JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH

Accepted on 16 September 2013
Journal of Zoological Systematics and Evolutionary Research Published by Blackwell Verlag GmbH J Zoolog Syst Evol Res doi: 10.1111/jzs.12044

Short Communication

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New data on the phylogeny of Ariantinae (Pulmonata, Helicidae) and the systematic position of *Cylindrus obtusus* based on nuclear and mitochondrial DNA marker sequences

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Abstract

The phylogenetic relationships among genera of the subfamily Ariantinae (Pulmonata, Helicidae), especially the sister-group relationship of *Cylindrus obtusus*, were investigated with three mitochondrial (*12S rRNA*, *16S rRNA*, *Cytochrome c oxidase subunit I*) and two nuclear marker genes (*Histone H4* and *H3*). Within Ariantinae, *C. obtusus* stands out because of its aberrant cylindrical shell shape. Here, we present phylogenetic trees based on these five marker sequences and discuss the position of *C. obtusus* and phylogeographical scenarios in comparison with previously published results. Our results provide strong support for the sister-group relationship between *Cylindrus* and *Arianta* confirming previous studies and imply that the split between the two genera is quite old. The tree reveals a phylogeographical pattern of Ariantinae with a well-supported clade comprising the Balkan taxa which is the sister group to a clade with individuals from Alpine localities. Additional lineages representing samples from southern Alpine localities as well as from Slovakia split from more basal nodes, but their relationships are not clearly resolved. To achieve more definitive conclusions concerning the geographical origin of Ariantinae, still more sequence data are needed to obtain a tree with better resolution of basal nodes. The genetic data also provided new insights concerning the genus *Cepaea,* which was used as one of the outgroup taxa. *Cepaea vindobonensis* is only distantly related to *Cepaea nemoralis* and *Cepaea hortensis*, the latter two being more closely related to *Eobania vermiculata*. Thus, in our tree, the genus *Cepaea* is paraphyletic.

Key words: *Cylindrus obtusus* – phylogeny – phylogeography – Alpine land snails – shell morphology – histone genes – *Cytochrome c oxidase subunit 1* – rRNA

Introduction

The phylogenetic relationships of and within the land snail subfamily Ariantinae (Pulmonata, Helicidae), especially the closest relative of *Cylindrus obtusus*[a], have been discussed for a long time. The taxon Ariantidae was introduced first by Mörch (1864) in the rank of a family of Pulmonata. Schileyko (1991) defined Ariantinae as a subfamily of Helicidae and characterized it mainly by shell morphology and genital anatomy. According to Schileyko (2006), the shells of the Ariantinae are mainly globular to flat, and the mucus glands are tubular, simple or biramous. Steinke et al. (2004) classified the Ariantinae as sister group of the Helicinae based on sequence data from two mitochondrial (mt) genes (*COI*, *16S rRNA*) and two nuclear (nc) genes (*ITS-1*, *18S rRNA*). *C. obtusus* is one of the most conspicuous species within the Ariantinae. It is an endemic of the Eastern Alps in Austria (Klemm 1974) and is mainly restricted to high elevations (1600–2500 m – comp. Bisenberger et al. 1999). The species displays several interesting particulars regarding its biology and ecology (Kühnelt 1937; Sattmann et al. 1995; Freitag and Desch 1996; Bisenberger et al. 1999; Duda et al. 2010), anatomy of reproductive organs (Martens 1895; Sturany and Wagner 1915; Schileyko 1996; Schileyko et al. 1997; Zopp 2012), population genetics and reproduction biology (Luise Kruckenhauser, Michael Duda, Daniela Bartel et al., unpublished). The systematic position of *C. obtusus* was discussed controversially in the past. It was originally placed in the genus *Pupa* (as *P. obtusa*) by Draparnaud (1805) because of its cylindrical shell. The formerly broadly defined genus *Pupa* includes various members of the superfamily Pupillioidea according to modern classifications (Bouchet et al. 2005). Martens (1895) gave a first anatomical description and placed it 'closer to *Helix* […] than *Buliminus* or *Pupa'* (translated after Martens 1895). Sturany and Wagner (1915) published an enhanced drawing and a short description of the anatomy and consequently allocated *C. obtusus* within the subfamily Campylaeinae. Boettger and Wenz (1921) also placed *C. obtusus* in the subfamily Campylaeinae, which comprises most of the genera of current Ariantinae *sensu* Schileyko (2006). Consequently, Schileyko (2006) assigned *C. obtusus* to the newly erected monotypic tribus Cylindruini within the subfamily

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[a]Schileyko (2012) suggests using *Cochlopupa obtusa* (Draparnaud, 1805) instead of *Cylindrus obtusus*, because he claims that (1) *Cylindrus* Fitzinger, 1833 is the junior homonym of *Cylindrus* Bartsch, 1789 (see also Dubois and Bour 2010 and http://www.animalbase.uni-goettingen.de/zooweb/servlet/AnimalBase/home/genustaxon?id=5061), and (2) *Cylindrus* Fitzinger, 1833 is a junior objective synonym of *Cochlopupa* Jan, 1830. However, according to Edmund Gittenberger (pers. comm.) the assignment of the name *Cylindrus* Batsch, 1789 is still disputed. Until this question is settled, we stick to the well-established name, *C. obtusus*.

