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Article

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The Oracle of Delphi—a molecular phylogenetic approach to Greek *Cordulegaster* Leach *in* Brewster, 1815 (Odonata: Anisoptera: Cordulegastridae)

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Abstract

Taxonomy of the genus *Cordulegaster* Leach *in* Brewster, 1815 in Greece is not completely understood. The taxonomic status of the subspecies *C. helladica buchholzi* (Lohmann, 1993), *C. helladica kastalia* (Lohmann, 1993), and *C. heros pelionensis* Theischinger, 1979 was still unclear. We applied a molecular genetic approach using sequences of mitochondrial and nuclear DNA fragments—cytochrome c oxidase I (COI) and Internal Transcribed Spacer 1 (ITS1). This approach revealed that specimens presently assigned to *C. heros pelionensis* should be considered as conspecific to the nominate subspecific taxon making *C. heros* a monotypic species. Two major monophyletic lines were found within the Greek representatives of the species grouped around *C. bidentata* Selys, 1843: the clade of the European endemic *C. bidentata* and the clade composed of three species: *C. helladica* (Lohmann, 1993), *C. buchholzi* (stat. nov., raised to species level), and *C. insignis* Schneider, 1845. *Cordulegaster helladica* is restricted to the Peloponnese. *Cordulegaster buchholzi* is not restricted to the Cyclades as previously thought, but widespread from the Cyclades over the island Euboea to south-east mainland Greece reaching in the west near Mount Parnassos, where it hybridize with *C. bidentata*. Hybridization between *C. bidentata* and *C. buchholzi* was detected at the Castalian Spring, where in ancient times the Oracle of Delphi was located, and some kilometres east of the Castalian Spring. These hybrids had been formerly named *C. helladica kastalia*. In the case of *C. insignis montandoni* St. Quentin, 1971 we have investigated specimens some kilometres away from the type locality in Romania, which all revealed hybrids between *C. bidentata* and *C. insignis*. However, we do not know if specimens phenotypically looking like *C. insignis* from further west in the SE Balkans represent isolated population of *C. insignis* within the range of *C. bidentata* or belong to a broader hybrid zone between *C. bidentata* and *C. insignis*.

Key words: *Cordulegaster buchholzi* stat. nov., *Cordulegaster helladica*, *Cordulegaster helladica kastalia*, *Cordulegaster heros pelionensis*, hybrid zone*, Peloponnese, South mainland Greece

Introduction

Species of *Cordulegaster* Leach *in* Brewster, 1815 are remarkably similar in structure in the Western Palearctic region and the westernmost East Palearctic while the pattern of yellow markings on abdomen, thorax and occipital triangle may vary, even within a single taxon (Boudot 2001; Corso 2019). This has resulted in confusion regarding the identification, distribution and infraspecific division. A comprehensive molecular analysis of the Greek *Cordulegaster* can help to understand the complex taxonomic status of this genus in Greece. Two recent molecular

studies on Western Palaearctic *Cordulegaster* have already improved many taxonomic problems in this genus (Froufe *et al*. 2014; Schneider *et al*. 2021). However, both studies did not treat the geographical separation of the taxa (species rank and subordinate) assigned to *C. helladica* (Lohmann, 1993) and *C. insignis* Schneider, 1845 in Greece and left the status of *C. heros pelionensis* Theischinger, 1979 unresolved. Froufe *et al*. (2014) already recognized that *C. helladica buchholzi* (Lohmann, 1993) shares a haplotype with *C. insignis* and is separated in COI-analysis from *C. helladica*.

Lohmann (1993) introduced the genus *Sonjagaster* for the *S. helladica*, **sp. nov.** with two new subspecies *S. h. kastalia* and *S. h. buchholzi*. He also transferred *C. insignis* into this genus. However, since then, no support was found for *Sonjagaster*, including the molecular genetic analysis from Froufe *et al*. (2014) and our recent revision of the eastern *Cordulegaster* species of the Western Palaearctic and the westernmost East Palearctic (Schneider *et al*. 2021). In the original description of *C. h. kastalia* the main difference with the nominate form is the middle broader and rectangular lateral yellow stripe on the sides of the synthorax. This weak argument was already questioned by Lopau (2010).

The other subspecies described by Lohmann (1993), *C. h. buchholzi*, was thought to be restricted to the Cyclades (Boudot 2001; Lopau 2010; Boudot & Holuša 2015; van Pelt 1999, 2020). The nominate *C. helladica* is currently believed to occur on the Peloponnese, south mainland Greece and at the island Euboea (Evia) (Boudot 2001; Lopau 2010; Boudot & Holuša 2015; van Pelt 2020). At Mount Parnassos it is believed to meet *C. bidentata* Selys, 1843 (Boudot 2001; Lopau 2010; van Pelt 2020).

Cordulegaster insignis is currently believed to occur in several Balkan areas like South Serbia, N-Macedonia, Romania, Bulgaria, some Greek Aegean Islands, and in western Turkey (for overview see Boudot *et al*. 2015). From Romania a subspecies has been described as *C. insignis montandoni* St. Quentin, 1971 which Lohmann (1993) announced to raise to species level without giving further details. One of us (G.J.v.P.) found that the only discriminating character for *C. i. montandoni* given by St. Quentin (1971) is not significant to define a subspecies, as the mentioned small black marking on the frons may also be present in typical *C. insignis* (van Pelt 2020). The original finding place of *C. i. montandoni* was Comana in Romania, only about 20 km away from the Danube River and near to Bulgarian records of *C. insignis* (Marinov *et al*. 2007). Thus, the taxonomic status from Romania and possible Bulgaria remains so far doubtful and unresolved. We had the possibility to analyse some Bulgarian specimens, which were originally allocated to *C. insignis* by one of us (G.J.v.P.).

Without information on the locality from which single individuals derived from, it may be nearly impossible to separate *C. insignis*, *C. h. kastalia,* and *C. h. buchholzi* by morphology only (van Pelt 2020).

Cordulegaster heros pelionensis was added as a subspecies with the original description of *C. heros* Theischinger, 1979, the main discriminating character allegedly being a striking black marking along the upper carina of the frons (Theischinger 1979). All Greece *C. heros* are considered to belong to this subspecies (Lopau 2010). Meanwhile several sequences of *C. heros* from different countries have been published (Froufe *et al*. 2014; Galimberti *et al*. 2021; Schneider *et al*. 2021).

Cordulegaster are reophilic, mainly restricted to clean running springs, brooks and rivers. These habitats, where many taxa of the genus live, are under particular threat in the south-east of Europe (Boudot & Kalkman 2014). Therefore, some of the European *Cordulegaster* are currently classified as endangered like *C. helladica* (Boudot 2010a) or even critically endangered as the subspecies *C. h. kastalia* (Boudot 2010b). A better taxonomic understanding of these taxa and their exact distribution is therefore of interest from the view of conservation aspects.

The aim of this study was to give a DNA-based overview of the *Cordulegaster* taxa inhabiting Greece, including the mainland, the Peloponese peninsula and the national islands, because several taxa in this region are taxonomically unresolved at the moment.

Material and methods

Material

A total of 80 *Cordulegaster* specimens from Greece and the neighbouring countries were investigated by molecular genetic analysis (Table 1): the genetic data of 42 of these were already published (*C. heros*, *C. picta*, *C. helladica*, *C. h. buchholzi*, and *C. insignis*) (Froufe *et al*. 2014; Galimberti *et al*. 2021; Schneider *et al*. 2021; Solano *et al*. 2018) while 38 specimens are new analysed.

clade	species	country / locality	latitude	longitude	collector / reference	collection
bidentata	*bidentata*	Greece, Moni Vellas	39.8637	20.6199	leg. W. Lopau	Senckenbergmuseum-Frankfurt.1036
bidentata	*bidentata*	Greece, Karitsa	39.8398	22.7589	leg. W. Lopau	Senckenbergmuseum-Frankfurt.890
bidentata	*bidentata*	Bulgaria	42.0133	24.8784	leg. G.J. van Pelt	RMNH.INS.974924
bidentata	*bidentata*	Czech Republic	50.8100	14.7762	leg. M. Waldhauser	
bidentata	*bidentata*	Greece	39.7366	22.7825	Froufe *et al.* 2014	
bidentata	*bidentata*	Greece	38.7289	22.2433	Froufe *et al.* 2014	
bidentata	*bidentata*	Greece	38.7289	22.2433	Froufe *et al.* 2014	
bidentata	*bidentata*	Greece	38.6666	22.3500	Froufe *et al.* 2014	
bidentata	*bidentata*	Greece	38.6666	22.3500	Froufe *et al.* 2014	
bidentata	*bidentata*	France	43.7425	3.44760	Froufe *et al.* 2014	
bidentata	*bidentata*	Italy	45.9000	9.30000	Galimberti *et al.* 2021	
bidentata	*bidentata*	Italy	46.2000	11.3000	Galimberti *et al.* 2021	
bidentata	*bidentata*	Germany	48.8605	8.34417	Froufe *et al.* 2014	
bidentata	*bidentata*	Albania	41.9983	20.5232	Froufe *et al.* 2014	
bidentata	*bidentata*	Albania	41.6465	20.1898	Froufe *et al.* 2014	
bidentata	*bidentata*	Montenegro	42.2343	18.9066	Froufe *et al.* 2014	
bidentata	*bidentata*	Italy	40.0502	16.1243	Froufe *et al.* 2014	
bidentata	*bidentata*	Italy	44.2000	7.70000	Galimberti *et al.* 2021	
bidentata	*bidentata*	Italy	no data	no data	Solano *et al.* 2018	
bidentata	*bidentata*	Italy	40.0502	16.1243	Froufe *et al.* 2014	
bidentata	*bidentata sicilica*	Italy	37.9275	14.6859	Froufe *et al.* 2014	
bidentata	*bidentata sicilica*	Italy	37.9274	14.6700	Froufe *et al.* 2014	
bidentata	*bidentata sicilica*	Italy	37.9275	14.6859	Froufe *et al.* 2014	
bidentata	*helladica kastalia*	Greece, Castalian Spring	38.4841	22.5066	leg. W. Lopau	Senckenbergmuseum-Frankfurt.905
bidentata	*helladica kastalia*	Greece, Castalian Spring	38.4841	22.5066	leg. W. Lopau	Senckenbergmuseum-Frankfurt.906
bidentata	*helladica kastalia*	Greece, Castalian Spring	38.4841	22.5066	leg. W. Lopau	Senckenbergmuseum-Frankfurt.lopi1
bidentata	*helladica kastalia*	Greece, Castalian Spring	38.4841	22.5066	leg. W. Lopau	Senckenbergmuseum-Frankfurt.lopi2
bidentata	*helladica kastalia*	Greece, Castalian Spring	38.4841	22.5066	leg. W. Lopau	Senckenbergmuseum-Frankfurt.lopi3
bidentata	*helladica kastalia*	Greece, Boeotia	38.5149	22.7332	leg. G.J. van Pelt	RMNH.INS.974960

