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Molecular species delimitation in the genus *Eumerus* (Diptera: Syrphidae)

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Abstract

Eumerus is one of the most diverse genera of hoverfly worldwide. Species delimitation within genus is considered to be difficult due to: (a) lack of an efficient key; (b) non-defined taxonomical status of a large number of species; and (c) blurred nomenclature. Here, we present the first molecular study to delimit species of the genus by using a fragment of the mitochondrial cytochrome-c oxidase subunit I gene (COI) gene. We assessed 75 specimens assigned to 28 taxa originating from two biogeographic zones: 22 from the western Palaearctic and six from the Afrotropical region. Two datasets were generated based on different sequence lengths to explore the significance of availability of more polymorphic sites for species delimitation; dataset A with a total length of 647 bp and dataset B with 746 bp. Various tree inference approaches and Poisson tree processes models were applied to evaluate the putative 'taxonomical' vs. 'molecular' taxa clusters. All analyses resulted in high taxonomic resolution and clear species delimitation for both the dataset lengths. Furthermore, we revealed a high number of mitochondrial haplotypes and high intraspecific variability. We report two major monophyletic clades, and seven 'molecular' groups of taxa formed, which are congruent with morphology-based taxonomy. Our results support the use of the mitochondrial COI gene in species diagnosis of *Eumerus*.

Keywords: DNA barcoding, COI, species KeyDelimitation, hoverflies, *Eumerus*

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Introduction

Hoverflies (Diptera: Syrphidae), also known as flower flies, constitute a cosmopolitan and highly diverse insect group with more than 6100 taxa described globally (Thompson *et al.*, 2010). Hoverflies influence ecosystems in many ways, such as: (a) pollinating a wide range of flowering plants; (b) controlling plant pests of which they are effective predators; (c) having phytophagous larvae feeding on bulbs; and (d) effectively recycling nutrients from dead matter (Rotheray & Gilbert, 2011). Because of their heterogeneous character and their wide distribution, hoverflies constitute an important and diverse group for ecological and biogeographical studies.

One of the most species-rich of the hoverfly genera is *Eumerus* Meigen, 1822, with 256 species recorded worldwide (Pape & Thompson, 2015), of which 89 occur in the western Palaearctic region (total species number of the entire Palaearctic is 140: Peck, 1988; Speight, 2014) and 72 in the Afrotropical region (Pape & Thompson, 2015). The only existing comprehensive key for *Eumerus* is that of Stackelberg (1961) based on only some of the Palaearctic taxa, which was used by Speight (2014) to compile a list of European *Eumerus*. Due to the lack of an up-to-date European identification key, species delimitation is often not feasible, especially for poorly studied areas, e.g., the Mediterranean. In addition, the taxonomic status of a considerable number of taxa is uncertain, with confusing nomenclature and synonymies frequently present (Peck, 1988). It is often unclear to which taxon many names refer, and a broad revision of the genus is needed, perhaps employing more sophisticated taxonomical tools, e.g., molecular systematics.

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Over the last two decades, DNA barcoding has been introduced to taxonomy and has greatly expedited species identification, assisted in species delimitation and elucidated species evolution and biology (Rubinoff, 2006). DNA barcoding can be a fast and efficient way to identify species, to diagnose new species, and to provide molecular operational units for ecological and biodiversity studies (DeWalt, 2011). However, opposing opinions exist regarding the application of DNA barcoding as a primary means of species delimitation. Rubinoff (2006) claimed that mitochondrial DNA (mtDNA) is not adequate as a sole source of species-defining data because of reduced effective population size and introgression, maternal inheritance, recombination, inconsistent mutation rate, heteroplasmy and compounding evolutionary processes. As a consequence, there are cases where some sister taxa cannot be identified because they have identical or nearly identical DNA barcodes, giving a false negative signal of species differentiation (Moritz & Cicero, 2004). In contrast, taxa with a wide geographical distribution may exhibit relatively large genetic divergence and, thus, might present a false positive signal (i.e., incorrectly indicating the occurrence of differentiated taxa) (Avise, 2000). Notwithstanding the controversy about its effectiveness, DNA barcoding has been highly effective in species identification, especially for resolving insect taxonomy (Ball *et al.*, 2005; Smith *et al.*, 2005). To overcome any false phylogenetic signal, it is recommended to use multiple and complementary tools to better delimit biodiversity ('integrative taxonomy': Dayrat, 2005). This implies that morphological features, and molecular (e.g., DNA sequences) and biochemical (e.g., alloenzymes) data, as well as morphometric data should be utilized for species delimitation (Mengual *et al.*, 2006).

The mitochondrial cytochrome-c oxidase subunit I gene (COI, cox1) is widely used in species identification due to its phylogenetic signal, which can discriminate species (Hebert *et al.*, 2003*a*, 2010; Hebert & Gregory, 2005). A fragment of approximately 650 bp of the 5′ end of the COI, i.e., the 'Folmer' fragment (Folmer *et al.*, 1994), is the most frequently used gene fragment in DNA barcoding of animals, and this approach has been successfully applied to hoverflies (Pérez-Bañón *et al.*, 2003; Ståhls *et al.*, 2009; Gibson *et al.*, 2011; Marcos-García *et al.*, 2011; Radenković *et al.*, 2011). Furthermore, primers amplifying a fragment of the 3′ end of the COI have been effectively applied to obtain longer sequences, i.e., >800 bp. According to Hebert *et al.* (2003*b*) COI-3′ regions present similar sequence divergence profiles as COI-5′ fragments, which imply that COI-3′ can be used as an alternative DNA barcode. Indeed, the COI-3′ region has proven valuable for solving taxonomical uncertainties in various hoverfly genera (Milankov *et al.*, 2005, 2008*a*, 2009, 2010*a*, *b*, 2013; Mengual *et al.*, 2006; Vujić *et al.*, 2012; Francuski *et al.*, 2013).

Molecular and morphological studies have been carried out on many hoverfly genera such as *Merodon* (Milankov *et al.*, 2008*a*, *b*; Ståhls *et al.*, 2009), *Cheilosia* (Ståhls *et al.*, 2004; Milankov *et al.*, 2010*a*, *b*), *Chrysotoxum* (Nedeljković *et al.*, 2013) and tribe Pipizini (Vujić *et al.*, 2013). In these studies, an integrative taxonomic approach was applied, i.e., complementary use of molecular markers (mtDNA and/or nuclear gene fragments) and morphological characters, which often provided rather good taxonomic resolution (Milankov *et al.*, 2008*a*, *b*). However, in certain cases, different taxa shared the same haplotypes (Milankov *et al.*, 2008*b*, 2010*a*, *b*; Ståhls *et al.*, 2009) or possessed character differences of solely one nucleotide (Ståhls *et al.*, 2009; Milankov *et al.*, 2010*b*). In the latter cases, this outcome indicated the insufficiency of a COI-based identification system alone, to delimit species or species complexes within the genus *Merodon* (Milankov *et al.*, 2008*b*; Ståhls *et al.*, 2009), and underlined the importance of integrative taxonomic inference.

Here, our aim is to validate usage of COI-3′ region for species delimitation of the genus *Eumerus* using different sequence lengths in order to identify species and explore intra- and interspecific variability. We address two specific questions: (1) Can COI-3′ barcoding reveal intra- and interspecific genetic variation in *Eumerus*? (2) Is a short sequence of COI-3′ (647 bp) sufficient to accurately resolve species taxonomy compared to a more elongated one (746 bp)?