Ariantinae. The current systematic assignment is mainly based on the anatomy of reproductive organs. However, *C. obtusus* shows a conspicuously aberrant shell form within this group – all other representatives display globular or more or less depressed shells (Kerney et al. 1983). In fact, *C. obtusus* might be considered as one of the striking cases in which a member of a family has 'crossed the adaptive valley' between the two height/width categories (i.e. long versus globular) (see also Gittenberger 2010). The exceptional shell morphology of *C. obtusus* raised questions about the evolutionary history of the species and its relationships within Ariantinae. Schileyko (2012) summarized the hitherto discussion and hypothesized that the species was very young and originated within its present range at the end of the Würm glaciation splitting from a representative of the Ariantinae due to a mutation. This opinion is largely in accordance with former hypotheses by Boettger (1949) and contrary to Adensamer (1937, 1962), who considered *C. obtusus* as a representative of an old lineage, which survived the ice age in high alpine habitats. If Schileyko (2012) was right, *Cylindrus* would exemplify an extremely fast speciation event and would most probably render *Arianta* (which displays rather high intraspecific genetic distances; Haase et al. 2003; Haase and Misof 2009) paraphyletic.

Although a number of molecular genetic analyses dealing with different taxonomic levels within Helicoidea have been published (Wade et al. 2001, 2007; Steinke et al. 2004; Koene and Schulenburg 2005; Manganelli et al. 2005; Fiorentino et al. 2008, 2010; Groenenberg et al. 2011; Gómez-Moliner et al. 2013), the subfamily Ariantinae has not been investigated extensively so far. An exception to this is a number of studies on *Arianta arbustorum* (Haase et al. 2003; Gittenberger et al. 2004; Haase and Misof 2009), a widespread species frequently occurring in the Alps and often found syntopically with *C. obtusus*. A first molecular phylogenetic analysis of the Ariantinae based on fragments of the mt genes *Cytochrome b* (*cytb*), *Cytochrome c oxidase subunit 1* (*COI*) and *16S rRNA* (*16S*), as well as of the nc gene for *Histone 3* (*H3*), was performed by Groenenberg et al. (2012). In that study, the combined tree of all marker sequences displayed *Cylindrus* as the sister group of *Arianta*. The phylogenetic relationships among other genera of Ariantinae were, however, not well resolved. In a parallel study, our group analysed the phylogenetic relationships of *Cylindrus* within the Ariantinae using two additional marker sequences, the mt *12S rRNA* gene (*12S*) and the nc *Histone H4* gene (*H4*) besides *16S*, *COI* and *H3*. Here, we present phylogenetic trees based on these five marker sequences and discuss the position of *C. obtusus* and phylogeographical scenarios in comparison with the results of Groenenberg et al. (2012).

Methods

Sampling

Samples included both fresh specimens recently collected for the analyses (2005–2011) and old, ethanol-preserved material of the mollusc collection of the Natural History Museum of Vienna (NHMW), collected between 1920 and 1991. The present work is not issued for purposes of zoological nomenclature and is not published within the meaning of the ICZN (see Art 8.2). In general, we used the nomenclature of Bank (2012); however, in cases of some taxa, we followed the same practice as Groenenberg et al. (2012), and we used provisional names referring to a taxonomic revision of the Balkan Ariantinae by Péter Subai (in prep.). A total of 48 specimens belonging to 29 land snail species (21 genera) were included in this study (Table 1). In addition to the representatives of Ariantinae, we included species of the subfamily Helicinae (*Cepaea nemoralis*, *Cepaea hortensis*, *Ceapea vindobonensis*, *Eobania vermiculata*), as well as representatives of two other families, which were used as outgroup: *Euomphalia strigella, Monacha cantiana* (Hygromiidae) and *Helicodonta obvoluta* (Helicodontidae; Table 1). Freshly collected animals were prepared according to Kruckenhauser et al. (2011) and subsequently stored in 80% ethanol. For DNA extraction, a tiny piece was cut off the snail's foot without damaging the shell or the rest of the body. Voucher specimens are stored in the mollusc collection of the NHMW; DNA and tissue samples are stored in the tissue collection of the NHMW (Table 1).

DNA extraction, molecular markers, amplification and sequencing

Genomic DNA was extracted using the First-DNA all tissue kit (Gen-ial, Troisdorf, Germany) following the manufacturer's protocol. A negative control was used for each set of samples extracted. The final elution volume was 30 µl. A total of five molecular markers were used: partial sequences of three mt genes, *COI*, *12S* and *16S* as well as of two nc genes, *H3* and *H4*. These genomic regions were amplified and sequenced with the primers listed in Table 2. Annealing temperatures and resulting amplified fragment sizes are also listed in Table 2. Polymerase chain reactions (PCRs) were performed on a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) in a final volume of 25 µl, containing 2.5 µl of PCR buffer, 3 mM of Mg^{2+}, 0.2 mM of each nucleotide, 0.5 U of Roche Taq Polymerase and 0.5 µM of each primer. Optimal amounts of template DNA were determined empirically (2–5 µl of the DNA solution). PCR profiles comprised an initial heating step at 94°C for 3 min followed by 35 cycles (40 cycles in some old samples): 30 s at 94°C, 30 s at annealing temperature (Table 2) and 60 s at 72°C. After the last cycle, a final extension of 7 min at 72°C was performed. Control reactions to detect potential contaminations were carried out with: (1) control 'extractions' (without sample) instead of the template and (2) with distilled water instead of the template. PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced in both directions at LGC Genomics (Berlin, Germany). In a few cases, cloning was necessary, for which PCR products were generated with Phusion High Fidelity DNA Polymerase (Finnzymes), extracted from agarose gels with the QIAquick Gel Extraction Kit (Qiagen, Inc.) and subsequently cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Sequencing of the clones (both directions) was also performed at LGC Genomics.