TABLE 1. (Continued)

clade	species	country / locality	latitude	longitude	collector/ reference	collection
bidentata	helladica kastalia	Greece, Fokida	38.4800	22.5843	leg. G.J. van Pelt	RMNH.INS.974963
bidentata	helladica kastalia	Greece, Fokida	38.4800	22.5843	leg. G.J. van Pelt	RMNH.INS.974941
bidentata	insignis montandoni	Bulgaria	42.9087	26.4603	leg. G.J. van Pelt	RMNH.INS.1152966
bidentata	insignis montandoni	Bulgaria	42.9087	26.4603	leg. G.J. van Pelt	RMNH.INS.1152967
bidentata	insignis montandoni	Bulgaria	42.9087	26.4603	leg. G.J. van Pelt	RMNH.INS.1152969
bidentata	helladica buchholzi	Greece, Andros	37.8486	24.8304	leg. T. Schneider	
bidentata	helladica	Greece, Euboea	38.0246	24.5093	leg. T. Schneider	
bidentata	helladica	Greece, Euboea	38.5530	23.9886	leg. T. Schneider	
bidentata	helladica buchholzi	Greece, Tinos	37.6034	25.0745	leg. W. Lopau	Senckenbergmuseum-Frankfurt.949
bidentata	helladica buchholzi	Greece, Tinos	37.6034	25.0745	leg. W. Lopau	Senckenbergmuseum-Frankfurt.950
bidentata	helladica buchholzi	Greece, Tinos	37.5946	25.2267	leg. W. Lopau	Senckenbergmuseum-Frankfurt.955
bidentata	helladica	Greece, Kamena Vourla / Mainland	38.7719	22.7770	leg. G.J. van Pelt	RMNH.INS.974961
bidentata	helladica	Greece, Boeotia / Mainland	38.5149	22.7332	leg. G.J. van Pelt	RMNH.INS.974962
bidentata	helladica	Greece, Boeotia / Mainland	38.5149	22.7332	leg. G.J. van Pelt	RMNH.INS.974939
bidentata	helladica	Greece, Boeotia / Mainland	38.5149	22.7332	leg. G.J. van Pelt	RMNH.INS.974940
bidentata	helladica buchholzi	Greece, Naxos	37.1089	25.5154	leg. G.J. van Pelt	RMNH.INS.974938
bidentata	helladica buchholzi	Greece, Naxos	37.1089	25.5154	leg. G.J. van Pelt	RMNH.INS.974937
bidentata	helladica buchholzi	Greece, Naxos	37.1394	25.5299	Froufe et al. 2014	
bidentata	helladica	Greece, Peloponnese	37.8345	22.1808	leg. W. Lopau	Senckenbergmuseum-Frankfurt.925
bidentata	helladica	Greece, Peloponnese	38.0602	22.2142	leg. M. Waldhauser	
bidentata	helladica	Greece, Peloponnese	38.0946	22.1965	leg. M. Waldhauser	
bidentata	helladica	Greece, Peloponnese	36.8842	22.3032	Froufe et al. 2014	
bidentata	helladica	Greece, Peloponnese	37.0987	22.2805	Froufe et al. 2014	
bidentata	helladica	Greece, Peloponnese	38.0000	21.8333	Froufe et al. 2014	
bidentata	helladica	Greece, Peloponnese	38.0000	21.8333	Froufe et al. 2014	
bidentata	insignis	Greece, Samos	37.7086	26.8175	Schneider et al. 2021	
bidentata	insignis	Greece, Samos	37.7958	26.8147	Schneider et al. 2021	
bidentata	insignis	Greece, Icaria	37.5786	26.0405	Schneider et al. 2021	

TABLE 1. (Continued)

clade	species	country / locality	latitude	longitude	collector/ reference	collection
bidentata	*insignis*	Greece, Icaria	37.5786	26.0405	Schneider *et al.* 2021	RMNH.INS.974936
bidentata	*insignis*	Greece, Icaria	37.6333	26.2166	Schneider *et al.* 2021	Senckenbergmuseum-Frankfurt.993
bidentata	*insignis*	Greece, Lesbos	39.0689	26.3467	Schneider *et al.* 2021	
bidentata	*insignis*	Turkey	37.0286	28.7553	Froufe *et al.* 2014	
bidentata	*insignis*	Turkey	37.0286	28.7553	Froufe *et al.* 2014	
bidentata	*insignis*	Turkey	37.2196	28.3658	Froufe *et al.* 2014	
bidentata	*insignis*	Turkey, Edirne	41.6771	26.5557	Schneider *et al.* 2021	
bidentata	*insignis*	Turkey, Emet	39.3444	29.2578	Schneider *et al.* 2021	
boltonii	*heros pelionensis*	Greece, Flampoura	39.0608	20.7512	leg. W. Lopau	Senckenbergmuseum-Frankfurt.912
boltonii	*heros pelionensis*	Greece, Dorothea	40.9985	22.0604	leg. W. Lopau	Senckenbergmuseum-Frankfurt.1021
boltonii	*heros pelionensis*	Greece, Agathi	41.0165	22.0559	leg. W. Lopau	Senckenbergmuseum-Frankfurt.1023
boltonii	*heros pelionensis*	Greece	38.7289	22.2433	Froufe *et al.* 2014	
boltonii	*heros*	Bulgaria	42.5936	23.4102	Schneider *et al.* 2021	RMNH.INS.974925
boltonii	*heros*	Bulgaria	42.4928	27.4733	Schneider *et al.* 2021	RMNH.INS.974930
boltonii	*heros*	Bulgaria	42.7338	25.4858	Schneider *et al.* 2021	RMNH.INS.974934
boltonii	*heros*	Italy	46.2000	13.3000	Galimberti *et al.* 2021	
boltonii	*heros*	Italy	45.6400	13.8600	Galimberti *et al.* 2021	
boltonii	*heros*	Italy	45.9000	13.6000	Galimberti *et al.* 2021	
boltonii	*heros*	Montenegro	42.2000	19.1000	Galimberti *et al.* 2021	
boltonii	*heros*	Montenegro	42.1837	19.2914	leg. G. De Knijf	
boltonii	*picta*	Greece, Thasos	40.6909	24.6208	leg. G.J. van Pelt	RMNH.INS.974955
boltonii	*picta*	Greece, Thrace Province	41.3093	26.1147	Froufe *et al.* 2014	
boltonii	*picta*	Greece, Samos	37.7958	26.8147	Schneider *et al.* 2021	

Among these 38 specimens we included specimens collected by Wolfgang Lopau at the *locus typicus* at the Castalian Spring in Delphi and those collected by one of us (G.J.v.P.) some kilometres north-east of the Castalian Spring, which both represent the only known localities of this putative subspecies. In addition we added further sequences of supposed *C. h. pelionensis* collected by Wolfgang Lopau (leg. 1999 and 2007, and stored in Senckenberg Museum, Frankfurt) and will compare them with the nominate species. *Cordulegaster picta* Selys, 1854 reaches its western distribution limits in eastern Greece and Bulgaria (van Pelt 2020), therefore specimens from this region were included in this study.

General Methods

We used a molecular genetic approach analysing fragments of the COI and ITS1 genes for constructing bootstrap maximum likelihood trees, Bayesian inference trees and haplotype networks. In addition, evolutionary distance between a pair of sequences was measured by the p-distance and the Kimura 2-P distance (K2-P). Together with geographical and phenotypical characters, the latter methods can be translated into taxonomic ranks. In zoology, about 2–3% difference is considered as good limit for species difference (Hebert *et al.* 2003a, b). For Odonata a difference between 2 and 3% are discussed for species discrimination and may be lower in closely related species (Rach *et al.* 2008; Koroiva *et al.* 2017; Pimenta *et al.* 2019; Galimberti *et al.* 2020; Ge *et al.* 2021; Geiger *et al.* 2021). A recent analysis for Western Palaearctic Odonata revealed for Anisoptera a K2-P distance above 1.96% for a good threshold (Galimberti *et al.* 2020). This barcoding analysis on a large set of Italian Odonata showed that genetic K2-P distance variation within morphospecies ranged from 0% to 9.17% (mean ± SD= 0.48 ± 0.62 %) in Zygoptera and from 0% to 2.64% (mean ± 326 SD = 0.33 ± 0.29 %) in Anisoptera. Interspecific K2-P distance values ranged from 0% to 27.29% (mean ± SD= 19.41 ± 3.71%) and from 0% to 25.28% (mean ± SD= 18.25 ± 3.18 %) in Zygoptera and Anisoptera, respectively (Galimberti *et al.* 2020). Therefore, we used beside the Bayesian inference trees and haplotype networks a K2P-distance above 2% as an orientation for species allocation. We observed no relevant difference between p- and K2-P distances.

Possible hybrids were interpreted when specimens with their maternally inherited COI corresponding to a different species than their ITS.

Molecular genetic analysis

We analyzed a fragment of the cytochrome c oxidase subunit I (COI) gene from the mtDNA and the ITS1 from the rDNA of the nucleus. For details of the origin of specimens see Table 1. For the COI gene, the sequences are 568 bp long, for the ITS1 the sequences are between 308 and 386 bp long, depending on the species.

DNA extraction, PCR methods, and sequencing

Per specimen, a 1.0 mm section of a leg was transferred to a tube with 20 μl 0,05N NaOH and 2 μl 5 % Tween 20. This was heated for 15 minutes at 95°C and cooled on ice. 100 μl sterile water was added to the tube and mixed. 1 to 5 μl of this solution was used in a PCR reaction. We amplified and sequenced a fragment of mtDNA (the barcoding segment of the cytochrome c oxidase subunit I (COI) gene) and the ITS1 between the SSU and LSU region of the 18S nuclear rDNA operon, using the PCR on a 2720 Thermal Cycler of Applied Biosystems, CA, USA. Primers used for PCR were CO1490F (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and CO2198R (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.* 1994) for the COI fragment. Cycle conditions were 95°C for 3 min followed by 45 cycles of 95 °C for 30 sec, 48 °C for 30 sec and 72 °C for 1 min. Primers used for amplifying the rDNA fragment are Vrain2F (5'-CTT TGT ACA CAC CGC CCG TCG CT-3') and 28R1 (5'-TGA TAT GCT TAA NTT CAG CGG GT-3') primers. Cycle conditions were 95°C for 3 min followed by 45 cycles of 95 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min. PCR products were sequenced on an ABI 3130XL automatic sequencer from Applied Biosystems with the BigDye 3.1 kit according to manufacturer instructions.