Materials and methods

Taxon sampling

We used dry pinned specimens deposited in two entomological collections: the Melissotheque of the Aegean located at the University of the Aegean, Greece (M-UAegean; Petanidou *et al.*, 2013); and the collection of the Faculty of Sciences at the Department of Biology and Ecology of the University of Novi Sad, Serbia (FSUNS). The specimens used in this study were collected from 2009 to 2014. In total, 75 specimens were used from two geographical areas: 69 derived from the western Palaearctic and six from Afrotropical region (RSA) (table S1). Initial species identification was based on morphology. Specimens were assigned to 28 taxa (table S1) of which three had been previously undescribed (*Eumerus* aff. *barbarus* Coquebert, 1804; *E.* aff. *rubiginosus* Lyneborg, in litt. and *E.* aff. *tarsatus* Lyneborg, in litt.). The selected taxa are mainly of Palaearctic origin (22 taxa), while six taxa originated from the RSA. Identifications were carried out by Dieter Doczkal (Afrotropical taxa) and Ante Vujić (Palaearctic taxa). Taxa identification for specimens derived from the RSA was based on Lyneborg's revision (Lyneborg, in litt.). A list of the specimens used in the analysis, together with their GenBank accession numbers and collection data, is given in table S1.

To detect intraspecific variation, *Eumerus amoenus* Loew, 1848 was analyzed, since its broad distribution in the Mediterranean appears to be the widest within the genus. Two populations from the Aegean islands of Lesvos (three specimens) and Samos (seven specimens) were assayed. Measurements of interspecific variation were determined through the taxa, *E. pulchellus* Loew, 1848 and *E. pusillus* Loew, 1848 by comparing the genetic distances between adjacent and distant populations. For *E. pulchellus*, molecular diversity indices were calculated by using one specimen for each geographical area (i.e., Chios, Dadia, Lesvos, Limnos, Rhodes, Samos and Sardinia). Molecular diversity indices were also calculated for *E. pusillus*, but in this case, two or more samples per geographical area (Chios, Crete, Karpathos and Naxos) were selected.

DNA extraction and PCR amplification

Total genomic DNA was extracted using the head and/or two to three legs from each specimen. We used the protocol by Chen *et al.* (2010) for Sodium Dodecyl Sulfate (SDS) extraction with the following modifications: (a) RNase A solution was not added; (b) the concentration of proteinase K solution was 40 mg ml^{-1}; and (c) an additional step of chloroform/isoamyl alcohol (24:1) was applied. Samples were re-suspended in 30 µl of TAE buffer.

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PCR amplifications of the COI-3′ were performed in a total volume of 25 µl, containing 25 ng µl^{-1} template DNA, 5 pmol µl^{-1} of each primer, 0.08 mM of dNTPs, 1× Reaction Buffer (Thermo Scientific, USA) and 1.25 units of Polymerase (Taq poly or Dream Taq poly, Thermo Scientific, USA). Amplifications were performed in an Authorized PCR Thermal Cycler (Mastercycler® personal, Eppendorf, Germany). Thermocycling conditions consisted of initial denaturation at 95°C for 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 49°C, 2 min extension at 72°C, followed by a final extension of 8 min at 72°C (Vujić *et al.*, 2013). We employed universally conserved primers to amplify and sequence the COI-3′: forward primer C1-J-2183 (5′-CAACATTTATTTTGATTTTTTGG-3′) (alias JERRY) and reverse primer TL2-N-3014 (5′-TCCAAT GCACTAATCTGCCATATTA-3′) (alias PAT) (Simon *et al.*, 1994). Amplified products were run on 1.5% agarose gels for visual inspection. Purification of the PCR products was done with the ExoSap-IT kit (USB, Cleveland, OH, USA) and clean products were thereafter Sanger sequenced in both directions on an ABI 3730 DNA analyzer (Applied Biosystems, USA) at the Sequencing Service laboratory of the Finnish Institute for Molecular Medicine (http://www.fimm.fi).

Sequence alignment

Two datasets of different sequence length were produced. Dataset A comprised sequences of 647 nucleotides in total length, obtained by forward sequencing of the COI-3′ region, from 75 *Eumerus* specimens and four outgroups. We chose *Platynochaetus setosus* Fabricius, 1794 (Accession No. KM224512), *Merodon erivanicus* Paramonov, 1925 (Accession No. KT157919) and two species of the genus *Megatrigon* Johnson, 1898 (Accession No. KT157920 and KT157921) as outgroups. Dataset B consisted of full length of the COI-3′ (746 nucleotides), acquired through bidirectional sequencing, for the aforementioned 75 *Eumerus* taxa and outgroups. All trees were rooted based on *P. setosus* sequence.

As required, sequences were edited by eye using BioEdit 7.2.5 (Hall, 1999). For multiple sequence alignment we employed the L-INS-i algorithm, which is considered to be more accurate as an iterative refinement method incorporating local pairwise alignment information (Katoh *et al.*, 2005). Alignments were implemented using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/index.html). Both datasets were trimmed to their final lengths using BioEdit 7.2.5 (Hall, 1999). Polymorphic sites, DNA polymorphism and basic molecular diversity indices were calculated using DnaSP 5.10.01 (Librado & Rozas, 2009), which also generated nexus files. Sequences were checked for possible presence of stop codons (Buhay, 2009) using the Mesquite 2.75 system for phylogenetic computing (Maddison & Maddison, 2011). All sequences were translated using the invertebrate mitochondrial code. The evolutionary models used for maximum-likelihood (ML) and Bayesian inference (BI) analyses were implemented in the HIV sequence database (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) (Posada & Crandall, 2001).

Molecular data analysis

PTP models

Several studies have highlighted the ability of Poisson tree processes (PTP) models to reveal and resolve taxonomic issues (Leasi & Norenburg, 2014; Soldati *et al.*, 2014; Tang *et al.*, 2014). Because this approach does not require ultrametrization of trees (and its associated biases), it constitutes a reasonable alternative to other species delineation models such as the General mixed Yule coalescent model (Pons *et al.*, 2006). In PTP models, the numbers of substitutions (branch lengths) represent speciations or branching events and, therefore they only require a phylogenetic input tree. PTP models have previously been implemented to reveal putative molecular species clusters (Zhang *et al.*, 2013). PTP analyses were conducted on the web server for PTP (available at http://species.h-its.org/ptp/) using the best ML tree resulting from the RA × ML analysis (see below).

Maximum parsimony (MP)

MP analyses were performed in the program NONA (Goloboff, 1999), spawned in WINCLADA version 1.00.08 (Nixon, 2002). A heuristic search algorithm with 1000 random addition replicates (mult × 1000) was performed, holding 100 trees per round (hold/100), max trees set to 100,000, and applying TBR branch swapping.

Maximum likelihood

ML trees were generated using RAxML 8.0.9 (Stamatakis, 2006; Stamatakis *et al.*, 2008) in the Cipres Science Gateway (Miller *et al.*, 2010) under the general time-reversible (GTR) evolutionary model with a gamma distribution (GTR + G) (Rodriguez *et al.*, 1990) and 1000 bootstrap replicates.

