the most of species, with no pattern of particular symbiont infecting a particular species or population occupying a particular area. Detected *Wolbachia* and *Rickettsia* strains were previously found in other weevils (Malloch and Fenton 2005; Merville et al. 2013) and other beetles (Roehrdanz et al. 2019), respectively.

The lack of Microsporidia might be surprising due to its detection in the earlier study (Ovcharenko et al. 2013). However, the detection was based on one population from the contact zone between *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus* in the Dunajec and Poprad Valley, so we can assume that Microsporidia infection is occasional in this subgenus.

The hypothesis of past hybridization and subsequent introgression seems to be supported by the detection of evidence of heterozygosity: double peaks in chromatograms, mostly at polymorphic and diagnostic sites of 28S-D2 and ITS2 sequences of specimens occupying contact zones (Fig. S4). The lack of ecological differences (here confirmed during collecting) along with similar endosymbiont infections and low genetic interspecific distances also suggest that *Liophloeodes* species may consist of lineages that have not fully sorted yet. Further support to this hypothesis is provided by the morphometric study, which did not show clear differences between *Liophloeodes* species, suggesting that they have not differentiated morphologically yet. Great caution should be taken when concluding about distinction between *Liophloeus* (*Liophloeodes*) *herbstii* and *Liophloeus* (*Liophloeodes*) *pupillatus* based on morphometric data, as only a few specimens were examined in our study.

As for the status of *Liophloeus tessulatus*, this species strongly differs from all *Liophloeodes* species morphologically, ecologically (it occurs both in wet/cold as in dry/warm biotopes, whereas *Liophloeodes* is restricted only to the former habitat type) and sexually (so far only bisexual populations of *Liophloeodes* have been detected). However, phylogenetic analyses based on nuclear markers consistently placed it within *Liophloeodes* clades (Figs 4–6) and heterozygosity was also detected in this species. To sum up, traditional systematics of *Liophloeodes* species seems to be strongly supported by ribosomal phylogenies, but the integration of different types of data along with the status of *Liophloeus tessulatus* that is not congruent with traditional systematics lead to some questions that we attempt to address.

Incongruence of mtDNA and nuclear DNA phylogeny

Lack of congruence between mtDNA and nuclear DNAs is often found in phylogenetic studies on many groups of organisms (Roca et al 2005; Larmuseau et al. 2010; Lumme et al. 2017; Thielsch et al. 2017; Wallis et al. 2017; Weigand et al. 2017) including insects (Linnen and Farrell 2007; Gompert et al. 2008; Hinojosa et al. 2019). Two main causes of this problem are: a) incomplete lineage sorting and b) introgression following hybridization. When the incongruence between two markers has a geographical structure (for example, when one mtDNA haplotype is shared by two species or by geographically close populations of those species), the incongruence is suspected to be the result of hybridization. When populations under speciation were divided by some geographic barrier for a long time and then came into secondary contact, accumulated mutations may not allow successful mating and genetic mixing of the two species. However, in some cases, a stable hybridization zone might emerge and introgression of mtDNA from one species to another might occur on a large scale (Toews and Brelsford 2012).

This can even lead to the replacement of the "native" haplotypes by the introgressed ones (Babik et al. 2005). Disagreement between the nuclear and mitochondrial DNA phylogenies may be strongly influenced by natural selection (Boratyński et al. 2011; Toews et al. 2014), infection by bacterial endosymbionts (Hurst and Jiggins 2005; Whitworth et al. 2007; Gompert et al. 2008), sexual selection, unequal survival of hybrids, differences in survival and dispersion between sexes (Bonnet et al. 2017). Moreover, invasions of populations to new areas are also often followed by stronger introgression from the local population to the invading one (Currat et al. 2008; Phuong et al. 2017).