Phylogenetic Methods

MAFFT (Multiple Alignment with Fast Fourier Transform)

Multiple sequence alignments were made with the online version of MAFFT (Katoh *et al.* 2019).

jModelTest

The model of DNA evolution that best fitting the data was determined with JMODELTEST version 2.1.10 (Darriba

et al. 2012). Based on the Bayesian information criteria (BIC), the best model was chosen for Bayesian inference in MRBAYES 3.2.7a (Ronquist *et al.* 2012) and Maximum Likelihood analysis in PAUP 4.0a168 (Swofford 2003).

Tree construction with MrBayes and PAUP

The model parameters from JMODELTEST were used in MRBAYES (nst=2 and rates=gamma for COI; nst=6 and rates=equal for ITS1). The settings were: 10 million generations, a sample frequency of 1000 and a burnin value of 5000 trees.

PAUP 4.0a168 was used for constructing the bootstrap Maximum Likelihood trees. The model parameters from JMODELTEST were used as model in PAUP HKY + G base=(0.3189 0.1331 0.1272) nst=2 tratio=4.1842 rates=gamma shape=0.1250 ncat=4 pinvar=0 for COI, TIM3+G base=(0.2469 0.2495 0.3203) nst=6 rmat=(0.6873 2.2209 1.0000 0.6873 3.2096) rates=gamma shape=0.1200 ncat=4 pinvar=0 for ITS1). 100 bootstrap replicates (starting with a Neighbor Joining tree) was performed with a branch swapping limit set to 100000 or 3 hours (what comes first) per bootstrap replicate. For creating trees, *Cordulegaster* DNA-sequences from the West Palaearctic, from America and from E-Asia were used. Genbank was searched for credible sequences. Available sequences for COI and the spacer region approximately the same length as our own were added to the alignment. Their accession numbers are listed next to the names in the trees.

StarBeast

In addition StarBeast, a multi-individual multi-locus species tree estimation program, using Bayesian coalescent analysis, as implemented in the BEAST package was applied for both genes (Drummond & Rambaut 2007; Drummond *et al.* 2012). Xml input files were created in BEAUTI v2.6.3 (Drummond *et al.* 2012), using the HKY+Γ+I model for both markers. The following settings were used for all analyses: base frequencies 'empirical' clock model 'Strickt clock Clock.rate = 1'; tree prior 'default values (Yule Model)'; The analyses were run in BEAST v.2.6.3 (Drummond & Rambaut 2007; Drummond *et al.* 2012). Analyses were run for 50 million generations, sampling every 5000th generation. Tracer v. 1.7.1 (Drummond & Rambaut 2007) was used for examining the effective sample size (ESS) for parameters and determining the burn-in. Trees and posterior probabilities were summarized using TreeAnnotator v. 2.6.3 (Drummond & Rambaut 2007) and showed on the Maximum clade credibility tree, with Posterior probability limit = 0.5 and Burnin percentage = 0. The trees were drawn in FigTree v.1.4.4 (Rambaut 2021).

BPP

As a third program, we used BPP v. 4.3.8 (Flouri *et al.* 2018) for coalescent species delimitation. For COI, the input species tree contained 21 species and 151 sequences. For ITS1, the input species tree contained 12 species and 53 sequences. For the combined COI and ITS1, the input species tree contained 12 species and 47 sequences.

1. Running species delimitation:
Settings were Speciesdelimitation = 1 0 2, Speciestree = 1, speciesmodelprior = 1, burnin = 16 000, samplefreq = 2, nsample = 500 000.

2. Running Species tree Estimation:
Settings were Speciesdelimitation = 0, Speciestree = 1, speciesmodelprior = 1, burnin = 16 000, samplefreq = 2, nsample = 500 000.

F_{ST}

Arlequin v. 3.5.2.2 (Excoffier *et al.* 2010) was used to calculate the Fixation Index (F_{ST}) between the species based on the COI gene. F_{ST} is the proportion of the total genetic variation of a subpopulation relative to the total genetic variation of the total population. A value close to zero means almost complete sharing of genetic material, a value close to one means no sharing.

Haplotype analysis

Haplotype network were built in POPART with the TCS network interference method from the COI alignment (Leigh & Bryant 2015). A haplotype network is the evolutionary sum of mutations that defines the current haplotypes through the DNA lineages that connect the current DNA molecules to the common ancestral DNA molecule.

Evolutionary distance analysis

The evolutionary distance between a pair of sequences is usually measured by the number of nucleotide or amino acid substitutions between them: p-distance. This distance is merely the proportion (p) of nucleotide sites at which the two sequences compared are different. This is obtained by dividing the number of nucleotide differences (nd) by the total number of nucleotides compared (n). This is one of the more simple methods to evaluate differences and to calculate relationships between taxa. Another established technique for evaluating DNA sequences is the Kimura 2-parameter (K2-P) index, which is conveniently expressed in percent differences between sequences. The p- and K2-P distance matrix between groups was constructed in MEGA X (Kumar *et al*. 2018). Transitions and transversions were included, uniform rates among sites and gaps were pairwise deleted.

Distribution maps

The distribution maps were created as follows: Collecting localities of the specimens were first georeferenced using Google's Geocoding API (Application Programming Interface). Subsequently, the resulting coordinates were plotted in ARCGIS version 10.2.2 using the geographic coordinate system WGS 1984. The source of the background map for reference is the U.S. National Park Service, public available through Esri services.

Results

Our results identified the presence within the investigated area of species which have evolved in two main clades, one of which split further to two clades called for convenience monophyletic lines (ML) (Table 2). The clades are represented by the following species: *C. boltonii* (Donovan, 1807), *C. heros*, *C. picta* (Clade I), and *C. bidentata*, *C. buchholzi*, *C. helladica*, *C. insignis* (Clade II). The latter is composed of two monophyletic lines (ML) formed by the following species: *C. bidentata* (ML1), and *C. buchholzi*, *C. helladica*, *C. insignis* (ML2).
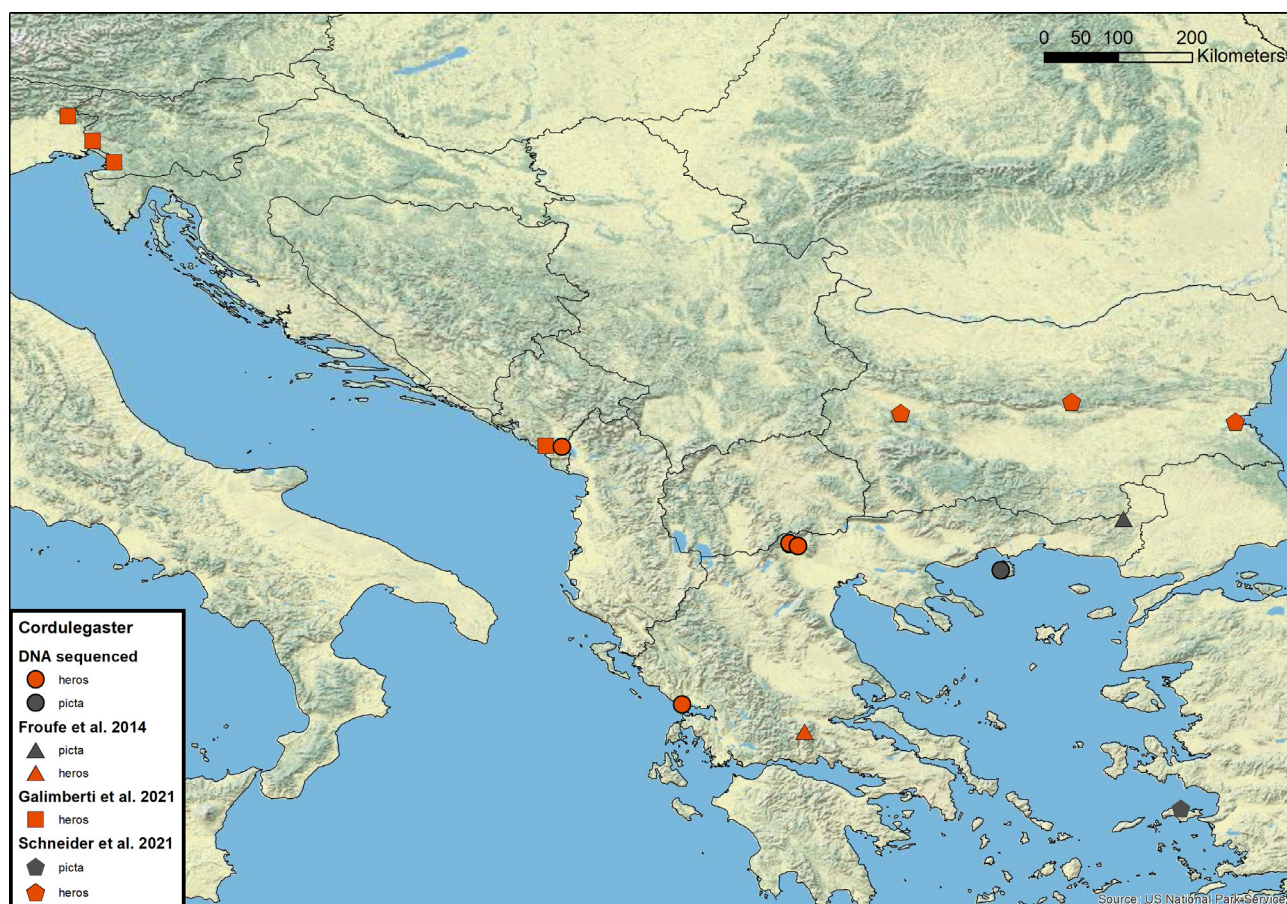

FIGURE 1. Map indicating the geographical distribution of investigated specimens of the Clade I in Greece and south-east Europe. For details see Table 1.