Bayesian inference

BI topologies were assessed using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) in the Cipres Science Gateway (Miller *et al.*, 2010) with the GTR + G nucleotide substitution model (Rodriguez *et al.*, 1990) proposed by the Akaike information criterion (AIC). For BI, two analyses were run based on codon partition models: (1) partitioned (MBP), i.e., each codon position was treated separately, as they are subject to different evolutionary rates, and (2) non-partitioned (MBUP). The settings for the Bayesian Markov chain Monte Carlo (MCMC) process for the non-partitioned dataset A and partitioned datasets A and B included two runs of 10×10^6 MCMC generations (×4 chains) with a sampling frequency of 1000 generations. For the non-partitioned dataset B, the same settings were applied except that the number of MCMC generations, i.e., 15×10^6, was increased in order to diminish the autocorrelation. The relative burn-in was 10%. MCMC results were checked with the program Tracer 1.6.0 and all trees were displayed using FigTree 1.4.0 (both available at http://tree.bio.ed.ac.uk/software/).

Median-joining (MJ) network

The software NETWORK 4.6.1.2 (Bandelt *et al.*, 1999) (http://www.fluxus-engineering.com/sharenet.htm) was applied to construct the MJ networks aiming to multistate the characters. For MJ reconstructions the mitochondrial haplotypes of outgroups were excluded.

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Table 1. Results generated for dataset A (i.e., forward sequencing of the COI-3′ region) and dataset B (i.e., bidirectional sequencing) in DNaSP 5.10.01, after excluding the outgroups.

	Dataset A	Dataset B
1. Polymorphic sites		
Number of sites	647	746
Total number of sites (excluding sites with gaps/missing data)	629	717
Invariable (monomorphic) sites	421	474
Variable (polymorphic) sites	208	243
Total number of mutations	268	325
Singleton variable sites	*30*	*37*
Parsimony informative sites	*178*	*206*
2. DNA polymorphism		
2.1 Without gaps		
Number of Haplotypes (*h*)	53	56
Haplotype (gene) diversity (Hd)	0.976	0.983
Variance of Haplotype diversity	0.00008	0.00005
SD of Haplotype diversity	0.009	0.007
Nucleotide diversity (Pi)	0.07111	0.07350
Theta (per site) from (Eta)	0.08717	0.09273
Average number of nucleotide differences (*k*)	44.731	52.699
2.2 With gaps		
Number of pairwise comparisons	2775	2775
Average number of sites analyzed	646.31	745.07
Average number of differences	48.586	57.865
Nucleotide diversity (Pi)	0.07517	0.07766
Analysis at individual sites (*column by column*)		
Number of sites analyzed	647.00	746.00
Number of polymorphic sites (S)	221	261
Average number of differences	48.720	58.055
Nucleotide diversity (Pi)	0.07530	0.07782
Theta-W, per sequence	45.22285	53.40954
Theta-W, per site	0.06990	0.07159

Results

The proportion of gaps and completely undetermined characters in the alignment as generated in RA × ML was 0.09 and 0.06% for datasets A and B, respectively; the relevant distinct alignment patterns were 250 and 274. The genetic polymorphism of datasets A and B is shown in table 1.

Molecular analyses vs. morphological delimitation

Initial assignment to species level was based on morphology, with the 75 study specimens classified into 28 taxa. PTP models included the *Eumerus* taxa plus the four outgroups and predicted 32–49 taxa for dataset A and 31–46 taxa for dataset B. MP (figs 1 and 2), ML (figs S1 and S2) and BI analyses (figs 3, 4, S3 and S4) yielded similar tree topologies for both A and B datasets, with two main clusters and the nodes of the putative taxa strongly supported. Bootstrap values in MP and ML trees were generally low for both datasets, whereas posterior probability values were much higher for BI trees.

In all trees, i.e., those generated by MP, ML and BI, the two major clades comprised the same groups of taxa in each dataset (table 2; figs 1–4 and S1–S4). One clade consisted of taxa belonging to the *tricolor* group (six taxa) with the second comprising the remaining 22 taxa. Our molecular-derived topologies revealed seven different groups of taxa, with all but eight of the 28 taxa assignable to one of these groups. We named these seven groups *basalis*, *minotaurus*, *ornatus*, *pulchellus*, *strigatus*, *sulcitibius* and *tricolor* (for more details see table 2). The eight 'ungrouped' taxa were *E. torsicus* Grković et Vujić, 2015, *E.* aff. *rubiginosus*, *E.* aff. *tarsatus*, *E. alpinus* Rondani, 1857, *E. argenticornis* Lyneborg, in litt., *E. clavatus* Becker, 1923, *E. hungaricus* Szilady, 1940, and *E. tarsatus* Lyneborg, in litt. (table 2). Slight differences were apparent in the topologies generated by datasets A and B. In dataset B, *E. argenticornis*, *E. tarsatus*, *E.* aff. *rubiginosus* and *E.* aff. *tarsatus* clustered together, close to the *ornatus* group (see figs 2, 4, S2 and S4), but in dataset A they appeared altogether, dispersed or clustered differently (see figs 1, 3, S1 and S3). Other observed discrepancies were as follows: (a) *E. clavatus* was in the *sulcitibius* group in all but the MP analysis of dataset B (fig. 2); (b) the position of *E. alpinus* differed in each analysis, and (c) branching topology for the *tricolor* group was only similar in MP, MBP and MBUP analysis of dataset A (figs 1, 3 and S3) and ML and MBUP analyses of dataset B (figs S2 and S4). Branching topology for the *ornatus* group was consistent across all analyses.

Among the 28 taxa, three were previously undescribed, but were closely related to known taxa and, thus, were named *E.* aff. *barbarus* (collected in Morocco), *E.* aff. *rubiginosus* and *E.* aff. *tarsatus* (both collected in South Africa). Both molecular (high nodal support) and morphological (clear diagnostic features) aspects strongly supported the species delimitation of these three taxa. The *E.* aff. *barbarus* was included in the