Data analysis

Editing of sequences was performed using BioEdit v. 7.0.1 (Hall 1999). Sequences were aligned using ClustalX (Larkin et al. 2007) with default settings and inspected by eye using Either BioEdit v. 7.0.1 or (as this software was already mentioned before) only BioEdit. The *12S* and *16S* alignments contained long and varied gaps among the different taxa, which made the alignments in these regions difficult and unreliable. To remove the poorly aligned stretches, we performed an automated trimming procedure with the software TrimAl 1.3 (Capella-Gutiérrez et al. 2009), implemented in the Phylemon 2.0 web tool (Sánchez et al. 2011). Lengths of alignments are provided in Table 2. We tested two different settings the software provides: 'no gap' and 'strict'. The 'no gap' method deletes all columns in the alignment with at least one gap in them. The 'strict' method involves a first trimming step based on the distribution of gaps in the alignment, followed by a second trimming step based on the similarity of the remaining aligned sequence (see Capella-Gutiérrez et al. 2009 for details). The 'strict' option yielded in general the best trees (i.e. with the highest node support). In the (few) cases where the 'no gap' option yielded better support, we provide the corresponding data (support values, mt tree, respectively; see Results). Also, for the *COI* data set, we tested two options, one with the complete sequences and another one where the third codon positions were coded according to the IUPAC nucleotide ambiguity codes as purine (R) or pyrimidine (Y) residuals, respectively ('COI3rdRY' coding), to reduce the saturation problem, because saturation affects mostly 3rd codon positions.

It has to be mentioned that the *H3* sequences of *C. vindobonensis* are highly diverged from the other taxa. We repeated the amplification and sequencing of both individuals and obtained the same sequences. As there are no frameshift mutations and no peculiar amino acid substitutions, we assume that this is the authentic *H3* sequence and no

Table 1. Specimens used for the phylogenetic study and the sequences analysed. Voucher numbers of the mollusc collection of the NHMW are provided. Numbers in the first column (individual) refer to numbers in the trees. * = from these individuals the *COI* sequence could not be obtained

Species/Individual	Geographical origin	Voucher number
Arianta arbustorum (Linnaeus, 1758) 1	AT, Lower Austria, Maria Schutz	NHM109000/AL/00500/2098
Arianta arbustorum/2	AT, Upper Austria, Höllengebirge Mts	NHM109000/AL/00499/2102
Arianta chamaeleon (L. Pfeiffer, 1868) 1*	SI, Kamniško-Savinjske Alps	NHMW90396
Arianta schmidti (Rossmässler, 1836) 1	SI, Kamniško-Savinjske Alps	NHMW90401
Campylaea illyrica (Stabile, 1864) 1	SI, Kamniško-Savinjske Alps	NHMW90785
Cattania cf. faueri (Subai, 1990) 1	GR, Kentriki Makedonia, Agio Pnevma	NHM109000/AL/00501/6998
Cattania cf. faueri/2	GR, Kentriki Makedonia, Agio Pnevma	NHM109000/AL/00501/6999
Cattania haberhaueri (Sturany, 1897) 1	RS, Pčinjski okrug, Bosilegrad, Božička Reka	NHM109000/AL/00502/7013
Cattania haberhaueri 2	RS, Pčinjski okrug, Bosilegrad, Božička Reka	NHM109000/AL/00502/7014
Causa holosericea (Studer, 1820) 1	AT, Lower Austria, Dürrenstein	NHM109000/AL/00504/2106
Causa holosericea 2	AT, Upper Austria, Dachstein Mt	NHM109000/AL/00503/2107
Chilostoma achates cingulina (Deshayes, 1839) 1	AT, Styria, Leobener Mt	NHM109000/AL/00507/2100
Chilostoma achates cingulina 2	AT, Salzburg, Hochtor Mts	NMHW90823
Chilostoma achates cingulina 3	AT, Lower Austria, Sonnwendstein Mt	NHM109000/AL/00506/2103
Chilostoma cingulatum baldense (Rossmässler, 1839) 1*	GE, Bayern, Bad Staffelstein	NMHW87065
Cylindrus obtusus (Draparnaud, 1805) 1	AT, Styria, Totes Gebirge, Elm	NHM109000/AL/00008/87
Cylindrus obtusus 2	AT, Lower Austria, Dürrenstein Mt	NHM109000/AL/00352/164
Dinarica pouzolzii (Deshayes, 1830) 1	ME, Herceg Novi, Rose	NHM109000/AL/00508/6557
Faustina faustina (Rossmässler, 1835) 1	SK, Žilinský kraj, Malá Fatra Mts	NHM109000/AL/00510/2110
Faustina faustina 2*	CZ, Moravskolezský Kraj, Zimrovice	NMHW74422
Helicigona lapicida (Linnaeus, 1758) 1	AT, Lower Austria, Gars am Kamp	NHM109000/AL/00511/2104
Isognomostoma isognomostomos (Schröter, 1784) 1	AT, Lower Austria, Maria Schutz	NHM109000/AL/00515/2099
Isognomostoma isognomostomos 2	AT, Salzburg, Salzberg	NHM109000/AL/00514/2105
Josephinella byshekensis (Knipper, 1941) 1	AL, Gjirokastër, Pacori	NHM109000/AL/00516/7015
Josephinella byshekensis/2	AL, Gjirokastër, Pacori	NHM109000/AL/00516/7018
Josephinella hemonica (Thiesse, 1884) 1	GR, Kentriki Makedonia, Veroia	NHM109000/AL/00517/6568
Josephinella hemonica 2*	GR, Epirus	NMHW102948
Kosicia intermedia (Férussac, 1832) 1	IT, Friuli-Venezia Giulia, Passo di Monte Croce Carnico	NHM109000/AL/00520/2108
Kosicia intermedia 2	AT, Carinthia, Bärental	NHM109000/AL/00519/2114
Kosicia intermedia 3	AT, Carinthia, Tscheppaschlucht	NHM109000/AL/00518/2115
Kosicia ziegleri (Rossmässler, 1836) 1	SI, Kamniško-Savinjske Alps	NHMW90400
Liburnica cf. dunjana (Knipper, 1941) 1	AL, Dibër, Shkopet	NHM109000/AL/00521/6565
Liburnica setosa setosa (Férussac, 1832) 1	HR, Splitsko-dalmatinska županija, Blato na Cetini	NHM109000/AL/00522/6566
Thiessea cf. sphaeriostoma (Bourguignat, 1857) 1	GR, Sterea Ellada, Euboea, Paralimni	NHM109000/AL/00526/6560
Thiessea cf. sphaeriostoma 2	GR, Sterea Ellada, Euboea, Mantoudi	NHM109000/AL/00525/6562
Vidovicia coerulans (C. Pfeiffer, 1828) 1	HR, Ličko-senjska županija, Velebit Mts	NHM109000/AL/00525/6562
Vidovicia coerulans 2	HR, Ličko-senjska županija, Velebit Mts	NHM109000/AL/00527/6458
Vidovicia coerulans 3	HR, Ličko-senjska županija, Velebit Mts	NHM109000/AL/00527/6459
Outgroup species		
Cepaea hortensis (O.F. Müller, 1774) 1	ES, Spanish Pyrenees	NHMW87004
Cepaea nemoralis (Linnaeus, 1758) 1	ES, Asturias	NHMW87010
Cepaea vindobonensis (C. Pfeiffer, 1828) 1	SK, Trenčiansky kraj, Strážovské vrchy	NHM109000/AL/00505/2109
Cepaea vindobonensis 2	AT, Lower Austria, Perchtoldsdorf	NHMW51761
Eobania vermiculata (O.F. Müller, 1774) 1	GR, Notio Egeo, Makri island	NHMW88046
Euomphalia strigella (Draparnaud, 1801) 1	AT, Lower Austria, Hundsheimerkogel Mt	NHM109000/AL/00509/2096
Helicodonta obvoluta (O.F. Müller, 1774) 1	AT, Lower Austria, Maria Schutz	NHM109000/AL/00513/2097
Helicodonta obvoluta 2	AT, Lower Austria, Heiligenkreuz	NHM109000/AL/00512/2101
Monacha cantiana (Montagu, 1803) 1	IT, Friuli-Venezia Giulia, Passo di Monte Croce Carnico	NHM109000/AL/00524/2112
Monacha cantiana 2	SI, Bovec, Soča valley	NHM109000/AL/00523/2113

