



PHYLOGENETIC RELATIONSHIPS OF THE EMMERICIIDAE (CAENOGASTROPODA: RISSOOIDEA)

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ABSTRACT: The phylogenetic relationships of the monogeneric rissoid family Emmericiidae Brusina, 1870 are unclear. The single genus *Emmericia* Brusina, 1870 occurs along the Adriatic coast from NE Italy to southern Croatia. It is characterised by the peculiar anatomy of the male genitalia (tri-lobed penis, bifurcate flagellum and penial gland). Mitochondrial cytochrome oxidase subunit I (COI) gene sequences, analysed together with nuclear 18S ribosomal RNA gene sequences, showed Bithyniidae and Bythinellidae as the sister taxa of the Emmericiidae, and confirmed the homology of the flagellum and penial gland in the Emmericiidae, Bythinellidae, Amnicolidae and Bithyniidae.

KEY WORDS: molecular phylogeny, cytochrome oxidase, 18S rRNA, Bayesian analysis, flagellum, penial gland, homology

INTRODUCTION

Emmericia Brusina, 1870, the type species *E. patula* (Brumati, 1838), is found along the Adriatic coast from North-East Italy to the south of Croatia. Apart from this range, isolated localities are known from France and Germany, but the latter are due to introductions (BRUSINA 1870, BOURGUIGNAT 1880, BOETERS & HEUSS 1985, MOUTHON 1986, KABAT & HERSHLER 1993, GLÖER 2002, GARGOMINY et al. 2011). The representatives of the genus inhabit rivers and springs (GIUSTI & PEZZOLI 1980, RADOMAN 1983, BOETERS 1998, GLÖER 2002). *Emmericia patula* (Brumati, 1838) is known from Monfalcone in Italy to the Neretva River in Croatia, not exceeding an altitude of about 70 m a.s.l. RADOMAN (1967, 1968, 1970, 1983) lists three more species inhabiting Croatia, Serbia and Bosnia. One of them occurs in the Neretva River, the other two are found locally in springs.

The genus is characterised by a unique, tri-lobed penis with a bifurcating flagellum and a penial gland, and a radula with no basal cusps on the rhachidian teeth. The phylogenetic relationships of *Emmericia* are enigmatic. BRUSINA (1870) established a monogeneric subfamily Emmericiinae, within the Rissoidae, and further discussed its position (BRUSINA 1874a, b).

BOURGUIGNAT (1877, 1880) placed the genus *Emmericia* in the Melaniidae (Cerithioidea). THIELE (1929–1935) placed Emmericieae, with *Emmericia* as the only genus, in the Hydrobiidae, subfamily Hydrobiinae, not far from the Lithoglyphaeae, Benedictieae, and Amnicoleae. RADOMAN (1967, 1968, 1970) reviewed the genus *Emmericia*, considering it anatomically most similar to *Lithoglyphus* Hartmann, 1821. GIUSTI & PEZZOLI (1980) and MOUTHON (1986) redescribed the genus, placing it in the family Emmericiidae, superfamily Pyrguloidea. PONDER & WARÉN (1988) included the subfamily Emmericiinae in the Hydrobiidae, placing the former close to the Baicaliinae, Benedictiinae and Tateinae, and far from the Lithoglyphinae and Amnicolinae. SZAROWSKA (2006a) inferred a phylogeny of *Emmericia*. Unfortunately, since several efforts of amplifying mitochondrial cytochrome oxidase subunit I (COI) gene had given no results (SZAROWSKA 2006a, WILKE personal communication), the phylogeny was based on the nuclear 18S rRNA gene sequence alone. Despite those limitations, it showed *Emmericia* as belonging neither to the Hydrobiidae nor to the Pomatiopsidae, Cochliopidae, and Tateidae, but clustering

in a big group together with *Bythinella*, Lithoglyphidae, Amnicolidae, and Bithyniidae. It was impossible to assess which of these taxa was the closest relative of *Emmericia*. In an appendix of SZAROWSKA (2006b) the literature data on the morphology of *Emmericia* were summarised, and SEM photographs of the shell surface and radula, morphology of the penis and female reproductive organs, and histology of the penis and flagellum (penial gland) were presented. The maximum-parsimony phylogenetic analysis of all

those morphological characters showed *Bithynia* Leach, 1818 as the sister taxon of *Emmericia*, and *Bythinella* Moquin-Tandon, 1855 and *Amnicola* Gould et Haldeman, 1840 as its close relatives.