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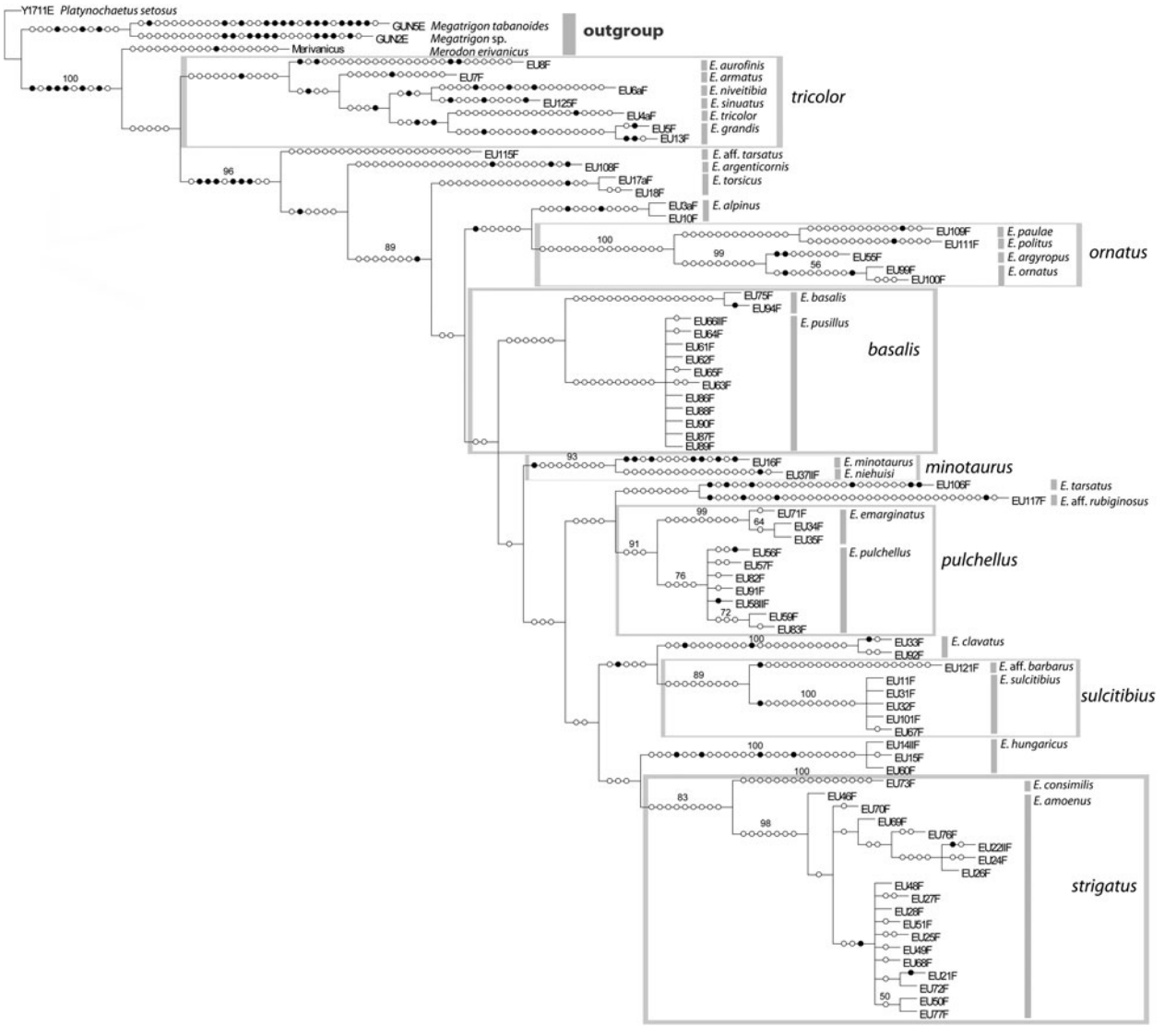

Fig. 1. Maximum parsimony analysis for dataset A produced 72 equally parsimonious trees; the strict consensus tree is illustrated here. Length 989 steps, Consistency index (CI) = 33, Retention index (RI) = 71; filled circles denote unique changes, open circles non-unique. Bootstrap support values (>50) are illustrated above the branches.

sulcitibius group, whereas the other two taxa were not collapsed to any of the seven supported groups (table 2).

We noted artifacts of Long Branch Attraction (LBA) artifacts in the ML and BI analyses of both datasets, but not in MP. LBA was due to sequences EU106, EU108, EU109, EU111, EU115 and EU117 (figs 1–4 and S1–S4). MJ network reconstructions supported our other analyses with sequences from both datasets grouping similarly to the clusters present in MP, ML and BI trees. Furthermore, the number of mutational steps between haplotypes was consistent with our phylogenetic reconstructions (figs 5 and 6).

Intra- and interspecific variability

In both datasets A and B, we recorded a high number of mtDNA COI haplotypes and very rich haplotype (Hd > 0.95) and nucleotide diversity (Pi > 0.005) (table 3). No shared haplotypes between delimitated species were obtained (table 3).

The basic molecular diversity indices for *E. amoenus*, *E. pulchellus* and *E. pusillus* were calculated and are shown in table 3. For *E. amoenus*, the MJ network for dataset A showed one to nine mutational steps among haplotypes detected in the Lesvos population, and one to three mutational steps among haplotypes in the Samos population (fig. 5). The MJ network constructed using dataset B revealed one to eight mutational steps among *E. amoenus* haplotypes from Lesvos and one to four among haplotypes found in the Samos population (fig. 6). When selecting one specimen of *E. pulchellus* from each of seven geographical origins the MJ network analysis of dataset A showed one to six mutational steps, and dataset B one to seven mutational steps (figs 5 and 6). For *E. pusillus*, two or more samples from four different geographic origins were analyzed and MJ network reconstructions showed one

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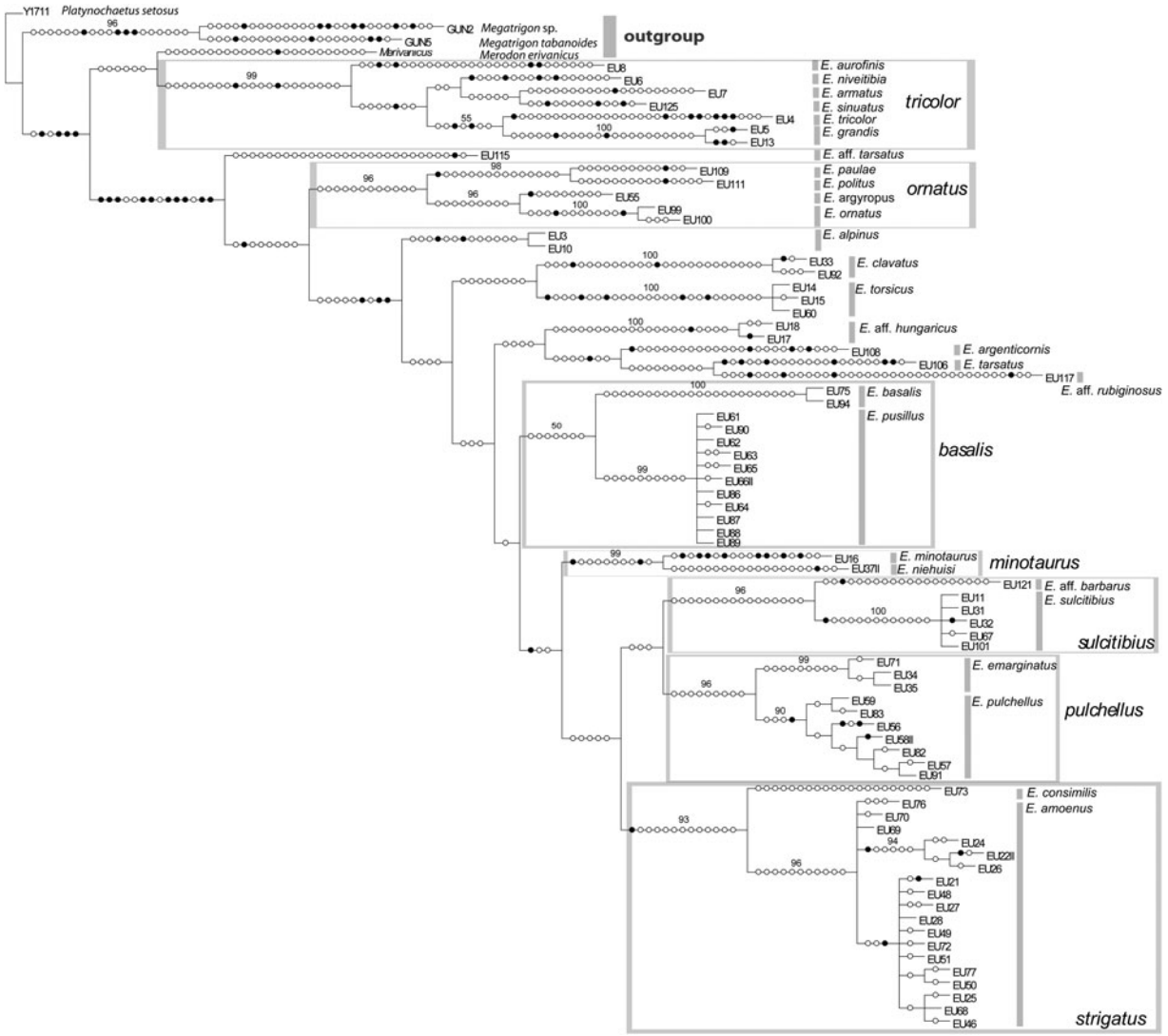