pseudogene. Furthermore, we computed the trees both including and excluding *C. vindobonensis* sequences, but the resulting topologies were the same.

The substitution models for the Bayesian inference (BI) of phylogenetic relationships, selected with jModelTest 0.1.1. (Posada 2008) and applying the Akaike information criterion, were as follows: *12S* (both no gap and strict): nst = 6, rates = gamma; *16S* (no gap): nst = 2, rates = gamma; *16S* (strict): nst = 6, rates = gamma; *COI* (both for complete sequence and 'COI3rdRY'): nst = 6, rates = gamma; *H3*: nst = 2, rates = gamma; *H4:* nst = 6, rates = equal. BI trees were calculated using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) using two runs (each with three heated chains and one cold chain) and 5 million MCMC generations (sample frequency 100). Convergence of runs was assessed by visual inspection of plotted log-likelihood values using the software Tracer 1.4 (Rambaut and Drummond 2007). In a conservative approach, 25% of the sampled trees were discarded as burn-in, although the stationary phase in the two runs was reached before. We calculated BI trees using the various options for *12S*, *16S* and *COI* as well as combining the three mt and the two nc markers and finally all five marker sequences into one comprehensive alignment. A maximum likelihood (ML) tree was calculated with Garli 2.0 (Zwickl 2006) using the same alignments as for the combined BI tree (*H3*, *H4*, *12S* 'strict', *16S* 'strict' and COI3rd position RY coded). The substitution models obtained from jModelTest were applied to each of the five data partitions, and a bootstrap search was performed with 500 replicates. The starting trees were calculated using the stepwise algorithm. The trees were rooted using *Euomphalia strigella, Monacha cantiana* (Hygromiidae) and *Helicodonta obvoluta* (Helicodontidae). The *COI* sequence could not be obtained for four individuals. Therefore, they were not used for the concatenated alignment, which, accordingly, comprised 44 sequences. The *12S*, *16S H3* and *H4* sequences of the remaining four individuals cluster close with their conspecifics (data not shown). The sequences determined in the course of the present study are registered under the following GenBank accession numbers: *COI*: KF596870–KF596913, *12S*: KF596774–KF596821, *16S*: KF596822–KF596869, *H3*: KF596914–KF596961, *H4*: KF596962 – KF597009.

