PHYLOGENETIC RELATIONSHIPS OF THE EMERRICIIDAE (CAENOGASTROPODA: RISSOOIDEA)

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ABSTRACT: The phylogenetic relationships of the monogeneric rissooid 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, GŁOER 2002, GARGOMINY et al. 2011). The representatives of the genus inhabit rivers and springs (GIUSTI & PEZZOLI 1980, RADOMAN 1983, BOETERS 1998, GŁOER 2002). Emmericia patula (Brumati, 1838) is known from Monfalcone in Italy to the Neretva River in Croatia, not exceeding an attitude of about 70 m a.s.l. 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 & WAREN (1988) included the subfamily Emmericiinae in the Hydrobiidae, placing the former close to the Baicaliinae, Benedictiinae and Tateinae, and far from the Lithoglypheae and Amnicolineae. 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 *Bythynia* 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 (5 × 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'-GGTCAACAAATCATAAATGTTG-3') (FöLmer 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                      |
|-----------------------------|-----------|-----------|---------------------------------|
| Adriohydrobia gagatinella   | AF367657  | AF317881  | WILKE & FALNIOWSKI (2001)       |
| Adriinsulana conovula       | AF367656  | AF367628  | WILKE et al. (2001)             |
| Alzoniella finalina         | AF367686  | AF367650  | WILKE et al. (2001)             |
| Amnicola limosa             | AF212916  | AF213348  | WILKE et al. (2000b)            |
| Bithynia tentaculata        | AF367675  | AF367643  | WILKE et al. (2001)             |
| Bithynella austriaca        | AF212917  | FJ545132  | FALNIOWSKI et al. (2009)        |
| Bithiospeum sp.             | AF367664  | AF367634  | WILKE et al. (2001)             |
| Dianella thiesseana         | AY676125  | AY676127  | SZAROWSKA et al. (2005)         |
| Emmericia patula            | KC810057  | KC810059  | present study                   |
| Emmericia patula            | KC810058  | KC810060  | present study                   |
| Graziana alpestris          | AF367673  | AF367641  | WILKE et al. (2001)             |
| Heleobia dalmatica          | AF367661  | AF367631  | WILKE et al. (2001)             |
| Horatia kleakiiana           | AF367669  | AF367637  | WILKE et al. (2001)             |
| Hydrobia acuta              | AF367680  | AF278808  | WILKE & Davis (2000)            |
| Islamia piristoma           | AF367671  | AF367639  | WILKE et al. (2001)             |
| Lithoglyphus naticoides     | AF367674  | AF367642  | WILKE et al. (2001)             |
| Marstoniopsis insubrica     | AF367676  | AY092783  | FALNIOWSKI & WILKE (2001)       |
| Pomatiopsis lapidaria       | AF367666  | AF367636  | WILKE et al. (2001)             |
| Pyrgula annulata            | AY676124  | AY341258  | SZAROWSKA et al. (2005)         |
| Radomaiola callosa          | AF367685  | AF367649  | WILKE et al. (2001)             |
| Rissoa labiosa              | AY676126  | AY676128  | SZAROWSKA et al. (2005)         |
| Saderiana fluminensis       | AF367683  | AY273996  | WILKE et al. (2001)             |
| Tricula wumingensis         | EF394892  | EF394901  | GUAN et al. (2008)              |
| Tricula pingi               | EF394901  | EF394901  | GUAN et al. (2008)              |
| Ventrosia ventrosa          | AF367681  | AF183305  | WILKE & DAVIS (2000)            |
(5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the mitochondrial cytochrome oxidase subunit I (COI) gene, and SWAM18SF1 (5'-GAATGGCTCATTAAATCGTAGGTTCCTTAGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAAGT GTACCTATCCAATTACGGAGC-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 MACCLADE 4.05 (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 Akaikie Information Criterion (Akaikie 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 & HUELENBECK 2003). We selected the best model of sequence evolution for each data set using MrModeltest 2.2 (Nylander 2004), applying the Akaikie Infor-

![Phylogenetic relationships of the Emummericidae](image)

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 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 rissooid 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.

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 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. 2005).

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 hydrobid genus Agrafia Szarowska et Falniowski, 2011 (SZAROWSKA & FALNIOWSKI 2011). So far, the homology of those structures in Agrafia remains enigmatic.

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