Fig. 2. Maximum parsimony analysis for dataset B produced 30 equally parsimonious trees; the strict consensus tree is illustrated here. Length 1153 steps, Consistency index (CI) = 32, Retention index (RI) = 71; filled circles denote unique changes, open circles non-unique. Bootstrap support values (>50) are illustrated above the branches.

mutational step for both datasets among different *E. pusillus* haplotypes (figs 5 and 6).

Discussion

A COI gene-based system has been successfully employed for species delimitation in various hoverfly genera such as the *ruficornis* group of the genus *Merodon* (Milankov *et al*., 2008*b*; Vujić *et al*., 2012), the *Cheilosia vernalis* complex (Ståhls *et al*., 2008), the genus *Chrysotoxum* (Suk & Han, 2013; Nedeljković *et al*., 2015), the Afrotropical hoverflies (Jordaens *et al*., 2015) and the genus *Platycheirus* (Young *et al*., 2016). Our study is the first implementation of molecular tools to infer species delimitation in the genus *Eumerus*. We assessed the feasibility of using COI-3′ fragment for *Eumerus* taxonomic inference on 75 specimens assigned to 28 putative 'taxonomical' taxa clusters. Various tree inference approaches on genetic data conformed to morphological species assignment. Since species delimitation (through conventional classical taxonomy) of *Eumerus* has proven challenging in the past, generation of barcode sequences to diagnose species within this genus can prove very useful. A DNA barcode library for *Eumerus* is currently under construction, with more than 130 sequences having been generated in the last 6 months. The present study contributes to enriching accessible barcode records; an assessment of GenBank records on 11 March 2016 revealed that this study has provided more than 50% of the available *Eumerus* sequences and species to date. In addition, our study extends representation of both the number and geographic distribution of *Eumerus* species.

We generated two datasets that differed in terms of sequence length in order to test whether longer sequences improved taxonomic resolution by support values for nodes. Dataset B was 99 nucleotides longer and possessed 28 more

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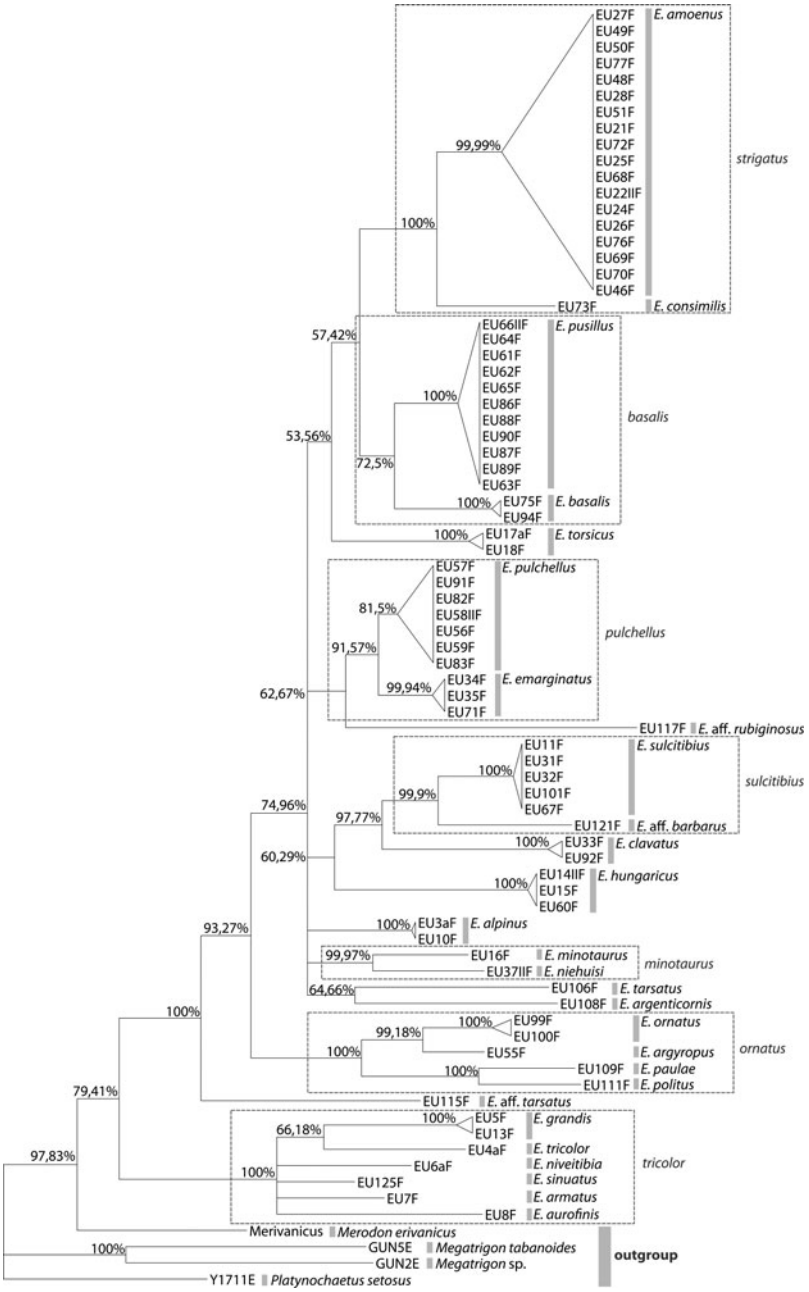

Fig. 3. Bayesian analysis of the dataset A (partitioned data). Values indicate Bayesian probability.

parsimony-informative sites compared to dataset A. However, dataset A still provided high taxonomic resolution, confirming the efficacy of a COI system based on approx. 650 bp for species delimitation. Due to sample unavailability arising from fieldwork limitations (and the absence of available sequences in barcoding databases), few sequences – in some cases even only one – were obtained for some taxa. Ahrens *et al.* (2016) discussed the issue of the singletons' (the only representative sequence of a species) issue in DNA-based species delimitation studies and ascertained that 'a high proportion of singletons has little impact on the accuracy of inferred species limits, and thus rarity (and singletons) should not be conflated with the much more pertinent population genetics parameters'.