The aim of the present paper is to establish phylogenetic relationships of *Emmericia* (to find its sister-clade) and to test homology of the flagellum and penial gland, applying cytochrome oxidase subunit I sequences, recently obtained in our laboratory.

MATERIAL AND METHODS

Material was collected from the Zrmanja River at Berberi, Croatia, 44°11'48.6"N, 15°46'04.6"E, 11 m a. s. l., in June 2011, with the use of a sieve of 0.5 mm mesh size. Snails were washed twice in 80% ethanol and left to stand in it for around 12 hours. Then the ethanol was changed twice more within 24 hours and finally, after a few days, the 80% solution was replaced with a 96% one, in which the samples were stored at -20°C.

DNA was extracted from foot tissue of two snails. The tissue was hydrated in TE buffer (3 × 10 min.); then total genomic DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in 20 µl TE buffer. The PCR reaction was performed with the following primers: LCO1490 (5'-GGTCAACAAATCATAAAGATAT TGG-3') (FOLMER et al. 1994) and COR722b

Table 1. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references

Species	18S GB#	COI GB#	References
<i>Adriohydrobia gagatinella</i> (Küster, 1852)	AF367657	AF317881	WILKE & FALNIOWSKI (2001)
<i>Adrioinsulana conovula</i> (Frauenfeld, 1863)	AF367656	AF367628	WILKE et al. (2001)
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
<i>Amnicola limosa</i> (Say, 1817)	AF212916	AF213348	WILKE et al. (2000b)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367675	AF367643	WILKE et al. (2001)
<i>Bythinella austriaca</i> (Frauenfeld, 1857)	AF212917	FJ545132	FALNIOWSKI et al. (2009)
<i>Bythiospeum</i> sp.	AF367664	AF367634	WILKE et al. (2001)
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676125	AY676127	SZAROWSKA et al. (2005)
<i>Emmericia patula</i> (Brumati, 1838) 6E66	KC810057	KC810059	present study
<i>Emmericia patula</i> (Brumati, 1838) 6E52	KC810058	KC810060	present study
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
<i>Heleobia dalmatica</i> (Radoman, 1974) 1	AF367661	AF367631	WILKE et al. (2001)
<i>Horatia klecakiana</i> Bourguignat, 1887	AF367669	AF367637	WILKE et al. (2001)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
<i>Lithoglyphus naticoides</i> (C. Pfeiffer, 1828)	AF367674	AF367642	WILKE et al. (2001)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF367676	AY027813	FALNIOWSKI & WILKE (2001)
<i>Pomatiopsis lapidaria</i> (Say, 1817)	AF367666	AF367636	WILKE et al. (2001)
<i>Pyrgula annulata</i> (Linnaeus, 1767)	AY676124	AY341258	SZAROWSKA et al. (2005)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676126	AY676128	SZAROWSKA et al. (2005)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
<i>Tricula wumingensis</i> Hu et al., 1994	(<i>P. lapidaria</i>)	EF394892	GUAN et al. (2008)
<i>Tricula pingi</i> Kang, 1984	(<i>P. lapidaria</i>)	EF394901	GUAN et al. (2008)
<i>Ventrosia ventrosa</i> (Montagu, 1803)	AF367681	AF118335	WILKE & DAVIS (2000)