Table 2. Primers used to amplify and sequence three mt and two nc genes. For *H3,* an alternative reverse primer was used in some cases. Alignment lengths of *12S* and *16S* are given for the two options used to trim ambiguous regions ('no gap'/'strict') (see text)

Region sequenced and primer names	Primer sequences (5′–3′)	Ta (°C)	Fragment (and alignment) length (bp)
12S			
12SGast_fwd2	AGTGACGGGCGATTTGT	55	628–653 (441/398)
12SGast_rev3	TAAGCTGTTGGGCTCATAAC		
16S			
16S_schneck_fwd	CGCAGTACTCTGACTGTGC	55	332–356 (283/250)
16S_schneck_rev	CGCCGGTCTGAACTCAGATC		
COI			
COI_folmer_fwd	GGTCAACAATCATAAAGATATTGG	48/50	654 (654)
COI_schneck_rev	TATACTTCTGGATGACCAAAAAATCA		
H3			
AriaH3_fwd	ATGGCTCGAACCAAGCAGACC	57	316 (316)
AriaH3_rev	GGTGACACGCTTGGCGTGG		316
OrcH3_right1	TGGGCATGATGGTGACACGCT		
H4			
AriaH4_fwd	AACCTCCGAAGCCGTACAGGGT	57	258 (258)
AriaH4_rev	GAAGAGGTAAAGGCGGCAAGGG		

Ta, annealing temperatures.

Results

The BI tree based on the five concatenated marker sequences using the 'strict' trimming option for *12S* and *16S* and the 'COI3rdRY' coding for *COI* provided in general the highest node support values. This tree comprising 44 individuals is presented in Fig. 1. Using other options resulted in the same overall topology, albeit with less support. There were only two nodes that provided higher support with the 'no gap' option for *12S* and *16S.* The corresponding values are also indicated in Fig. 1. In addition, performing a ML analysis with the partitioned data set did not result in better resolved nodes, either (Fig 1). Regarding the analysis of the mt data set, the tree with the highest support values was based on the alignments of the entire *COI* and the *12S* and *16S* with all gaps excluded ('no gap' option; Figure S1; Supporting information). The tree based on concatenated nc sequences is shown in Figure S2.

The comprehensive tree based on all concatenated sequences (Fig. 1) is divided into three clades, two of them containing species not belonging to Ariantinae: the representatives of the Hygromiidae and Helicodontidae, which were used to root the tree (clade A, black in Fig. 1), and the species of the subfamily Helicinae (B; green), which appear as the sister group of Ariantinae. Within clade B, *C. vindobonensis* and the sister taxa *C. nemoralis* + *C. hortensis* are separated by surprisingly high distances. *Eobania vermiculata* is more closely related to the latter two rendering *Cepaea* paraphyletic. This result is highly supported and also observed in both the concatenated mt and nc genes trees, as well as in most of the single gene trees (except for *16S* and *COI;* data not shown). The species of the subfamily Ariantinae (C; red, orange and blue in Fig. 1) are divided into three main clades: (1) Two species of *Kosicia* (*intermedia* and *ziegleri*) from the Southern Alps and *Faustina faustina* (from the Northern Carpathians). This clade (orange) obtained high support only in the tree where the 'no gap' option was used for the mt *12S* and *16S* genes. It is also found in the concatenated mt and nc gene trees (highly supported in the latter). (2) The second group (blue) is highly supported and comprises taxa distributed in the Balkan region: *Josephinella*, *Thiessea*, *Cattania*, *Dinarica*, *Liburnica* and *Vidovicia*. This clade is also found in the concatenated mt tree, but only when the 'no gap' option was used. Within this Balkan clade, most nodes are highly supported, for example the sister-group relationship between *Josephinella* and *Thiessea* and their clustering with *Cattania* as well as the clustering of *Liburnica* and *Dinarica*. (3) The remaining taxa are combined in a clade (red) with very low support, which is also found in the concatenated mt tree. It contains specimens from Alpine localities: *Chilostoma achates*, *Helicigona lapicida*, *Causa holosericea*, *Isognomostoma isognomostomos, Campylea illyrica* and the highly supported sister genera *Arianta* and *Cylindrus.* Within this 'Alpine' clade, most internal nodes obtained maximum support, and there are three well-supported lineages: a subclade combining *Arianta* + *Cylindrus*, another one comprising *C. achates*, *H. lapicida*, *C. holosericea*, and *I. isognomostomos*, and finally *C. illyrica* as a separate lineage.

In the tree based on the concatenated nc sequences (Figure S2), some relationships are very well supported, such as the *Kosicia* + *Faustina* clade and the differentiation between Helicinae and Ariantinae. However, other groups within the Ariantinae appear disrupted and obtained only low support.

Discussion

Our comprehensive tree based on five marker sequences provides strong support for the sister-group relationship between *Cylindrus* and *Arianta* confirming the results of Groenenberg et al. (2012). In addition, the tree presented here reveals a phylogeographical pattern within Ariantinae with some well-supported clades each combining either western (mainly Alpine and Carpathian) or eastern (Balkan) taxa, respectively. The distances between lineages imply several quite old radiations. However, there is still some ambiguity in the clustering of deeper branches. The hypothesis of Groenenberg et al. (2012) that the origin of Ariantinae, which they dated about 30 million years ago (mya), lies in the Balkan Peninsula is not supported in our analysis, as the species with a distribution in the Balkan region are united within one clade that does not split from the base of the Ariantinae. Yet, the even lower support values in the trees of Groenenberg et al. (2012) do not lend credence to the assumption of a Balkan origin either. In spite of the high amount of sequence data in both investigations (Groenenberg et al. 2012; this study), it seems that additional sequences will be necessary to resolve the deeper nodes within Ariantinae and to test any presumed phylogeographical pattern.