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TABLE 2. Taxa of *Cordulegaster* specimens after genetic investigation.

clade	taxon before genetic investigation (see Table 1)	taxon after genetic investigation	country / locality	PCR COI	PCR ITS	genbank COI	genbank ITS
bidentata	bidentata	bidentata	Greece, Moni Vellas	15595	15632	MW353693	MW363795
bidentata	bidentata	bidentata	Greece, Karitsa	15596	failed	MW353694	no data
bidentata	bidentata	bidentata	Bulgaria	11568	11620	MK779815	MK861454
bidentata	bidentata	bidentata	Czech Republic	10949	failed	MK779814	no data
bidentata	bidentata	bidentata	Greece			KF584925	KF584995
bidentata	bidentata	bidentata	Greece			KF584926	no data
bidentata	bidentata	bidentata	Greece			KF584927	KF584995
bidentata	bidentata	bidentata	Greece			KF584931	KF584995
bidentata	bidentata	bidentata	Greece			KF584928	KF584995
bidentata	bidentata	bidentata	France			KF584963	KF584984
bidentata	bidentata	bidentata	Italy			MT298385	no data
bidentata	bidentata	bidentata	Italy			MT298384	no data
bidentata	bidentata	bidentata	Germany			KF584958	KF584980
bidentata	bidentata	bidentata	Albania			KF584923	no data
bidentata	bidentata	bidentata	Albania			KF584924	no data
bidentata	bidentata	bidentata	Montenegro			KF584922	KF584995
bidentata	bidentata	bidentata	Italy			KF584929	no data
bidentata	bidentata	bidentata	Italy			MT298386	no data
bidentata	bidentata	bidentata	Italy			MH304666	no data
bidentata	bidentata	bidentata	Italy			KF584930	no data
bidentata	bidentata sicilica	bidentata	Italy			KF584972	no data
bidentata	bidentata sicilica	bidentata	Italy			KF584971	no data
bidentata	bidentata sicilica	bidentata	Italy			KF584947	no data
bidentata	helladica kastalia	bidentata x buchholzi	Greece, Castalian Spring	15614	15690	MW353695	MW363782
bidentata	helladica kastalia	bidentata x buchholzi	Greece, Castalian Spring	15589	15696	MW353691	MW363784
bidentata	helladica kastalia	bidentata x buchholzi	Greece, Castalian Spring	15642	15697	MW353698	MW363783
bidentata	helladica kastalia	bidentata x buchholzi	Greece, Castalian Spring	15643	15630	MW353699	MW363793
bidentata	helladica kastalia	bidentata x buchholzi	Greece, Castalian Spring	15594	15631	MW353692	MW363794

TABLE 2. (Continued)

clade	taxon before genetic investigation (see Table 1)	taxon after genetic investigation	country / locality	PCR COI	PCR ITS	genbank COI	genbank ITS
bidentata	*helladica kastalia*	*bidentata x buchholzi*	Greece, Boeotia	12297	12321	MK779818	MK861480
bidentata	*helladica kastalia*	*bidentata x buchholzi*	Greece, Fokida	12300	12324	MK779817	MK861483
bidentata	*helladica kastalia*	*bidentata x buchholzi*	Greece, Fokida	11756	11637	MK779816	MK861479
bidentata	*insignis montandoni*	*bidentata x insignis*	Bulgaria	15489	15497	MT811768	MT812012
bidentata	*insignis montandoni*	*bidentata x insignis*	Bulgaria	15490	15498	MT811769	MT812013
bidentata	*insignis montandoni*	*bidentata x insignis*	Bulgaria	15492	15500	MT811770	MT812014
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Andros	15528	15536	MW353688	MW363790
bidentata	*helladica*	*buchholzi*	Greece, Euboea	15529	15537	MW353689	MW363791
bidentata	*helladica*	*buchholzi*	Greece, Euboea	15530	15538	MW353690	MW363792
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Tinos	15653	failed	MW353700	no data
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Tinos	15636	failed	MW353697	no data
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Tinos	15654	failed	MW353701	no data
bidentata	*helladica*	*buchholzi*	Greece, Kamena Vourla / Mainland	12298	12322	MK779853	MK861481
bidentata	*helladica*	*buchholzi*	Greece, Boeotia / Mainland	12299	12323	MK779854	MK861482
bidentata	*helladica*	*buchholzi*	Greece, Boeotia / Mainland	11583	11635	MK779851	MK861488
bidentata	*helladica*	*buchholzi*	Greece, Boeotia / Mainland	11601	11636	MK779852	MK861489
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Naxos	11582	11634	MK779849	MK861478
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Naxos	11802	11633	MK779850	MK861485
bidentata	*helladica buchholzi*	*buchholzi*	Greece, Naxos			KF584939	KF584979
bidentata	*helladica*	*helladica*	Greece, Peloponnese	15617	failed	MW353696	no data
bidentata	*helladica*	*helladica*	Greece, Peloponnese	10934	10939	MK779855	MK861455
bidentata	*helladica*	*helladica*	Greece, Peloponnese	10935	11063	MK779856	MK861456
bidentata	*helladica*	*helladica*	Greece, Peloponnese			KF584938	KF584986
bidentata	*helladica*	*helladica*	Greece, Peloponnese			KF584937	KF584983
bidentata	*helladica*	*helladica*	Greece, Peloponnese			KF584935	KF584986
bidentata	*helladica*	*helladica*	Greece, Peloponnese			KF584936	KF584986
bidentata	*insignis*	*insignis*	Greece, Samos	15525	15533	MW353685	MW363787

TABLE 2. (Continued)

clade	taxon before genetic investigation (see Table 1)	taxon after genetic investigation	country / locality	PCR COI	PCR ITS	genbank COI	genbank ITS
bidentata	*insignis*	*insignis*	Greece, Samos	15526	15534	MW353707	no data
bidentata	*insignis*	*insignis*	Greece, Icaria	15527	15535	MW353686	MW363788
bidentata	*insignis*	*insignis*	Greece, Icaria	11580	11632	MW353687	MW363789
bidentata	*insignis*	*insignis*	Greece, Icaria	15656	failed	MK779847	MK861484
bidentata	*insignis*	*insignis*	Greece, Lesbos	15725		MW353702	no data
bidentata	*insignis*	*insignis*	Turkey		failed	KF584943	KF584979
bidentata	*insignis*	*insignis*	Turkey			KF584941	KF584979
bidentata	*insignis*	*insignis*	Turkey			KF584942	no data
bidentata	*insignis*	*insignis*	Turkey, Edirne	11567	11619	MK779846	MK861486
bidentata	*insignis*	*insignis*	Turkey, Emet	11807	11624	MK779848	MK861487
boltonii	*heros pelionensis*	*heros*	Greece, Flampoura	15715	15727	MW353705	MW363785
boltonii	*heros pelionensis*	*heros*	Greece, Dorothea	15716	15728	MW353706	MW363786
boltonii	*heros pelionensis*	*heros*	Greece, Agathi	15701	failed	MW353703	no data
boltonii	*heros pelionensis*	*heros*	Greece	11770	11712	KF584940	KF584991
boltonii	*heros*	*heros*	Bulgaria	11772	failed	MK779812	MK861451
boltonii	*heros*	*heros*	Bulgaria	11578	failed	MK779810	no data
boltonii	*heros*	*heros*	Bulgaria			MK779811	no data
boltonii	*heros*	*heros*	Italy			MT298391	no data
boltonii	*heros*	*heros*	Italy			MT298392	no data
boltonii	*heros*	*heros*	Italy			MT298393	no data
boltonii	*heros*	*heros*	Montenegro			MT298390	no data
boltonii	*heros*	*heros*	Montenegro	11552	11701	MK779813	MW353703
boltonii	*picta*	*picta*	Greece, Thasos	12230	failed	MK779802	no data
boltonii	*picta*	*picta*	Greece, Thrace Province			KF584944	KF584992
boltonii	*picta*	*picta*	Greece, Samos	15524	failed	MW353684	no data

Molecular genetic analysis of the COI and ITS gene fragments

Clade I

In Greece two species of this clade are found by our molecular approach (Fig. 1). All Greek *C. heros* supposed to be *C. h. pelionensis* grouped with all other *C. heros* from Italy, Bulgaria and Montenegro in the COI (Fig. 2) as well as in the ITS (Fig. 3) analysis, and in the combination of both genes (Fig. 4) by MRBAYES alignments. The haplotype analysis revealed no evidence for subspecies level either (Fig. 5). Coalescent based method StarBEAST and species delimitation BPP revealed similar results (data not shown).

All *C. picta* from Bulgaria and Greece group together with those from Turkey and the Black Sea region of Russia.

The K2-P distances were above 3% for the species: *C. boltonii*, *C. heros*, *C. picta*, but not for *C. h. pelionensis* (Table 3). For the latter the K2-P distance was below 1% in comparison to the nominate *C. heros* (Table 3).

TABLE 3. Genetic distance, estimated by the Kimura 2-parameter method, between the different taxa in the Clade I.

	boltonii	*heros*	*heros pelionensis*	average distance within the group
boltonii				0.0077
heros	0.0794			0.0112
heros pelionensis	0.0814	0.0098		0.0092
picta	0.0773	0.0588	0.0604	0.0066

Clade II

Within the Clade II, two clades, called here for convenience *monophyletic lines* (ML), are recognized by the COI, ITS, and combined analysis with all three programmes used, which are represented by the following species: *C. bidentata* (MLI), *C. buchholzi, C. helladica* and *C. insignis* (MLII). The distribution of the Greece members is represented on Figures 6 and 7. While *C. bidentata* is well settled there is some taxonomic uncertainty within the MLII.

Monophyletic line II (MLII)

In the MLII three closely related species could be defined in the COI analysis with the MRBAYES alignment: *C. buchholzi, C. helladica,* and *C. insignis*. (Figs. 2–4, 7). The alignments of COI, with the MRBAYES (Fig. 2), and haplotype analysis supports the species level of *C. buchholzi*, **stat. nov**. StarBEAST-analysis of the COI-gene also supports species level for *C. buchholzi* (Fig. 8). Delimitation BPP methods (not shown) were less specific and did not separate closely related species as *C. buchholzi* and *C. helladica*. *Cordulegaster buchholzi* shares a haplotype with *C. insignis*.

The calculated F_{ST} values indicate, that there is nearly no interbreeding between the species. Interbreeding occurs only in rare hybrid zones as for example between *C. bidentata* and *C. insignis* (F_{ST} =0.03691) in Bulgaria, and between *C. bidentata* and *C. buchholzi* in SW mainland Greece (0.24985; Table 4).