The outcomes of our analyses were congruent for both datasets and indicated that the genus *Eumerus* is divided into two main lineages: the *tricolor* group and a lineage consisting of all the other taxa (both grouped and the 'ungrouped'). Tree topologies differed slightly within and between dataset(s). Although longer sequences improved phylogenetic resolution, they did not fully resolve the position of some taxa, e.g., *E. argenticornis*, *E. tarsatus*, *E.* aff. *rubiginosus* and *E.* aff. *tarsatus*. As singletons were used in a few cases, employment of replicate reference

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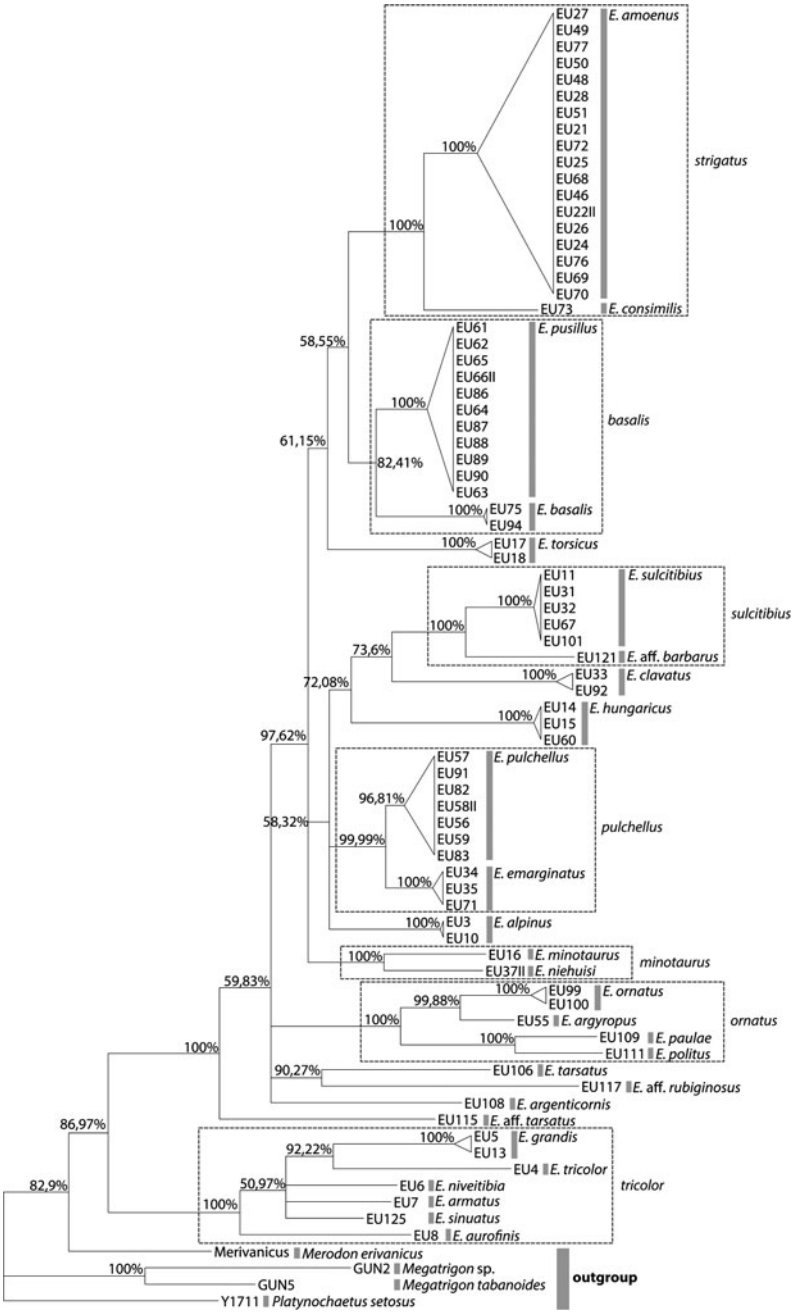

Fig. 4. Bayesian analysis of the dataset B (partitioned data). Values indicate Bayesian probability.

specimens could be beneficial in determining the phylogenetic positions of unresolved *Eumerus* taxa. Our is only the second study to date presenting a hypothetical *Eumerus* phylogeny; Doczkal & Pape (2009) found indications of the genus being paraphyletic based on morphological characters (although this was not corroborated nor further investigated by them). Even if it was not the purpose of this study to deliberate the genus' phylogeny, based on our results *Eumerus* could be monophyletic with two main lineages. In addition, the formation of several groups within the genus reveals certain affinities among the species. Such affinities have never been discussed before, except in Speight (2014) who only commented on the *strigatus* group that included the taxa *E. consimilis* Šimić et Vujić, 1996, *E. narcissi* Smith, 1928, *E. ruficornis* Meigen, 1822, *E. sogdianus* Stackelberg, 1952, and *E. strigatus* (Fallen), 1817. Based on our data, *strigatus* group is composed of at least two taxa (*E. amoenus* and *E. consimilis*) but we did not have sequences for the other taxa considered by Speight (2014) to incorporate them into our analyses. However, our findings do suggest that *E. amoenus* has a probable (genetic) affinity with the other taxa of Speight's (2014) *strigatus* group, but this needs further verification.

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Table 2. Taxa grouping as formed after the implementation of the MP, ML and BI analyses and the MJ network reconstructions.

'Molecular' taxa groups (supported by morphology)	Taxa
basalis	*E. basalis* Loew, 1848 and *E. pusillus* Loew, 1848
minotaurus	*E. minotaurus* Claussen et Lucas, 1988 and *E. niehuisi* Doczkal, 1996
ornatus	*E. argyropus* Loew, 1848, *E. ornatus* Meigen, 1822, *E. paulae* Herve-Bazin, 1913 and *E. politus* Lyneborg, in litt.
pulchellus	*E. emarginatus* Loew, 1848 and *E. pulchellus* Loew, 1848
strigatus	*E. amoenus* Loew, 1848 and *E. consimilis* Šimić et Vujić, 1996
sulcitibius	*E.* aff. *barbarus* Coquebert, 1804 and *E. sulcitibius* Rondani, 1868
tricolor	*E. aurofinis* Grković, Vujić et Radenković, 2015, *E. armatus* Ricarte et Rotheray, 2012, *E. grandis* Meigen, 1822, *E. niveitibia* Becker, 1921, *E. sinuatus* Loew, 1855 and *E. tricolor* (Fabricius), 1798
'Ungrouped' taxa	*E. torsicus* Grković et Vujić, 2015, *E.* aff. *rubiginosus* Lyneborg, in litt., *E.* aff. *tarsatus* Lyneborg, in litt., *E. alpinus* Rondani, 1857, *E. argenticornis* Lyneborg, in litt., *E. clavatus* Becker, 1923, *E. hungaricus* Szilady, 1940 and *E. tarsatus* Lyneborg, in litt.

The groups are in concordance with the taxa morphology. Taxa groups are separated with ≥19 mt steps.