Concerning the lineages of *Cylindrus* and *Arianta,* the high distance value implies that this split is quite old (e.g. mean p-dis-

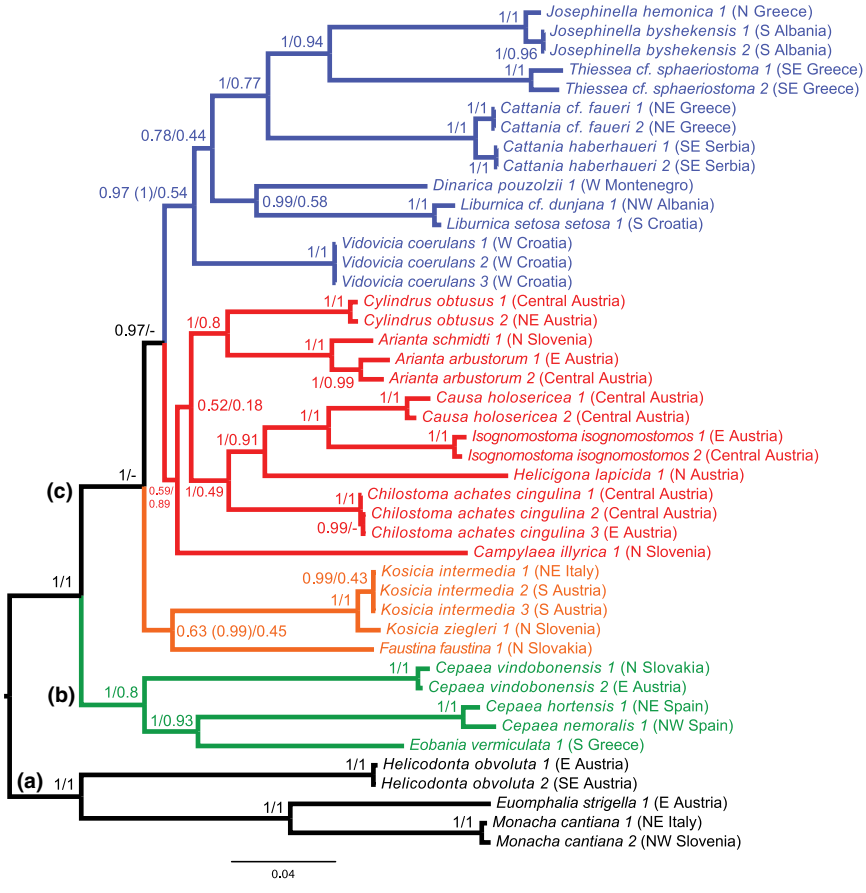

Fig. 1. Bayesian tree showing the phylogenetic relationships within Ariantinae and related taxa. The tree was calculated with the concatenated sequences of *12S-16S-COI-H3-H4*. Support values correspond to the analysis where the 'strict' option was used to trim the *12S* and *16S* sequences, and the 'COI3rdRY' option was used for *COI* (see text). Support values of the maximum likelihood (ML) analyses with the partitioned data set are also depicted (nodes that were absent in the ML analysis are indicated by '-'). Furthermore, two nodes obtained higher support in the analysis with the 'no gaps' option (indicated in brackets). The general geographical locations where the specimens were collected are provided

tance for *COI* between *Cylindrus* and *Arianta* is 20%, corresponding to a corrected distance of 24% – Tamura-Nei correction), which seems to be in contrast to the hypothesis of Schileyko (2012), who assumed a very young speciation of *Cylindrus*. Schileyko's argumentation was mainly based on the lack of fossils of *C. obtusus* and the fact that no other Pleistocene fossils resembling *C. obtusus* in shell morphology (which could be considered as a possible ancestor or sister taxon) are known. However, it needs to be emphasized that the environmental dynamics in these Alpine regions, which are characterized by changing conditions (temperature, humidity, wind, earthmoving, physical and chemical conditions of the environment, etc.), offers bad opportunities for shell fossilization. Especially, the black soil of the preferred habitat of *Cylindrus* is acidic and not appropriate for the fossilization of calcite shells. Thus, the lack of fossils in the Alpine region (which is also an argument against using this species for clock calibration; see below) is not a good argument to postulate a recent origin of *Cylindrus*. In any case, the supposed conflicting assumptions about old versus young origin of *Cylindrus* are not really contradicting, as the tree of extant species provides information about the *split of lineages*, but not about speciation or *origin of the phenotype* of *C. obtusus*. The latter could only be inferred from the fossil record.

Groenenberg et al. (2012) propose 17.5 my for the split between *Arianta* and *Cylindrus*. Yet, one has to keep in mind that a well-resolved tree is a prerequisite for dating splits. Although the distances between lineages within the subfamily Ariantinae are quite high, it remains open whether the datings of Groenenberg et al. (2012) are reasonable estimates. The fossils used for calibration and the low resolution of trees (probably partly due to sequence saturation in some genes) render their molecular clock approach still speculative. Using fossils of *C. obtusus* for calibration is not advisable, as almost no fossils of this species at all have been found up to now. There are only some rare and mostly young findings within the extant distribution range in the Eastern Alps (Frank 2006). Frank (2006) named five localities with fossil *Cylindrus* that she dated as late glacial ('Spätglazial'), during the Würm glaciation ('Würmeiszeitlich') or young Holocene ('Jüngstholozän'). However, no scientific dating is proved in Frank (2006) for these *C. obtusus* findings, and it is most probable that the existing fossils are not older than Würm. As the other datings used by Groenenberg et al. (2012) cover a very wide time range, those fossils are probably a too weak basis to reliably calculate the age of the split between *Cylindrus* and *Arianta*.