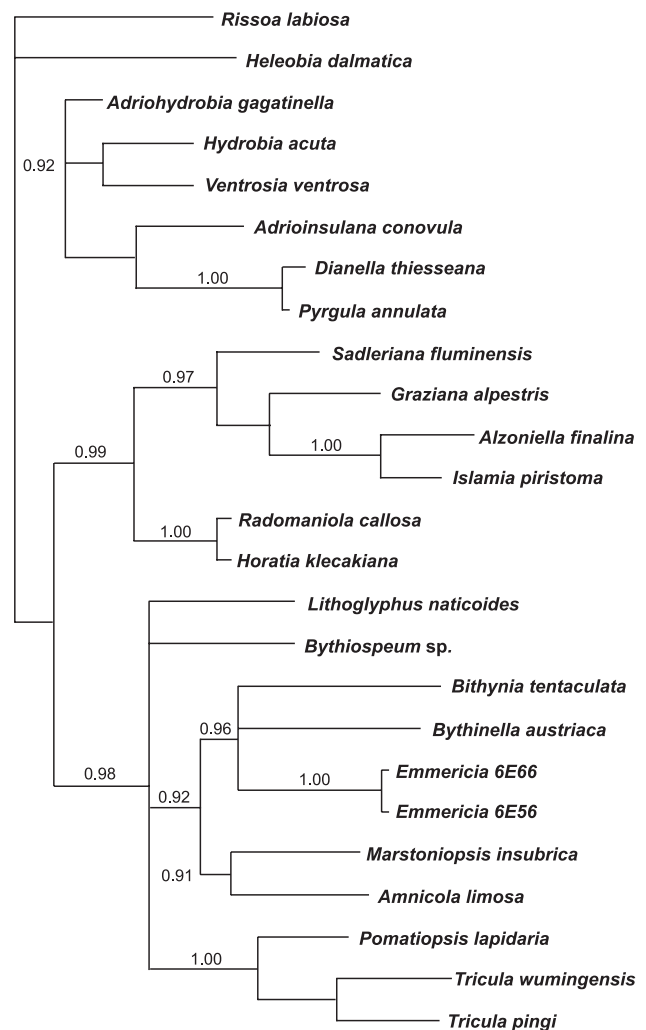
(5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the mitochondrial cytochrome oxidase subunit I (COI) gene, and SWAM18SF1 (5'-GAATGGCTCATTAATCAGT CGAGGTTCCCTTAGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAGT GTACTCATTCCAATTACGGAGC-3') for the nuclear 18S rRNA gene (PALUMBI 1996). The PCRs were run on Biometra TProfessional thermocycler. The PCR conditions were as follows: COI – initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 48°C, 2 min at 72°C, and a final extension of 4 min at 72°C; 18S – initial denaturation step of 4 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 51°C, 2 min at 72°C and, after all cycles were completed, an additional elongation step of 4 min at 72°C was performed. The total volume of each PCR reaction mixture was 50 µl. To check the quality of the PCR products 10 µl of the PCR product was ran on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and the purified PCR products were amplified in both directions (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer. All the sequences were deposited in GenBank (Table 1).

Four COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999). For 18S, an initial alignment was performed using CLUSTALX 1.82 (THOMPSON et al. 1997) and edited with MACCLADE4.05 (MADDISON & MADDISON 2002). Mutational saturation for the COI dataset was examined by plotting the numbers of transitions and transversions for all the codon positions together, and for the 3rd position separately, against the percentage sequence divergence, using DAMBE 5.2.9 (XIA 2000). We also used DAMBE 5.2.9 to perform the saturation test (XIA et al. 2003). It revealed a significant degree of saturation in the third position of the sequences. In rissooids, COI approaches saturation with about 18.6% or 120 nucleotide differences (DAVIS et al. 1998, WILKE et al. 2000a), which seems to happen after approximately 10 million years. However, to avoid a substantial loss of information in the case of closely related species, this position was not excluded from the dataset and it was used for the analysis.

The partition homogeneity test (FARRIS et al. 1995) was performed (1,000 replicates) with PAUP**4.0b10 (SWOFFORD 2002), to check whether the two genes could be analysed together. Since the results of the test were positive ($p=0.789$), the sequences were analysed together. For each maximum likelihood (ML) analysis, we used the best fit model of sequence evolution found by Modeltest v3.06

(POSADA & CRANDALL 1998, POSADA 2003). Following the recommendations of POSADA & BUCKLEY (2004) and SOBER (2002), the best model for each dataset was chosen using the Akaike Information Criterion (AKAIKE 1974). We performed ML analyses in PAUP* and used a heuristic search strategy with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping (SWOFFORD et al. 1996). Nodal support was estimated using the bootstrap (BS) approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 1,000 bootstrap replicates, the "fast" heuristic search algorithm, and the same model parameters as for each ML analysis. Additionally, we ran ML analysis in PAUP with option "estimate" for all the model parameters.