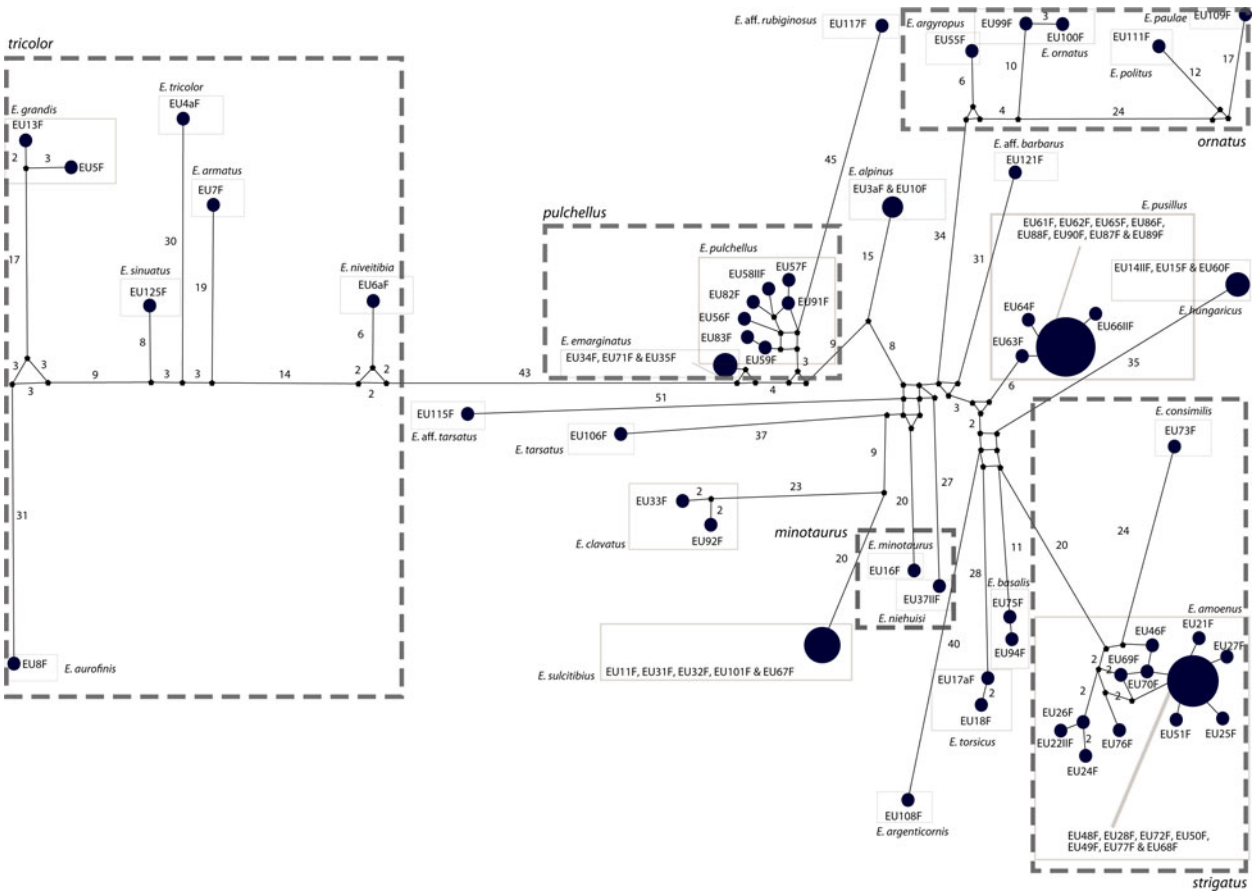

Fig. 5. The median-joining network of the haplotypes of the dataset A, as it was constructed by Network software ver. 4.6.1.2 (http://www.fluxus-engineering.com). Circle sizes are proportional to haplotype frequencies. The number of mutational steps is the one between each pair of haplotypes. When not stated, one mutational step interferes between the nodes/OTUs. Taxa and molecular taxa groups (supported by morphology) are depicted (apart of *basalis* and *sulcitibius* group).

The large number of mitochondrial haplotypes we generated underlines the high genetic diversity of *Eumerus*, despite the employment of only one genomic region. Species did not exhibit shared haplotypes and were well separated as shown by the number of mutational steps in the MJ networks. This is not always the case for hoverflies (e.g., the genus *Melanostoma* Schiner, 1860, Haarto & Stahls, 2014). Therefore, in *Eumerus*, a COI gene-based system can yield unique, distinguishing mtDNA haplotypes between species.

We also studied intraspecific variation for *E. amoenus*, *E. pusillus* and *E. pulchellus* for which we had more abundant sequences and geographic spread. These taxa are considered

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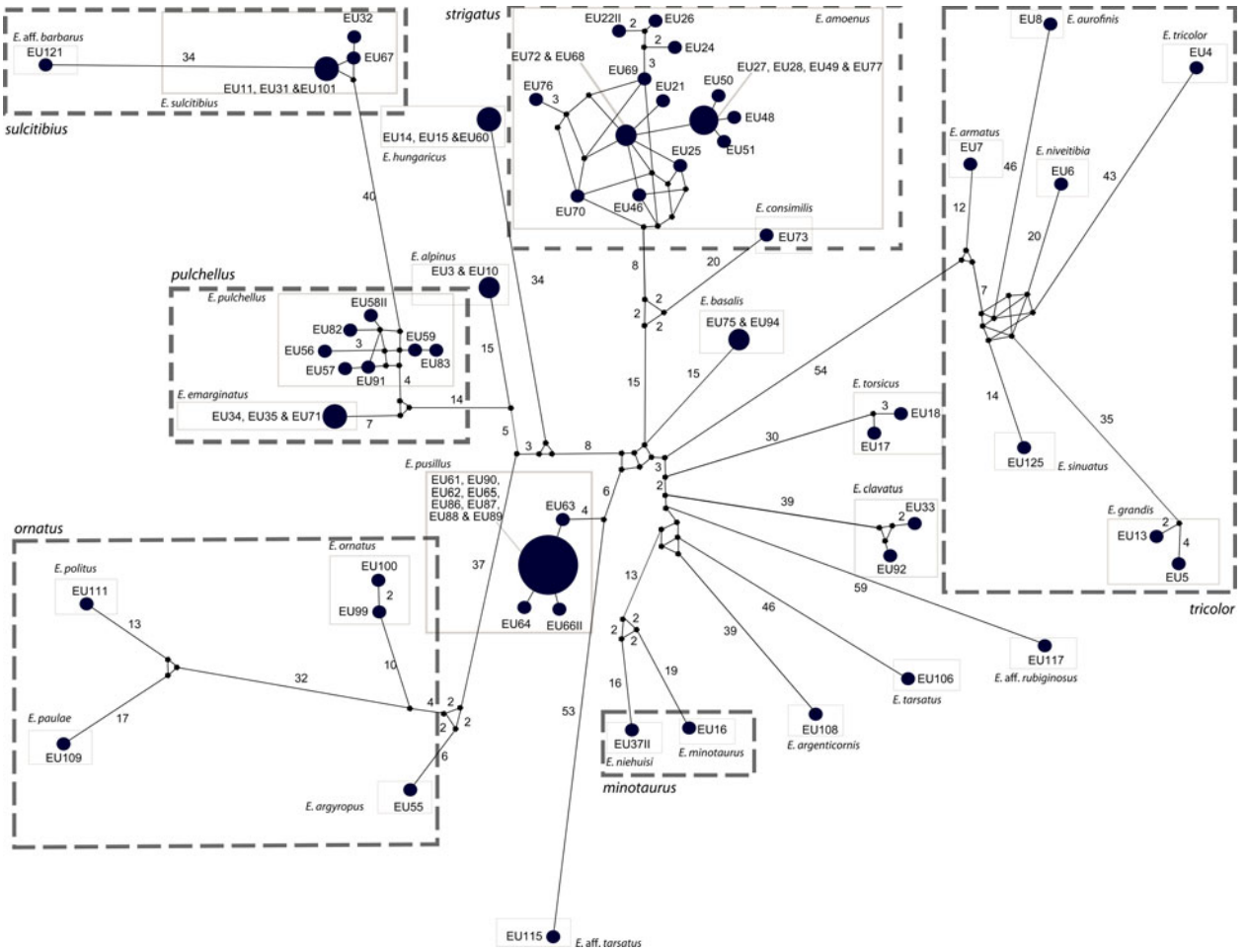

Fig. 6. The median-joining network of the haplotypes of the dataset B, as it was constructed by Network software ver. 4.6.1.2 (http://www.fluxus-engineering.com). Circle sizes are proportional to haplotype frequencies. The number of mutational steps is the one between each pair of haplotypes. When not stated, one mutational step interferes between the nodes/OTUs. Taxa and molecular taxa groups (supported by morphology) are depicted (apart of *basalis* group).