The K2-P distances (Table 5) between the species: *C. bidentata*, *C. helladica*, *C. buchholzi*, and *C. insignis* were well above 3% (far above the threshold of 1,96% calculated for Western Palaearctic Anisoptera by Galimberti *et al.* 2020). *Cordulegaster buchholzi* is found in south-east mainland Greece, Euboea, Andros, Tinos and Naxos (Fig. 7).
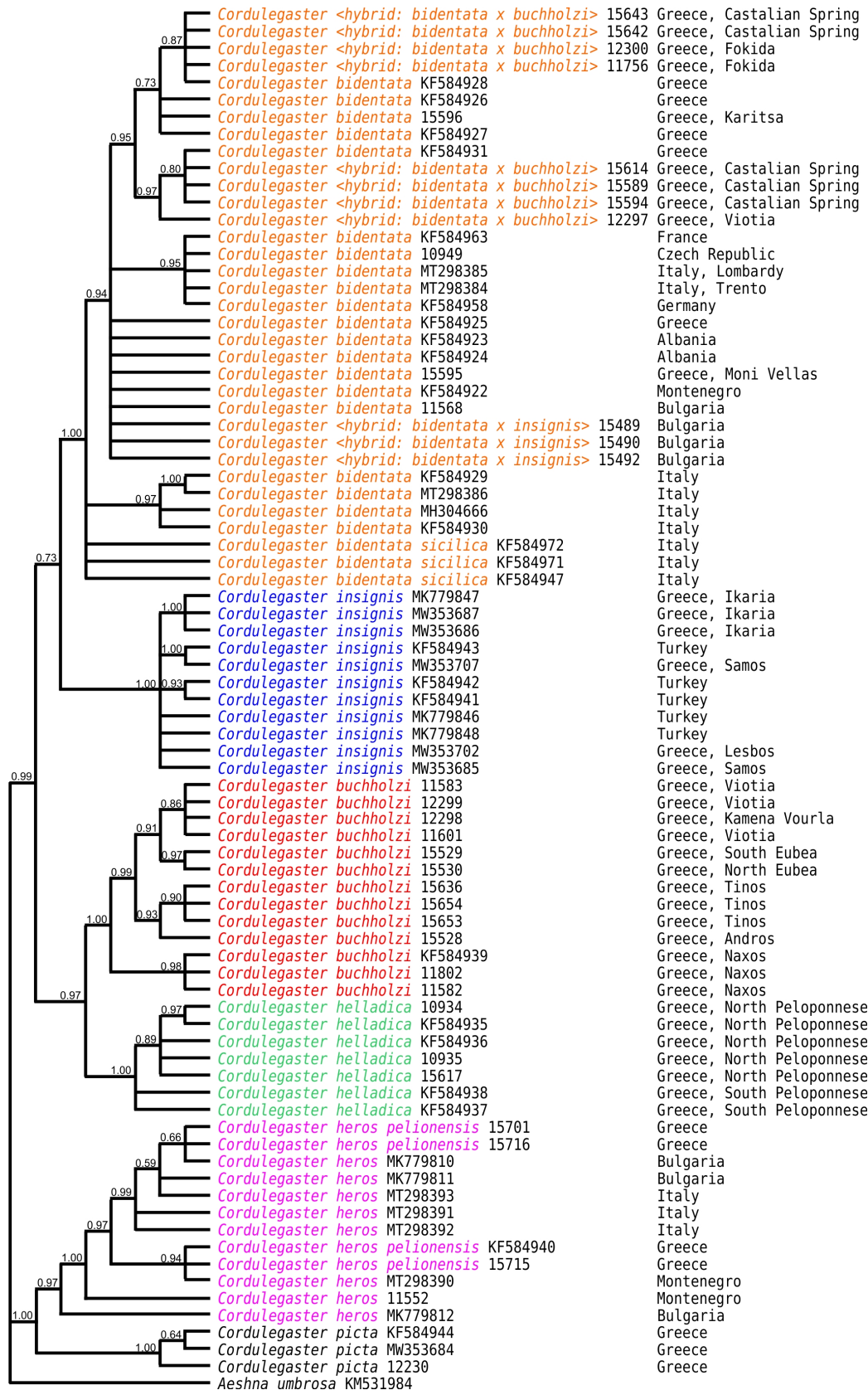
FIGURE 2. Overview tree for the COI gene fragment. Bayesian inference tree using MrBayes 3.2.7a using the best-fit model (GTR+I+G) identified with JModeltest 2.1.10. Bayesian posterior probabilities values are depicted at the nodes. Included are our own sequences (PCR number next to the name) and those retrieved from GenBank (accession numbers next to the name), if specimens identify different taxa in the COI and ITS analysis they are considered hybrids. Haplotype analysis (TCS-network made in PopART 1.7) is shown in Figs. 5 and 6.
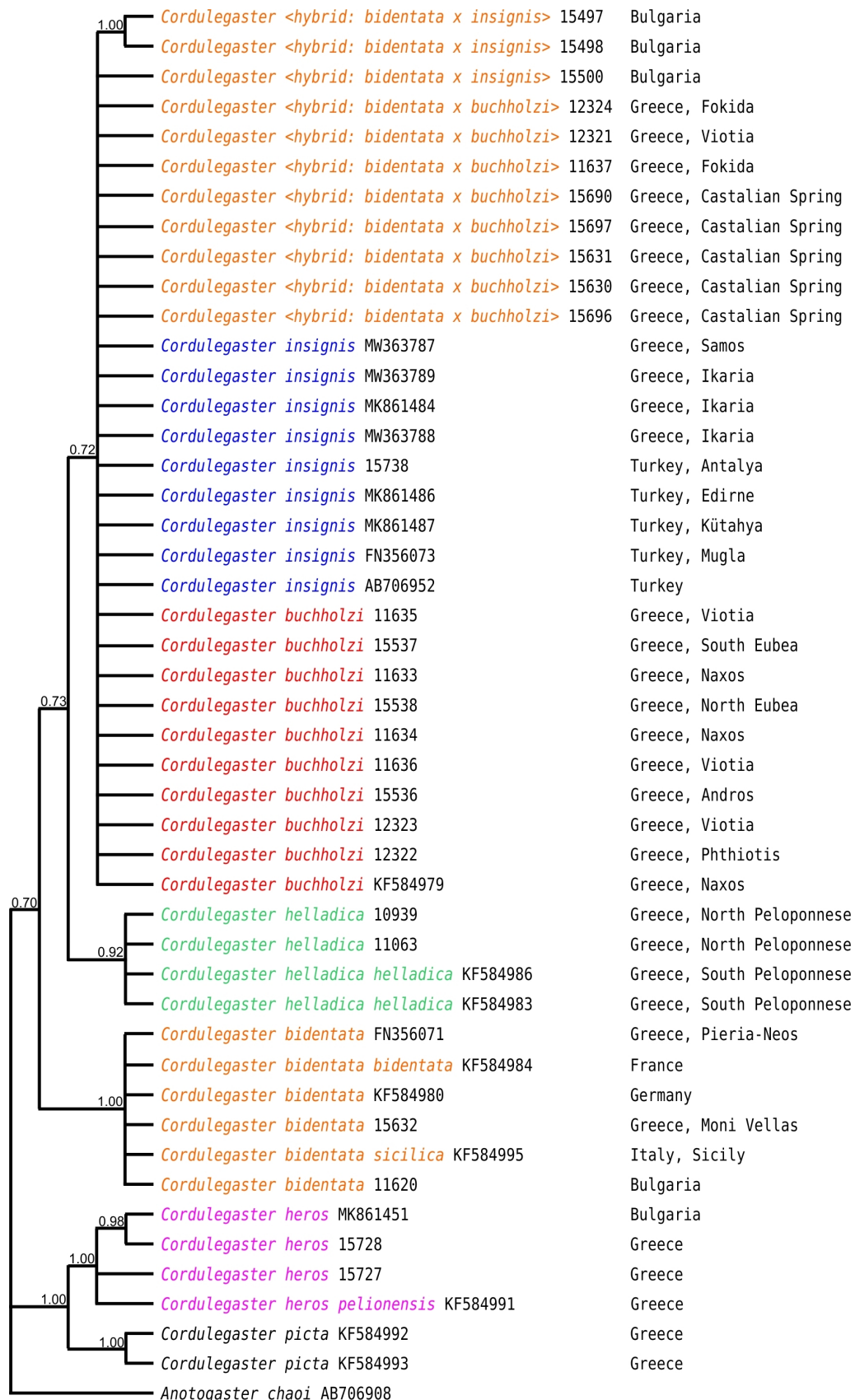
FIGURE 3. Overview tree from the ITS gene fragment. Bayesian inference tree using MrBayes 3.2.7a using the best-fit model (HKY+G) identified with JModeltest 2.1.10. Bayesian posterior probabilities values are depicted at the nodes. Included are our isolated sequences (PCR number next to the name) and those retrieved from GenBank (accession numbers next to the name), if specimens identify different taxa in the COI and ITS analysis they are indicated hybrids.
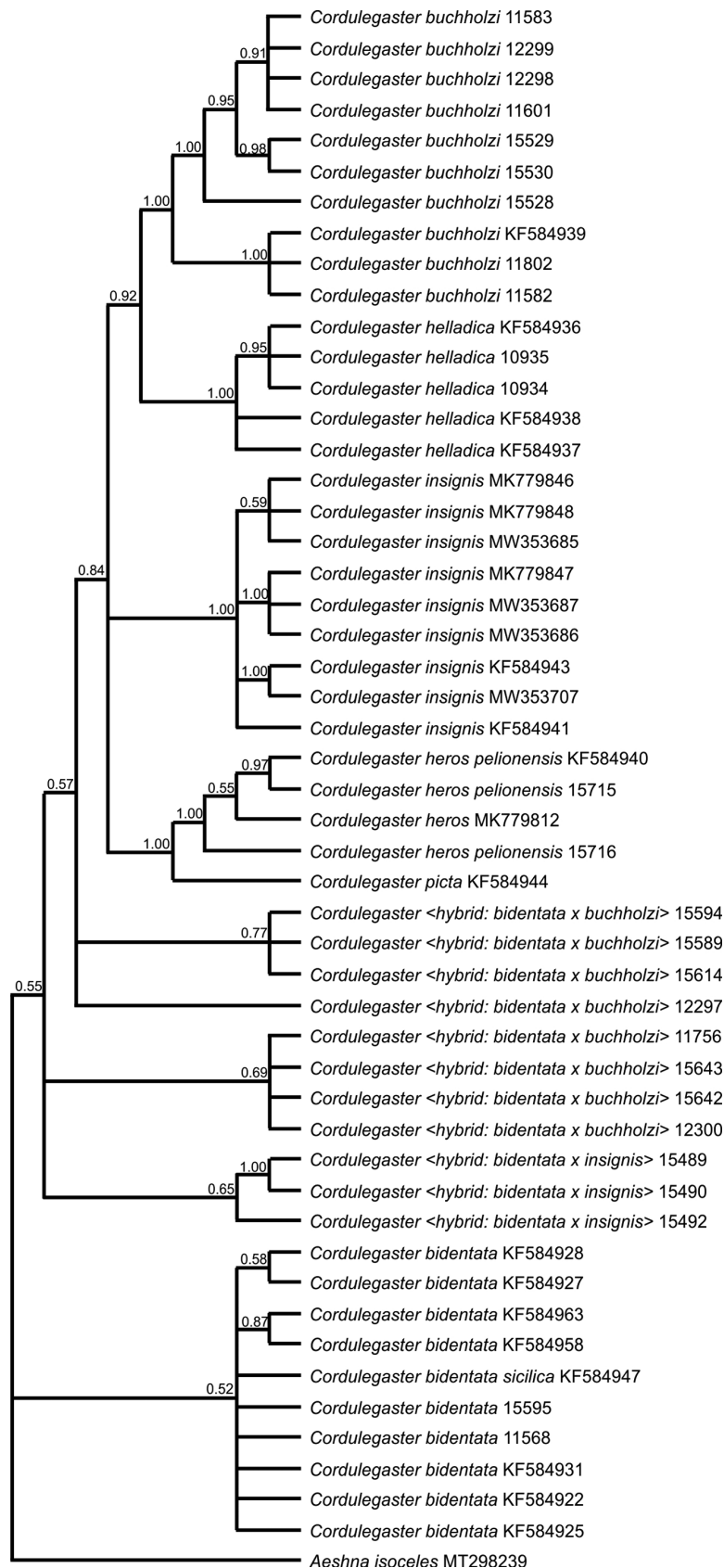
FIGURE 4. Overview tree from the COI- and ITS gene fragments combined. Bayesian inference tree using MrBayes 3.2.7a using the best-fit model (HKY+G) identified with JModeltest 2.1.10. Bayesian posterior probabilities values are depicted at the nodes. Included are our isolated sequences (PCR number next to the name) and those retrieved from GenBank (accession numbers next to the name), if specimens identify different taxa in the COI and ITS analysis they are indicated hybrids.
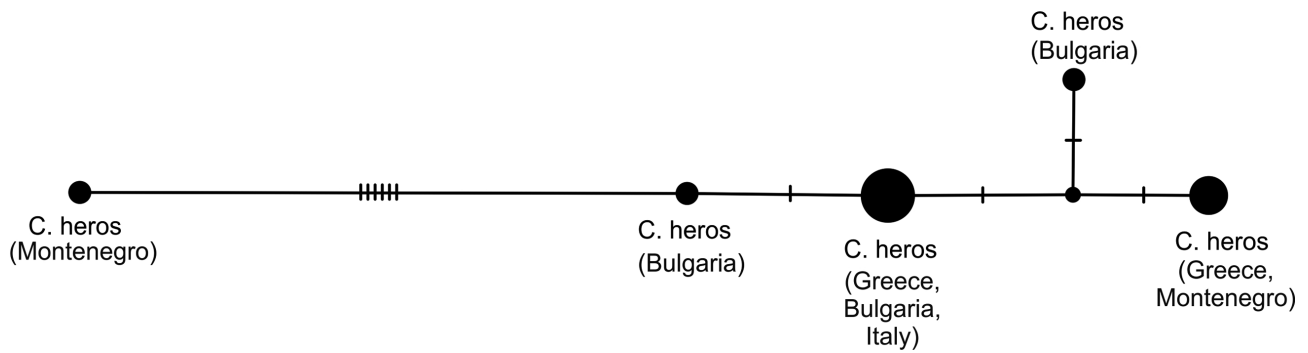
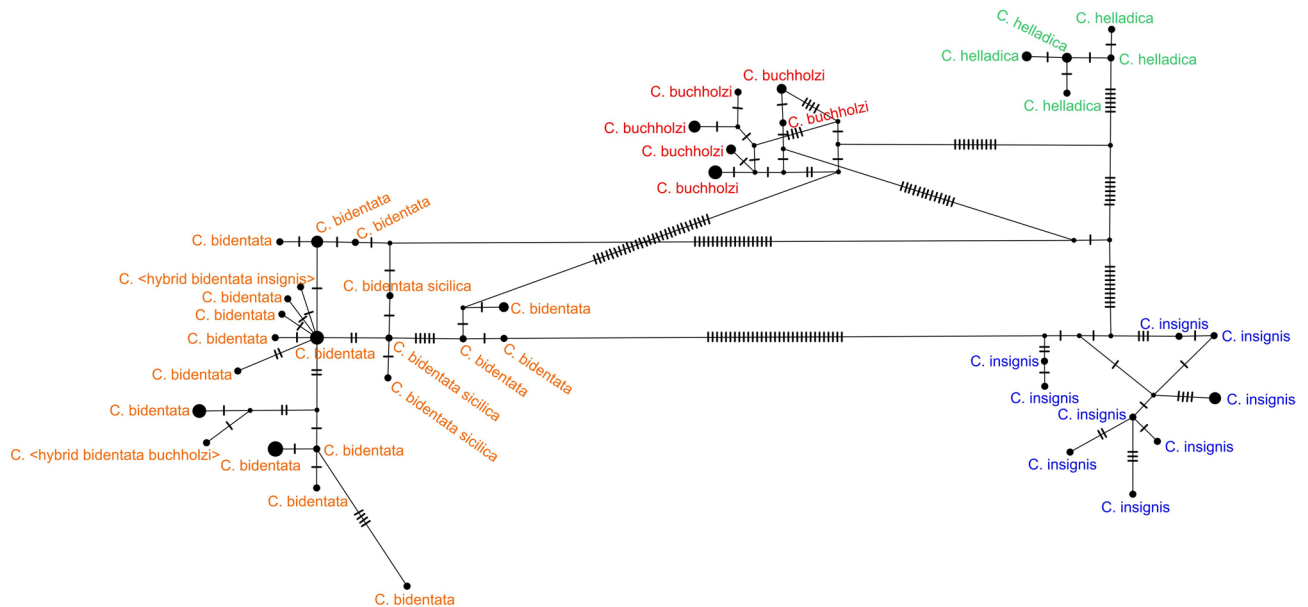**FIGURE 5.** Haplotype analysis for *C. heros* (TCS-network made in PopART 1.7).