A common practice in the absence of reliable fossil records and/or well-defined biogeographical events to calibrate a molecular clock is to apply fixed substitution rates (Wilke et al. 2009). However, the variety of rates reported in the literature is very high, ranging from the very conservative 1% 'standard mitochondrial clock rate' (e.g. Avise 2000; substitution rate for protein coding mt genes) to rates that are an order of magnitude higher (e.g. Pons et al. 2010; Kotsakiozi et al. 2012). Considering the 24% *COI* sequence divergence between *Arianta* and *Cylindrus*, the

inferred divergence time would – depending on the rate applied – range between 1.2-12 mya, which is not quite informative.

A problem concerning the systematics of Ariantinae is that the taxonomic assignment of species and subspecies is often based mainly on geographical information (sample localities and presumed distribution ranges) rather than on diagnostic traits. Our results imply that additional molecular data (i.e. additional markers) as well as samples from a broader geographical range, complementary to this and previous studies, are needed. This would allow to establish a phylogenetic scaffold to search for further diagnostic traits, to assign clades to described taxa and finally to assess the distribution ranges of the genetic groups. This will subsequently help to disentangle the complicated phylogeny of Ariantinae. Embedded in a sound phylogeny of Helicidae and combined with a higher number of reliable fossil data such a comprehensive analysis would allow to attempt a conclusive molecular clock analysis of this group.

The genetic data presented here revealed new insights concerning the outgroup taxa investigated. The distinct position of *C. vindobonensis* and the sister-group relationship of *C. nemoralis* + *C. hortensis* with *E. vermiculata*, rendering the genus *Cepaea* paraphyletic, require broader geographic sampling for a reasonable taxonomic solution. Thus, a comprehensive phylogenetic analysis of the genera *Cepaea*, *Eobania* and other possibly related taxa is intended to resolve their relationships.

Acknowledgements

Financial support for this study was given by the Austrian Science Fund (FWF): project number P 19592 B17 and by the Synthesys Project (AT-TAF-3166), which is financed by the European Community Research Infrastructure Action under the FP7 'Capacities' Program, as well as by the 'Friends of the Natural History Museum Vienna'. For assistance in the lab, we thank Barbara Däubl. We are grateful for collecting help to Helmut Baminger, Agnes Bisenberger, Barbara Däubl, Doris Kleewein, Ira Richling and Sabine Zwierschitz. Many thanks to Peter Reischütz and Alexander Reischütz who provided samples from the Balkans and to Anita Eschner who supported this study by making available the samples from the mollusc collection of the NHMW. For fruitful discussions, we are indebted to Anatoly Schileyko, Hans Kothbauer, Renate Kothbauer and especially Wilhelm Pinsker, who also critically overhauled the manuscript.

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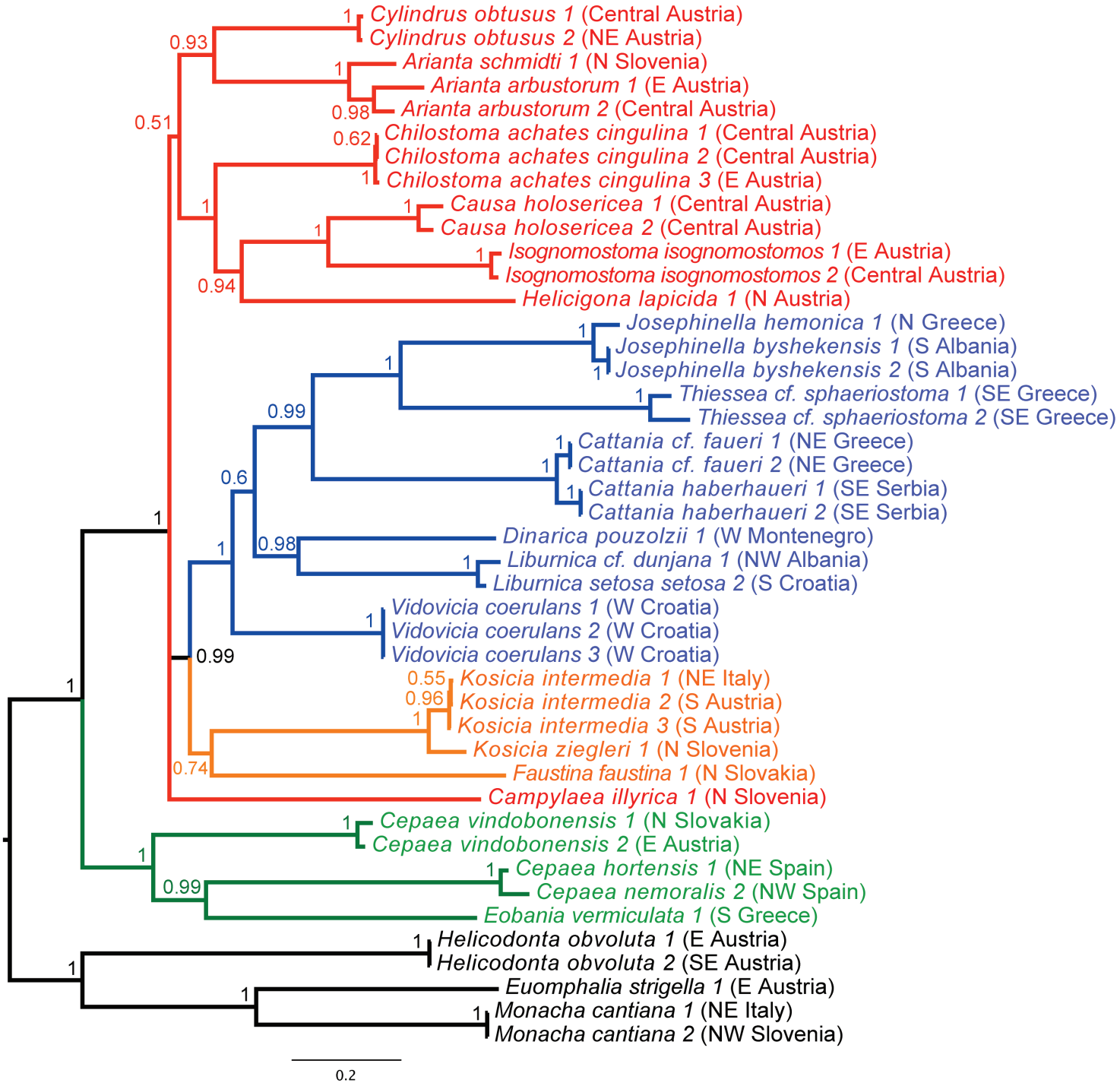
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. BI tree based on concatenated mt sequences: *12S* and *16S* ('no gap' option), *COI* (all positions) (44 sequences).