For Bayesian inference (BA) we used MRBAYES 3.1.2 (HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003). We selected the best model of sequence evolution for each data set using MrModeltest 2.2 (NYLANDER 2004), applying the Akaike Infor-



0.1
Fig. 1. Bayesian phylogenetic tree. Bayesian probabilities given where >0.90

mation Criterion (POSADA & BUCKLEY 2004). The Bayesian inference was performed with the following parameters: 4 chains in two parallel analyses (1 cold, three heated; T=0.15) Metropolis-Coupled Monte Carlo analysis run twice in parallel for 80,000,000 generations, trees sampled every 1,000 generations starting after a burn-in of 3,000,000 generations (the value chosen according to the log-likelihood values). The Bayesian inference was run unless the parallel runs

RESULTS

In both ML and BA trees *Emmericia* clustered outside the Hydrobiidae/Cochliopidae group, with Lithoglyphidae, *Bythiospeum*, Bithyniidae, Bythinellidae, Amnicolidae and Pomatiopsidae. ML analysis run with option "estimate" for all the model parameters resulted in more reliable trees, since in the trees inferred with the model found by the Modeltest *Bythinella* was outside all the flagellum-bearing taxa, close to the Pomatiopsidae. However, in all the ML trees *Lithoglyphus* appeared as the sister taxon of

achieved convergence (split frequency standard deviations <0.001). The partition was set, with COI treated as coding and 18S as noncoding. We inferred final consensus trees with Bayesian probabilities.

In the phylogeny reconstruction, we used 23 rissoid taxa sequences from GenBank (Table 1), selected to represent all the main lineages of the freshwater Rissooidea, with a better representation of the flagellum-bearing taxa.

Emmericia. As the bootstrap support for this grouping was less than 50%, we do not present those trees.

In the Bayesian tree (Fig. 1), the significantly supported (Bayesian probability 0.98) clade consisted of *Bithynia*, *Bythinella*, *Emmericia*, Amnicolidae and Pomatiopsidae. Close to significant (0.92) was the probability of the clade consisting of *Bithynia*, *Bythinella*, *Emmericia*, and Amnicolidae (Fig. 1). An unresolved trichotomy, with a significant probability (0.96), was formed by *Emmericia* and *Bithynia*, and *Bythinella* as the sister clade of the former.

DISCUSSION

It is evident that Emmericiidae are not closely related to the "Pyrgulidae" (the latter belong to the Hydrobiidae: SZAROWSKA et al. 2005), which contradicts GIUSTI & PEZZOLI (1980). Interestingly, the ML trees seemed to confirm the close relationships between *Emmericia* and *Lithoglyphus* as postulated by RADOMAN (1968) and (in part) by the 18S phylogeny in SZAROWSKA (2006a). However, all the ML-inferred relationships were weakly supported (bootstrap values less than 50%). As a general rule bootstrap supports are lower than the corresponding Bayesian probabilities (SUZUKI et al. 2002, DOUADY et al. 2003, ERIXON et al. 2003, WILCOX et al. 2003).

BA resulted in a grouping supported by significant Bayesian probabilities. The Bayesian probability 0.96 was found for the clade consisting of *Emmericia*, *Bithynia* and *Bythinella*, thus the latter two genera are putative (an unresolved, most probably soft trichotomy) sister taxa of *Emmericia*, which agrees with the morphology-based phylogeny presented by SZAROWSKA

(2006b). Recently, WILKE et al. (2013) inferred similar relationships of the Emmericiidae based on 18S and 16S rRNA sequences. The BA phylogeny confirms the homology of the flagellum and penial gland within the Rissooidea. It must be pointed out, however, that a flagellum and penial gland may be secondarily lost, as it is in the case of *Pseudobithynia* Glöer et Pešić, 2006 (SZAROWSKA 2006a). Recently a flagellum with a small but typical penial gland was found in the newly described hydrobiid genus *Agrafia* Szarowska et Falniowski, 2011 (SZAROWSKA & FALNIOWSKI 2011). So far, the homology of those structures in *Agrafia* remains enigmatic.

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