While the *Liophloeus tessulatus* clade in the COI tree consists only of specimens from this species (and there are no specimens from this species in other clades), the other three *Liophloeodes* clades include specimens from more than one species, with one dominating in each. All clades have a strong geographical structure (Fig. S2, Fig. 12b). Probably contact between those two species was followed by hybridization and introgression, which might have led to the removal of *Liophloeus* (*Liophloeodes*) *lentus* COI haplotypes from Polish populations. Furthermore, some specimens belonging to this clade live in the Sudetes, which is a separated mountain range in the west, almost 300 km from the contact zone. Thus, it can be speculated that the present contact zone is just one of the areas where hybridization and introgression occurred and/or that *Liophloeus* (*Liophloeodes*) *lentus* populations dispersed west. The other area where hybridization and introgression could have occurred or is currently occurring is the eastern part of Polish Carpathians, where *Liophloeus* (*Liophloeodes*) *liptoviensis* and *Liophloeus* (*Liophloeodes*) *gibbus* are often sympatric. Heterozygosity detected in specimens from this area may support the hypothesis that this is an ongoing process. The heterozygosity found in nuclear markers, and a COI phylogeny with clades of intermixed species suggests also other places where hybridization could occur, such as Tatra in Slovakia [*Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *liptoviensis*], Romania [*Liophloeus* (*Liophloeodes*) *herbstii* and *Liophloeus* (*Liophloeodes*) *liptoviensis*], and Pannonian Basin in Hungary and Serbia [different populations of *Liophloeus* (*Liophloeodes*) *lentus*]. One of the factors that could increase introgression is *Wolbachia* spread between the species and populations (hitchhiking effect), and this endosymbiont was detected mostly in areas of possible hybridization (Miyata et al. 2020). Incongruence between gene trees often occurs in species that underwent many geographical range shifts in the past and now occur sympatrically in several areas, often in montane environments (Rodríguez et al. 2010; Haines et al. 2017; Ortego et al. 2017; Tóth et al. 2017), a scenario that suits *Liophloeodes* well.

Status of *Liophloeus tessulatus*

Liophloeus tessulatus includes both parthenogenetic and bisexual populations, yet in areas where it is sympatric with *Liophloeodes* no bisexual populations were found. Based on this information and the unexpected position of *Liophloeus tessulatus* in the phylogenetic trees (Figs 4–6), we propose two possible scenarios for its evolutionary history. According to the first one, lineages of *Liophloeodes* and *Liophloeus sensu stricto* (including *Liophloeus tessulatus*) diverged long time ago and then both evolved and adapted to different environmental conditions. A consequence would be that the position of *Liophloeus tessulatus* in the phylogenetic trees is misleading, and its taxonomic position should be established based on genetic distances of all three markers (Tables S2–S4), morphology, and ecology. In this scenario, *Liophloeodes* populations, restricted to wet and cold biotopes, would shrink their ranges to mountain refugia during times of climate warming, which would lead to their diversification and subsequent speciation. The more cosmopolitan *Liophloeus sensu stricto* would be more resistant to climate change events and this would, along with its partially parthenogenetic mode of reproduction, lead to its present wide range of distribution. Because in populations sympatric with *Liophloeodes* those weevils are parthenogenetic, it is possible that divergence between the two lineages occurred somewhere inside present *Liophloeodes* range and the parthenogenetic type of reproduction evolved only once. The second hypothesis derives from the position of *Liophloeus tessulatus* in the phylogenetic trees, and according to it, *Liophloeus tessulatus* is one of *Liophloeodes* species that diverged during subgenus evolution along with other species. Thus, different morphology and ecological requirements are the results of different adaptations during climate change events and range shifts. However, the ancestor of *Liophloeus tessulatus* changed its ecological niche, expanded its range and a new type of reproduction evolved. Niche shifts were one of the key factors in weevil evolution and diversification (Marvaldi et al. 2002) and parthenogenetic forms often invaded new areas, also in Europe in glacial and interglacial periods (Kajtoch et al. 2009; Kajtoch et al. 2012) However, the parthenogenetic reproduction may be quite a new adaptation in this lineage. Heterozygosity found in *Liophloeus tessulatus* may be a result of recent hybridizations between bisexual populations. It is also worth mentioning that parthenogenetic populations of *Liophloeus tessulatus* are triploid (Suomalainen 1955), which, along with the 28S-D2 heterozygosity might indicate a hybrid origin of this species – hybridization is in fact likely the main cause of emerging parthenogenetic forms among weevils (Stenberg and Lundmark 2004; Neiman et al. 2009). The sequence of these events (emergence of parthenogenesis, niche shift, geographic expansion, possible hybridization) remains unsolved. This hypothesis suggests that bisexual, montane populations of *Liophloeus tessulatus* from Western Europe are a distinct species that diverged before the niche shift and evolution of parthenogenesis. However, without examining those populations, no definitive conclusions about the status of *Liophloeus tessulatus* can be derived. If the second hypothesis was true, *Liophloeus tessulatus* should be included in the *Liophloeodes* subgenus.

Low morphometric diversity

Results of the morphometric analysis suggest small shape differences in selected body parts between species, which seem to correspond with Smreczyński's suggestion (1958) that the aedeagal shape is the only trait that can be treated as diagnostic, and the other traits display a gradient of variation among species. To support (or reject) this conclusion, traits mentioned by Smreczyński such as the shape of the rostrum (how it expands to the end), tooth on the femur, the structure of the abdomen's margin were visually checked, and the validity of his opinion was confirmed. Given the ecological requirements of *Liophloeodes* species, these results are not surprising. All species prefer the same biotope and this ecological conservatism probably led to speciation – changing conditions caused the division of ranges and withdrawal to glacial refugia, which was likely followed by isolation and speciation. This association of diverged species to the same biotope, along with repeated hybridizations that may have occurred, might result in small morphometric diversity.