FIGURE 6. Haplotype analysis for the Clade II (TCS-network made in PopART 1.7).

TABLE 4. Matrix with F_{ST} values for the COI gene. Values were calculated with Arlequin v. 3.5.2.2 (Excoffier *et al*. 2010). Values close to one means no sharing of genetic material between populations, values close to zero means sharing a lot of genetic material.

	boltonii	*trinacriae*	*princeps*	*picta*	*heros*	*bidentata*
boltonii	0.00000					
trinacriae	0.88000	0.00000				
princeps	0.87277	0.92223	0.00000			
picta	0.89856	0.92563	0.93140	0.00000		
heros	0.88667	0.90933	0.89914	0.84560	0.00000	
bidentata	0.90477	0.90747	0.91681	0.90276	0.89198	0.00000
insignis/bidentata	0.92346	0.95014	0.96385	0.94154	0.90590	-0.03691
buchholzi/bidentata	0.92212	0.94109	0.95059	0.93428	0.91307	0.24985
C. insignis	0.91198	0.91393	0.92396	0.90333	0.87789	0.85388
C. buchholzi	0.90314	0.91761	0.91482	0.89964	0.88566	0.85799
C. helladica	0.92665	0.94974	0.96064	0.94136	0.90665	0.87613
Aeshna	0.94890	0.96986	0.97028	0.95518	0.93031	0.93541

TABLE 4. (Continued)

	Hybrid insignis/bidentata	Hybrid buchholzi/bidentata	insignis	buchholzi	helladica	Aeshna
boltonii						
trinacriae						
princeps						
picta						
heros						
bidentata						
insignis/bidentata	0.00000					
buchholzi/bidentata	0.51426	0.00000				
C. insignis	0.88247	0.88404	0.00000			
C. buchholzi	0.88068	0.88397	0.84495	0.00000		
C. helladica	0.96436	0.93600	0.86832	0.82601	0.00000	
Aeshna	0.99228	0.96434	0.93586	0.93082	0.98102	0.00000

TABLE 5. Genetic distance, estimated by the Kimura 2-parameter method, between the different species in the Clade II.