Figure S2. BI tree based on concatenated nc sequences (*H3* and *H4*) (48 sequences).

Cadahía L, Harl J, Duda M, Sattmann H, Kruckenhauser L, Fehér Z, Zopp L, Haring E (2013) New data on the phylogeny of Ariantinae (Pulmonata, Helicidae) and the systematic position of *Cylindrus obtusus* based on nuclear and mitochondrial DNA marker sequences.



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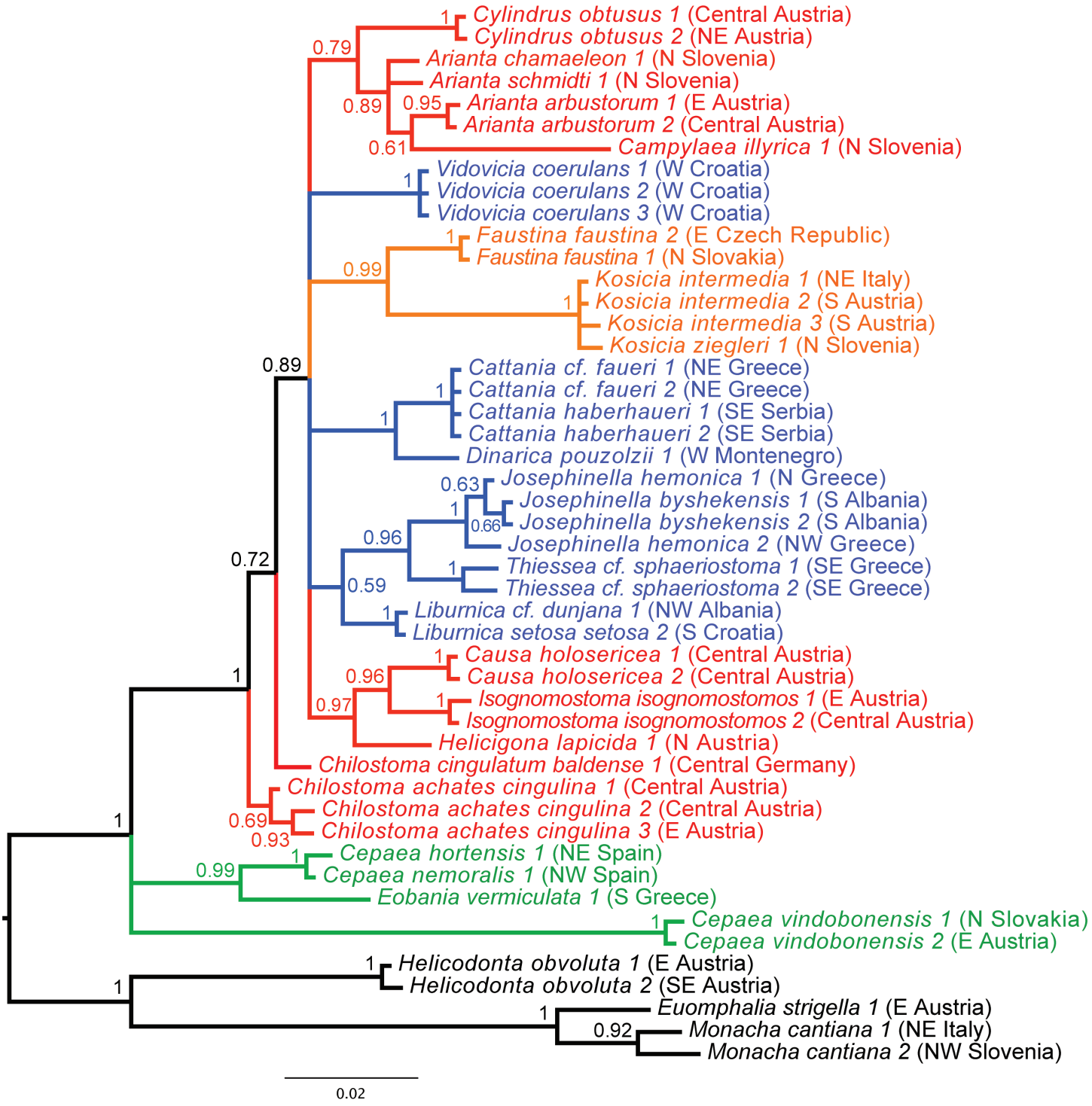

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