There is another interesting aspect of this problem: small interspecific diversity can be a result of high intraspecific diversity that probably evolved before speciation, so it can be considered a kind of ancestral polymorphism (Williams et al. 2015). However, it is important to remember that males of two species [*Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *liptoviensis*)] display significant diversity, which could be the result of an ongoing differentiation. The presence of hybrid specimens with intermediate features might have heavily affected the morphometric results, erasing the diversity between species groups, which may cause problems with species identification (Nugnes et al. 2017).

Inconsistency between phylogenies of nuclear markers

Lack of congruence between phylogenies derived from different nuclear markers happens more rarely than between nuclear and mitochondrial DNA, but their causes are similar: introgression and incomplete lineage sorting. The random distribution of specimens in the phylogenetic tree usually suggests the latter cause (Page and Charleston 1997; Buckley et al. 2006; Vaezi and Brouillet 2009). This is not the case of *Liophloeodes* because all species are clearly distinguished in all nuclear marker trees (Figs 4–6), and there are no signs of interspecific admixture (as it is in COI – Fig. S2). The main difference is the distribution of species in the trees and their histories.

Two things should be considered here. The first is the conservatism of the 28S-D2 rDNA marker, which can affect the phylogeny of young, recently derived species (Jordal and Kambestad 2014). ITS2 marker is far more sensitive to recent divergence processes (Jousselin et al. 2006). The second argument is that ITS2 phylogeny is more robust, based on the statistical support of nodes. The entire ITS2 tree is indeed fully resolved and strongly supported, whereas the placement of *Liophloeus* (*Liophloeodes*) *herbstii*, *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus* in the 28S rDNA tree is unsupported. Morevoer the topology of ITS2 tree is consistent with the tree from combined nuclear marker.

Evolutionary history of *Liophloeodes*

Based on the ITS2 phylogeny, there are two main phylogeographic groups of *Liophloeodes*. The first, consisting of *Liophloeus* (*Liophloeodes*) *lentus* and *Liophloeus* (*Liophloeodes*) *gibbus*, is the most frequent in the north of the Pannonian Basin, and surrounds it nearly completely through (in clockwise geographical order): West Carpathians, Podolian Upland, Apuseni, Milevska Planina/Vitosha, Dinaric Alps, the Sudetes, and Western Carpathians. On the east, there are more than 400 km of break between Apuseni and Vitosha. Among these two species, *Liophloeus* (*Liophloeodes*) *lentus* is the westernmost one (it occurs in the Alps, the Sudetes, Pannonian Basin, and Western Carpathians). It is interesting that in this tree, this clade is sister to generally more western *Liophloeus tessulatus*, which may support the hypothesis of divergence of this species by niche shifts.

The second ITS2 clade [(*Liophloeus* (*Liophloeodes*) *liptoviensis* + [(*Liophloeus* (*Liophloeodes*) *pupillatus* + (*Liophloeus* (*Liophloeodes*) *herbstii*]] has more eastern distribution, with one exception [*Liophloeus* (*Liophloeodes*) *liptoviensis* from Slovenia]. It can be hypothesised that the first strong divergence in the history of *Liophloeodes* was between populations occupying central Carpathians and populations from more western and southern mountain ranges.

Probably recurrent glacial and interglacial events caused recurrent changes in the ranges of distribution with expansions and reductions. Due to *Liophloeodes* ecological requirements, these weevils probably expanded their ranges during glaciations and withdrew to refugia during interglacial periods (which we can observe now, as they occur mostly in mountain valleys). Maybe climate changes caused the division of distinct species lineages and populations of diverged lineages came into secondary contact when their ranges expanded. Some of them might have also dispersed through mountain ranges, even during interglaciations because the climate was stable there. For example, sympatry of *Liophloeus* (*Liophloeodes*) *gibbus* and *Liophloeus* (*Liophloeodes*) *liptoviensis* in the eastern part of Polish Carpathians can be the result of the dispersion of both species through Carpathians (the first one to the west, the latter to the east).

Detailed *Liophloeodes* phylogeography is difficult to resolve. The topology of all phylogenetic trees suggests strong intraspecific gene flow between populations – we can observe small clades consisting of populations from wider areas such as Poland and Romania. Alternatively, many small clades consist of specimens restricted to very limited ranges. A possible explanation for this is that the evolution of this subgenus was influenced by repeating isolations of populations in refugia, which could lead

Table 2. Synthetic summary of the integrative approach with all independent lines of evidence listed. Y=starting hypothesis supported; N=starting hypothesis not supported; P=starting hypothesis partially supported; IPC=integration by partial congruence; species hypothesis stability: S=stable, U=unstable.