'common', i.e., they are widely distributed in all the Mediterranean peninsulas (Anatolian, Apennine, Balkan and Iberian). *Eumerus amoenus*, *E. pulchellus* and *E. pusillus* were analyzed further, for intraspecific genetic diversity. *Eumerus pulchellus* exhibited the highest haplotype diversity, with each single specimen per geographic origin having a different haplotype. Genetic distances among haplotypes inferred from the number of mutational steps and geographic distances between sampling locations (Greece, Italy and Montenegro), showed no clear pattern. *Eumerus amoenus* also presented very high haplotypic diversity, which was to be expected because sampling was performed over a wider geographical area. MJ network analysis of haplotypes generated a star-like pattern for this latter taxon, suggestive of past expansion (Bandelt *et al.*, 1995). Nucleotide diversity values were similar for *E. pulchellus* and *E. amoenus* for dataset A, whereas they appeared to be a bit higher for *E. amoenus* and *E. pusillus* based on dataset B. *Eumerus pulchellus* and *E. pusillus* were represented by more than two specimens from neighboring and remote geographical areas, and in general showed the lowest values for molecular diversity indices among the three analyzed taxa. The star-like patterns observed for MJ haplotype networks for both datasets for *E. pusillus* may indicate a recent expansion of this taxon, resulting in the observed lower genetic diversity. *Eumerus pusillus* specimens originating from Crete, Karpathos and Naxos (one sequence, EU66) shared the same haplotypes in datasets A and B. Specimens from Chios and others from Naxos presented different haplotypes in both datasets. Further conclusions from these discrepancies are limited due to the low sample sizes for the population genetic analyses and, in order not to be speculative, we encourage further intraspecific analyses.

LBA is a sensitive issue in phylogenetic analyses. We used maximum parsimony and likelihood based methods (including BI), with these latter having been proven to be less sensitive to LBA-artifacts compared with maximum parsimony (Bergsten, 2005). Nevertheless, long branches appeared in both ML- and BI-derived phylogenetic trees, but not for MP, with datasets A and B sharing the same LBAs. LBA-artifacts can arise due to several factors, such as poor taxon sampling and selection of highly divergent outgroups (for more details, see the review by Bergsten, 2005). Here, our MJ network

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Table 3. Results generated for dataset A (i.e., forward sequencing of the COI-3′ region) and dataset B (i.e., bidirectional sequencing) in DNaSP 5.10.01 for *E. amoenus* (18 sequences), *E. pulchellus* (seven sequences) and *E. pusillus* (11 sequences).

	Dataset A			Dataset B		
	E. amoenus	*E. pulchellus*	*E. pusillus*	*E. amoenus*	*E. pulchellus*	*E. pusillus*
1. Polymorphic sites						
Total number of sites (excluding sites with gaps/missing data)	636	645	646	737	746	746
Invariable (monomorphic) sites	636	636	642	716	736	740
Variable (polymorphic) sites	11	9	4	21	10	6
Total number of mutations	16	10	4	22	11	6
Singleton variable sites	*8*	*5*	*4*	*12*	*11*	*5*
Parsimony informative sites	*8*	*4*	*0*	*9*	*4*	*1*
2. DNA polymorphism						
2.1 Without gaps						
Number of Haplotypes (*h*)	13	7	5	16	7	6
Haplotype (gene) diversity (Hd)	0.928	1.000	0.618	0.987	1.000	0.727
Variance of Haplotype diversity	0.00268	0.00583	0.02701	0.00053	0.00583	0.02084
SD of Haplotype diversity	0.052	0.076	0.164	0.023	0.076	0.144
Nucleotide diversity (Pi)	0.00554	0.00568	0.00113	0.00616	0.00530	0.00166
Theta (per site) from Eta	0.00731	0.00633	0.00211	0.00868	0.00602	0.00275
Average number of nucleotide differences (*k*)	3.523	3.667	0.727	4.542	3.952	1.236
2.2 With gaps						
Number of pairwise comparisons	153	21	55	153	21	55
Average number of sites analyzed	645.27	646.43	646.82	744.59	746.00	746.00
Average number of differences	5.163	3.667	0.927	5.863	3.952	1.236
Nucleotide diversity (Pi)	0.00800	0.00567	0.00143	0.00787	0.00530	0.00166
Analysis at Individual Sites (*column by column*)						
Number of sites analyzed	647.00	647.00	647.00	746.00	746.00	746.00
Number of polymorphic sites (S)	22	9	5	26	10	6
Average number of differences	5.587	3.667	0.927	6.184	3.952	1.236
Nucleotide diversity (Pi)	0.00863	0.00567	0.00143	0.00829	0.00530	0.00166
Theta-W, per sequence	6.45560	3.67347	1.71915	7.60792	4.08163	2.04850
Theta-W, per site	0.00998	0.00568	0.00266	0.01020	0.00547	0.00275

reconstructions for each dataset explain the LBAs as those taxa having the highest numbers of mutational steps. In addition, the geographic origins of samples should be taken into account. It is important to clarify that the *ornatus* clade included taxa from very geographically distant areas, i.e., Greece (Dadia) and South Africa (KwaZulu-Natal), so LBAs were to be expected. Given that long branched taxa occurred in all trees, we claim that our topologies remain robust. Adding more samples for LBA taxa could lessen the impact of the LBAs. However, taxon sampling can remain an issue for molecular and other data. We felt that inclusion of as many taxa as possible in our analyses was paramount and so we chose not to exclude any taxa.

To conclude, the present study reveals the adequacy of the COI gene fragment to delimit species in the genus *Eumerus*. Forward and bidirectional sequencing datasets led to similar results; the forward sequencing dataset appeared to be sufficient to identify taxa within *Eumerus* and the more enriched sequence dataset, i.e., bidirectional, provided slightly more information and mitigated certain (though not all) analytical problems. Moreover, we reveal high intraspecific diversity and a high number of mitochondrial haplotypes. Our findings confirm the potential of an integrative approach – combined usage of a COI barcoding system and morphological characters – to diagnose and delimit species within the genus *Eumerus*. For a complete revision of the genus, including phylogenetic inferences, we endorse the usage of additional molecular markers and/or longer mitochondrial sequence (>800 bp). More taxa and more specimens per taxa should also be sought in order to overcome the drawbacks faced in the present study.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000729.

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Conflict of interest statement

We certify that there is no conflict of interest regarding the publication of this manuscript.

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