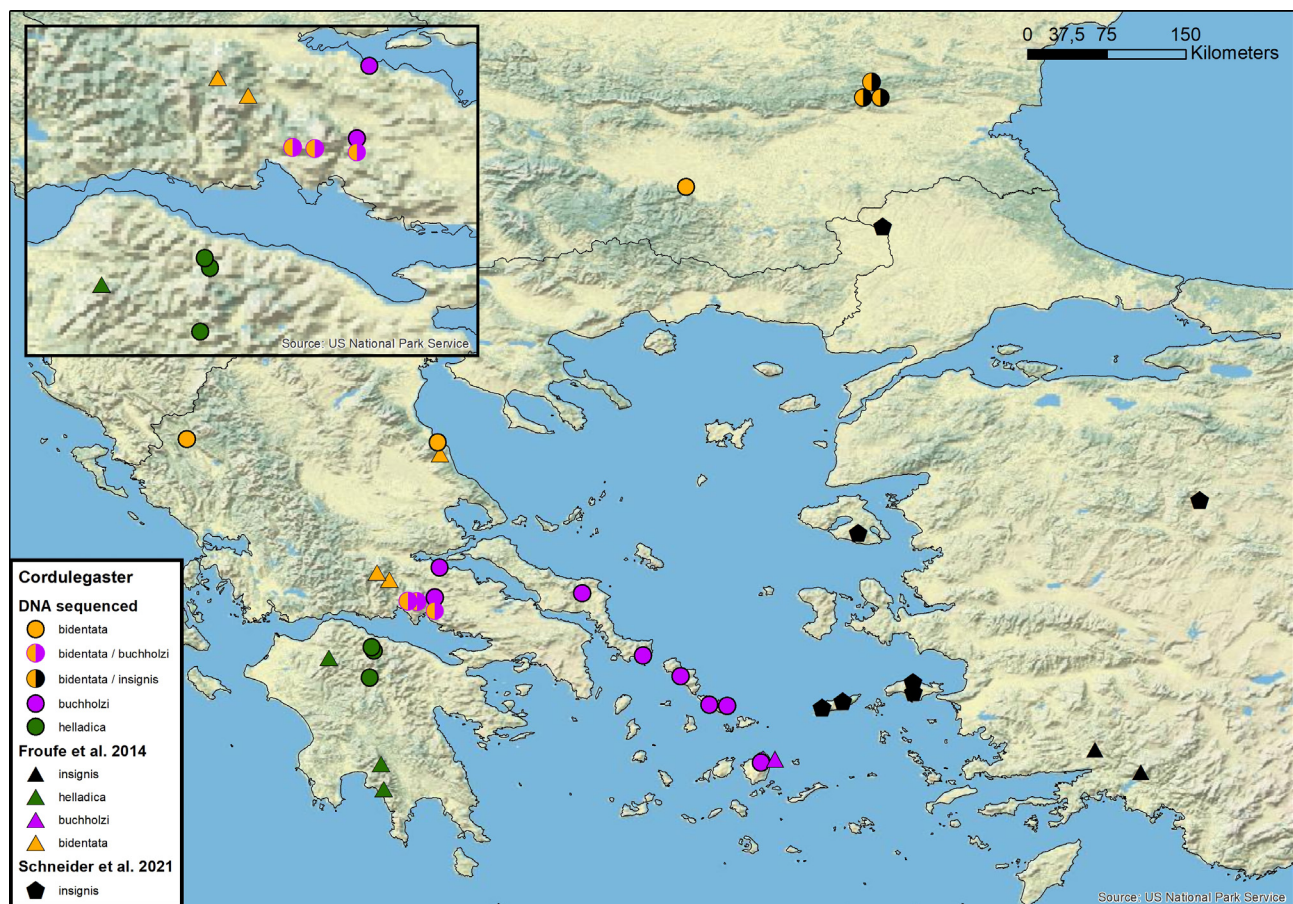|	bidentata	insignis	helladica	average distance within the group
bidentata				0.0098
insignis	0.0690			0.0093
helladica	0.0679	0.0528		0.0027
buchholzi	0.0723	0.0644	0.0430	0.0098

FIGURE 7. Map indicating the geographical distribution of investigated specimens of the Clade II in Greece and south-east Europe. For details see Table 1.

Cordulegaster insignis is found on the islands of Lesbos, Icaria and Samos (Fig. 7). However, three specimens from Bulgaria phenotypically assigned to *C. i. montandoni*, were identified as hybrids *C. bidentata* x *C. insignis* by DNA analysis (Figs. 2–4, 7). The supposed hybridisation was unidirectional in all three, as the COI fragments grouped with those from *C. bidentata* and ITS fragments with those from *C. insignis* (Figs. 2–4, 7). About 165 kilometres South-West of this locality *C. bidentata* was found (Fig. 7).

All five specimens regarded as *C. h. kastalia* from the Castalian Spring (COI PCR: 15614, 15589, 15642, 15643, 15594) and all from the second known locality (Fokida) some kilometres east of the Castalian Spring (COI PCR: 12300, 11756) grouped in the COI analysis with 23 *C. bidentata* and in the ITS1 analysis with *C. buchholzi* (Figs. 2–4, 6–7). At Boeotia some kilometres east of Fokida only one (COI PCR: 12297) out of four specimens (COI PCR: 11583, 11601, 12299) investigated was such a hybrid. The supposed hybridisation was unidirectional in all eight specimens reported here, as the COI fragments grouped with those from *C. bidentata* and ITS fragments with those from *C. buchholzi* (Figs. 2–4, 7). All other specimens collected here and further east revealed to be *C. buchholzi* (Figs. 2–4, 6–7). Thus, around the Castalian Spring in Delphi, where in ancient times the Oracle of Delphi was located, the European endemic *C. bidentata* meets the Asian derived *C. buchholzi*. Therefore, we regard *C. h. kastalia* as a hybrid between *C. bidentata* and *C. buchholzi*. This view is also supported by the F_{ST} values (Table 4). Specimens collected further west of the Castalian Spring, West of Mount Parnassos, belong to *C. bidentata* (Fig. 7). All specimens from the Peloponnese investigated by us and Froufe *et al.* (2014) revealed *C. helladica*, which is separated in the COI, haplotype and ITS from *C. buchholzi* and *C. insignis* (Figs. 2–4, 6–7). No hybridization was found in specimens regarded as *C. helladica* from the Peloponnese.
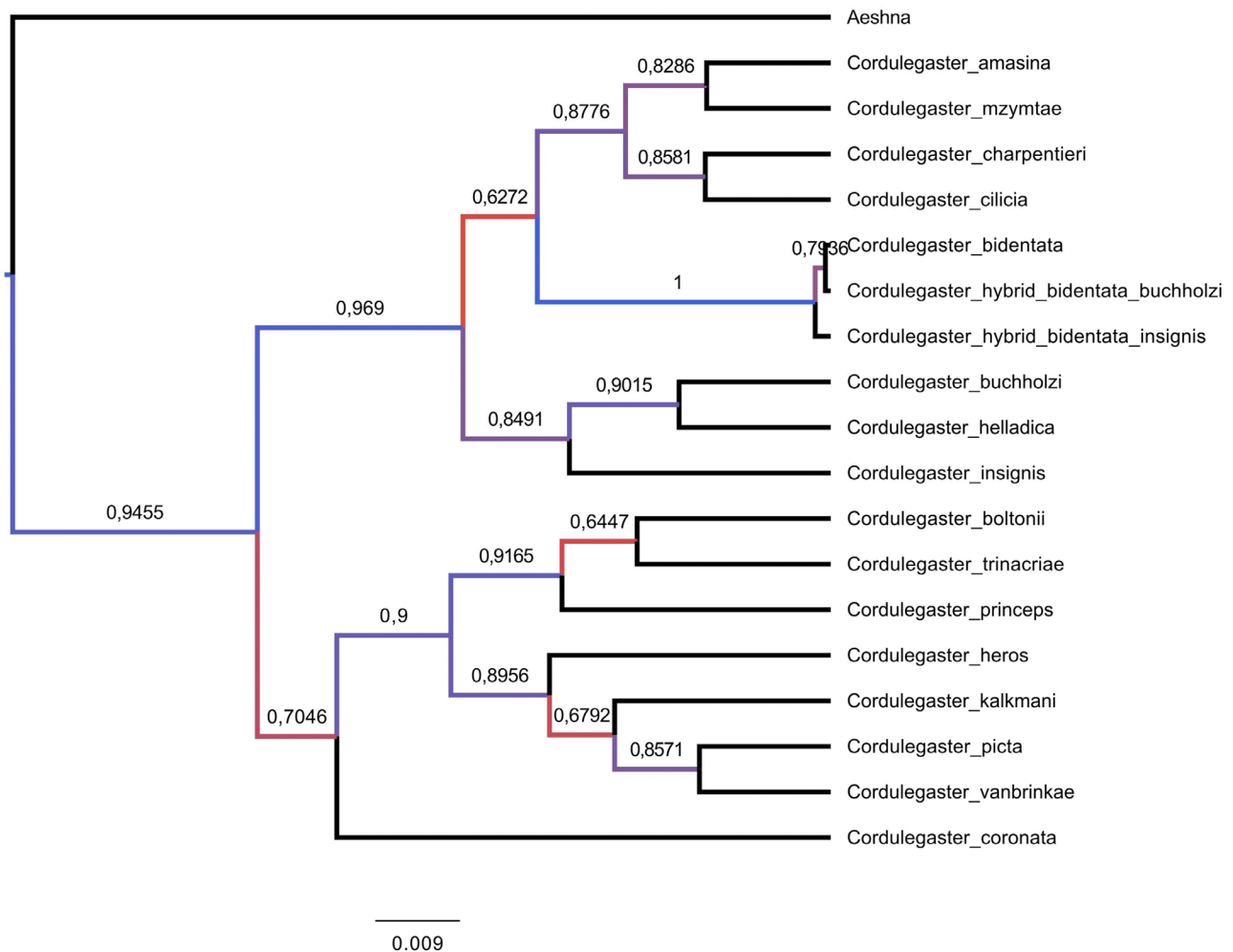

FIGURE 8. Overview tree for the COI gene fragment. Construction of the tree was done with StarBeast following the protocol from Barido-Sottani *et al.* (2018). Posterior probabilities are shown next to the nodes.

Discussion

There is an ongoing debate about the genus *Cordulegaster* in the Western Palaearctic since one and a half century (Selys 1850; Morton 1916; Fraser 1929; St. Quentin 1952; Lohmann 1993; Boudot & Jacquemin 1995; Boudot 2001; Froufe *et al.* 2014; Solano *et al.* 2018). In our recent two step approach, with a molecular analysis in the first step and a morphological analysis in a second step, we could establish a revision of the eastern members of the genus *Cordulegaster* of the West Palaearctic and the western East-Palaearctic (Schneider *et al.* 2021). In this analysis, as well as in the previous molecular phylogenetic analysis of the western members of the genus *Cordulegaster* of the West Palaearctic (Froufe *et al.* 2014; Schneider *et al.* 2021), the concept of two evolutionary clades (provisionally named in here Clade I and Clade II) was supported. In the Clade II three clades were revealed by molecular analysis in the eastern zone of the West Palaearctic composed by the following species: the well-known European endemic *C. bidentata* (MLI), *C. buchholzi*, *C. helladica*, *C. insignis* (MLII) in south-eastern Europe and western Turkey, and *C. charpentieri* (named in here as MLIII) from the eastern-Anatolia to the Levant, the Caucasus-countries and Iran (Schneider *et al.* 2021 and this paper).