***Liophloeodes* species**	**Morphology (H0)**	**28S-D2**	**ITS2**	**COI**	**Morphometry**	**Symbionts**	**IPC**
Liophloeus (*Liophloeodes*) *lentus*	Y	Y	Y	P	N	N	S
Liophloeus (*Liophloeodes*) *gibbus*	Y	Y	Y	P	N	N	S
Liophloeus (*Liophloeodes*) *liptoviensis*	Y	Y	Y	P	N	N	S
Liophloeus (*Liophloeodes*) *herbstii*	Y	Y	Y	N	N	N	U
Liophloeus (*Liophloeodes*) *pupillatus*	Y	Y	Y	N	N	N	U

to speciation, and by subsequent contacts of already diverged populations that resulted in hybridization.

Conclusions

Six independent lines of evidence were used: traditional morphology (as the starting hypothesis), phylogenies based on three molecular markers, morphometry and endosymbiont occurrence/phylogeny (Table 2). The latter two methods were inconclusive in supporting species differentiation. Phylogeny based on both ribosomal markers supported traditional morphology, whereas the different topology of the COI phylogeny can be explained by hybridization and introgression events. Examining all the lines of evidence considered we conclude that:

1. Although not supported by all lines of evidence, we argue that traditional systematics of *Liophloeodes* based on the shape of genitalia, an important trait for reproductive isolation (Langerhans 2016), should not be changed, lacking conclusive evidence to challenge taxonomic stability.
2. Species of *Liophloeodes* probably represent young lineages with evidence of hybridization and/or introgression detected in the COI phylogeny and the heterozygosity found in nuclear markers of specimens from contact zones.
3. The status of *Liophloeus tessulatus* and the *Liophloeus sensu stricto* subgenus remains uncertain. Morphology, morphometrics, ecology, reproduction, and genetic data suggest its distinctiveness from *Liophloeodes*, however phylogenetic analyses indicate that it may be another *Liophloeodes* species. For more sound conclusions on the taxonomic status of this species, and of the *Liophloeus* s.s subgenus, more populations (especially bisexual) and the remaining species of the subgenus, respectively, must be examined.

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Supplementary material 1

File 1

Authors: Wacławik et al. (2021)
Data type: .pdf
Explanation note: Figure S1. Morphometric measurements.
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Supplementary material 2

File 2

Authors: Wacławik et al. (2021)
Data type: .pdf
Explanation note: Figure S2. A Bayesian phylogenetic tree of the subgenus Liophloeodes based on COI sequences (GenBank accession: MT858362–MT858668), with Eusomus ovulum as an outgroup. Bayesian posterior probability values are given above branches. Scale bar represents 0.03 substitutions per site.
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Supplementary material 3

File 3

Authors: Wacławik et al. (2021)
Data type: .pdf
Explanation note: Figure S3. A Bayesian phylogenetic tree of Rickettsia sequences based on 16S rDNA sequences (GenBank accession MN621120–MN621139) with Orientia tsutsugamushi, Wolbachia-E. formosa, Wolbachia-N. vitripennis as outgroups. Bayesian posterior probability values are given above branches.
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Supplementary material 4

File 4

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Supplementary material 5

File 5

Authors: Wacławik et al. (2021)
Data type: .xlsx
Explanation note: Table S1. List of collected specimens. Species in bold (column D) have been recognized based on the morphological study of particular specimens, species with normal font have been recognized based on study of different males found in the same place.
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Supplementary material 6

File 6

Authors: Wacławik et al. (2021)
Data type: .xlsx
Explanation note: Table S2. Uncorrected p-distance values. Interspecific COI percentage genetic distances are below diagonal, with standard deviations above diagonal.
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Supplementary material 7

File 7

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Data type: .xlsx
Explanation note: Table S3. Uncorrected p-distance values. Interspecific 28S-D2 percentage genetic distances are below diagonal, with standard deviations above diagonal.
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Supplementary material 8

File 8

Authors: Wacławik et al. (2021)
Data type: .xlsx
Explanation note: Table S4. Uncorrected p-distance values. Interspecific ITS percentage genetic distances are below diagonal, with standard deviations above diagonal.
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Supplementary material 9

File 9

Authors: Wacławik et al. (2021)
Data type: .pdf
Explanation note: Table S5. Accession numbers of Rickettsia strains available from Genbank used in phylogenetic analysis.
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Link: https://doi.org/10.3897/asp.79.e64252.suppl9

Supplementary material 10

File 10

Authors: Wacławik et al. (2021)
Data type: .xlsx
Explanation note: Table S6. Endosymbionts detected in *Liophloeus*.
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