As widely accepted, *C. insignis* inhabits the eastern Greek islands and West Turkey. However, the situation in eastern Europe is less clear. *Cordulegaster insignis* is reported from several Balkan countries like southern Serbia, North Macedonia, Romania and Bulgaria (for an overview see Boudot *et al.* 2015). From Romania, a subspecies had been described as *C. i. montandoni*. However, the only discriminating character for *C. i. montandoni* given by St. Quentin (1971) does not justify separation of these populations at the subspecies level. The original type locality of *C. i. montandoni* was Comana in Romania, only about 50 km away from the location in Bulgaria where one of us (G.J.v.P.) collected the specimens, which entered our molecular analysis. Phenotypically these specimens looked like *C. insignis*, but the molecular analysis revealed for all three specimens hybridisation between *C. bidentata* and *C. insignis*. Thus, we think all specimens from eastern Europe, which are phenotypically assigned to *C. insignis* should be re-evaluated by a molecular phylogenetic approach to clarify its real distribution in Europe and the extension of the hybrid zone. Until such study has been carried out, we suggest supressing the taxon *C. i. montandoni*, because most likely it was established based on a hybrid specimen. The hybrid-zone may be possibly broad reaching hypothetically Serbia and North Macedonia. The F_{ST} value indicate a high degree of interbreeding in the hybrid zone.

Without information on the locality, from which single individuals derived from, it may be nearly impossible to separate the *C. insignis*, *C. buchholzi*, and *C. helladica* by only on morphological differences (van Pelt 2020) (Fig. 9), which explains why the *C. buchholzi* populations from Euboea Island and mainland Greece had been taken for the nominate *C. helladica* in the past. In most cases *C. insignis* has blue eyes, whereas *C. buchholzi* and *C. helladica* have green eyes (Fig. 9). However, *C. insignis* as well with rather green eyes are found on Icaria and in Turkey. *Cordulegaster helladica* is typically very yellow like *C. insignis*, while *C. buchholzi* on the Cyclades is darker than *C. helladica* and *C. insignis* (Fig. 7). On south-east mainland Greece very yellow *C. buchholzi* occur, which looks like *C. helladica*. Moreover, the most yellow forms, formerly described as the subspecies *C. h. kastalia*, occurring at the Castalian Spring are indeed hybrids between *C. buchholzi* and *C. bidentata*. The hybrid-zone seems to be restricted to a small corridor wide about 30–40 km in south-western Greece.

The male appendages of all three species are very similar. The lower appendage of *C. helladica* and *C. buchholzi* is rather tapering to the distal end, while in *C. insignis* this is more parallel-sided (Lohmann 1993; van Pelt 2020; Schneider *et al.* 2021). The phenotypical similarities of these closely related species and the occurrence of hybrids in contact zones were due to ambiguities regarding the exact differentiation and distribution of these taxa.

On the islands between Greece and Turkey, hybridization may have resulted in a new species *C. buchholzi*, as it shares still a haplotype with *C. insignis*, and was probably isolated during the post glacial by the Aegean Sea from *C. insignis* populations of Icaria, Samos and WestTurkey, when sea level increased by 115–120 m. This was already discussed by Froufe *et al.* (2014). East of Naxos on the island of Icaria and further east on the island of Samos we documented the presence of *C. insignis* (Fig. 7).

According to Froufe *et al.* (2014) we suggest to raise the subspecies *C. h. buchholzi* to the full species level. Our molecular phylogenetic analysis of specimens collected from Naxos, Tinos, Andros, Euboea and south-eastern mainland Greece revealed that they all belong to this species. All specimens assigned to *C. helladica* collected on the Peloponnese grouped together in COI and ITS1 analysis were well separated from *C. buchholzi*.
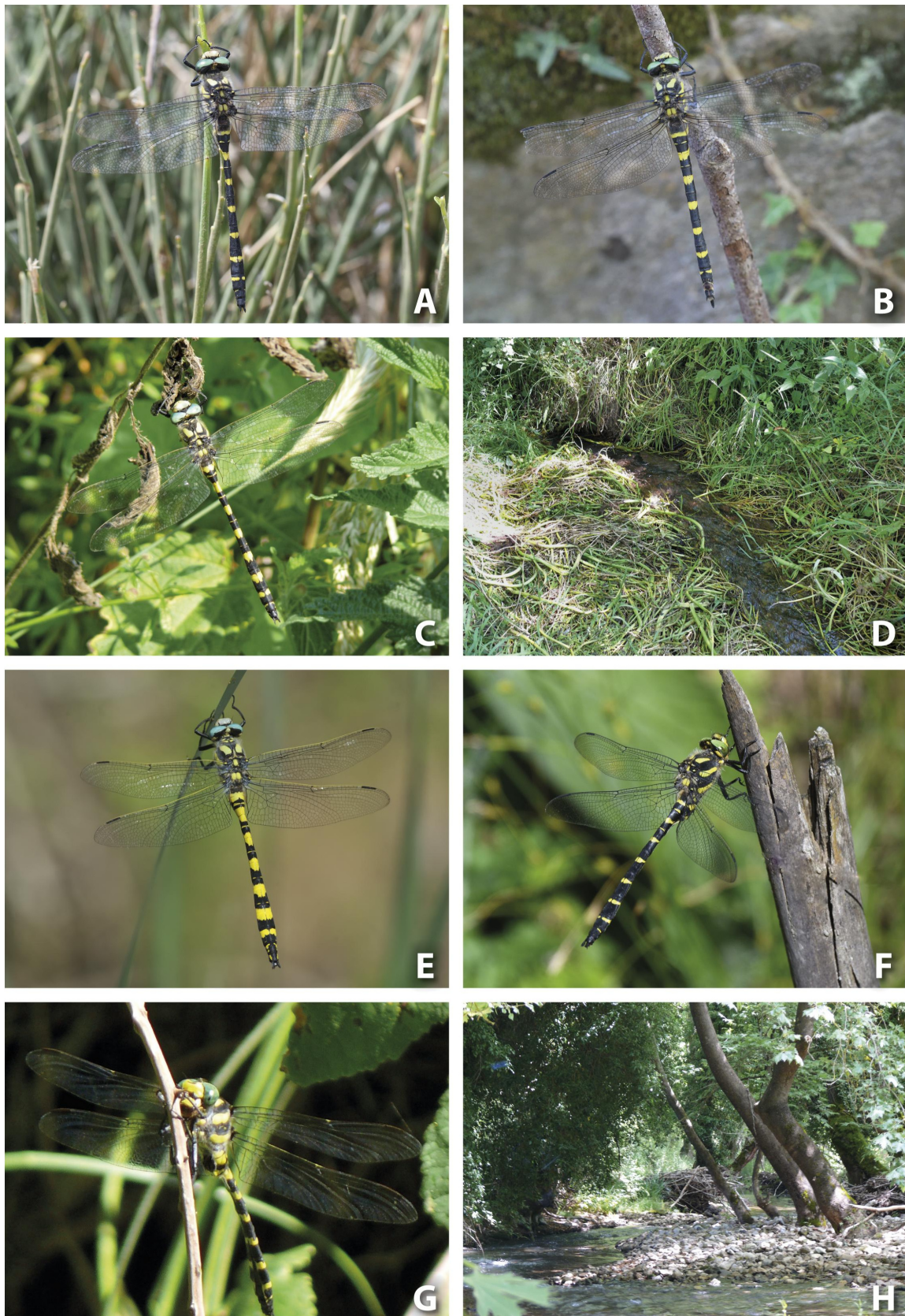
FIGURE 9. Some *Cordulegaster* species. A: *C. buchholzi* male from Andros, Greece. B: *C. buchholzi* female from Andros, Greece. C: *C. helladica* male from Peloponnese, Greece. D: Habitat of *C. helladica* near Kauteli, Peloponnese. E: *C. insignis* male from Saklikent, West Turkey. F: *C. bidentata* male, Westfalia, Germany. G: *C. heros* male, River Selimoundas, Peloponnese, Greece—note the black bar at the upper ventral frons. H: Habitat of *C. heros,* River Selimoundas, Peloponnese, Greece. Photographs by Dietmar Ikemeyer (A, B, E, F) and Elias Schneider (C, D, G, H).

Thus, *C. helladica* does not occur in south-eastern mainland Greece or in Euboea as believed before, but is restricted to the Peloponnese and separated by the Gulf of Corinth from *C. buchholzi* and *C. bidentata* (Fig. 7). In contrast, *C. buchholzi* is not restricted to the Cyclades as previously thought, but is widespread from the Cyclades over the island of Euboea to south-eastern mainland Greece, reaching in the west near Mount Parnassos, where it hybridizes with *C. bidentata*. Hybridization between *C. bidentata* and *C. buchholzi* was detected at the Castalian Spring, where in ancient times the Oracle of Delphi was located, and some kilometres east. These three localities were the only known areas where *C. h. kastalia* occurs. Our results therefore suggest not to use *C. h. kastalia* on subspecies level any longer, because this taxon was established based on hybrid specimens.

These data are also important for a better protection of some of the endangered taxa. *Cordulegaster helladica* is classified as endangered (Boudot 2010a). Now our data suggest that this species is restricted to the Peloponnese and may therefore be even more endangered than believed before. Even hybrids, like the critically endangered *bidentata* x *buchholzi*, are worth to be protected. They might be the nucleus of the genesis of new species.

On the other side we have *C. buchholzi*, which may have derived from an earlier hybridisation between *C. insignis* and an unkown ancestor of *C. helladica*. This species lives in a few places in south-eastern mainland Greece, on Euboea, and the Cyclades. If the enviromental disasters, which are increasingly frequently reported in the last years are considered (Earth Observation System 2021), such as for example the floods and fires in 2021 on Euboea, it can be imagined how fragile these populations may be.

Cordulegaster h. pelionensis was described based on minor discriminating characters like a strong black marking along the upper carina of the frons (Theischinger 1979). We could use published sequences of *C. heros* from Greece assigned to this subspecies and new sequences from supposed *C. h. pelionensis* collected by W. Lopau, and compare them with sequences of specimens from other regions (Froufe *et al.* 2014; Galimberti *et al.* 2020; Schneider *et al.* 2021). Our analysis does not support the status of the subspecies *C. h. pelionensis*. The K2-P distance was below 1%, and the specimens from Greece grouped together with those from Montenegro and Bulgaria. Thus, we would suggest that all specimens presently assigned to *C. h. pelionensis* should be considered as conspecific to the nominate subspecific taxon making *C. heros* a monotypic species.

Cordulegaster picta from Samos, Thasos and eastern mainland Greece, although looking phenotypically different—specimens from Samos are very yellow, those of eastern mainland Greece rather dark, belong to the same species as those from Bulgaria, Turkey and Russian Black Sea region (Schneider *et al.* 2021).

Acknowledgement

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