PHYLOGENETIC POSITION OF BOLEANA UMBILICATA (KUŠČER, 1932) (CAENOGASTROPODA: RISSOOIDEA)

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ABSTRACT: Phylogenetic position of Boleana umbilicata (Kuščer, 1932) from Močilnik spring in Slovenia, the type locality of the species, was inferred with ML technique using nucleotide sequences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear 18S rRNA genes.

KEYWORDS: Rissooidea, mtDNA, COI, 18S rRNA, phylogeny, spring, type locality

INTRODUCTION

Močilnik, the main source of the Ljubljanica river, Slovenia is inhabited by interesting representatives of the Hydrobiidae (BOLE 1967, 1985, RADOMAN 1983). It was from this locality that Kuščer (1932) described Belgrandiella umbilicata. RADOMAN (1973) described a new monotypic genus Boleana, with the type species Boleana umbilicalis (Kuščer, 1932). SZAROWSKA (2006) confirmed RADOMAN’s (1973) data concerning anatomy, and presented SEM photographs of the protoconch and radula. In the molecular phylogeny based on mitochondrial COI and nuclear 18S rRNA sequences (SZAROWSKA 2006: fig. 241) Boleana is placed close to Daphniola Radoman, 1973. Unfortunately, that phylogeny was based upon a very short (about 200 bp) COI sequence. The aim of this study is to correct the position of Boleana in the molecular phylogeny using a longer COI sequence, and to analyse the two loci together.

MATERIAL AND METHODS

Using a sieve, numerous specimens of Boleana umbilicata were collected from Močilnik (Fig. 1), the huge spring of the Ljubljanica river (45°57’15”N, 14°17’33”E, 313 m a.s.l.). Snails were washed twice in 80% ethanol and left to stand in it for about 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.

The shells were cleaned in an ultrasonic cleaner and photographed with a CANON EOS 50D digital camera. Two males and two females were dissected, using a NIKON SMZ-U stereomicroscope.

DNA was extracted from foot tissue of each snail. The tissue was hydrated in TE buffer (10 mM TRIS-HCl pH 8.0, 1 mM EDTA) (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’-GGTCAACAAATCATAAAGATATTGG-3’) (FOLMER et al. 1994) and COR722b (5’-TAACTTCTAGGGTGACCAAAAAATYAG-3’) (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene, as well as SWAM18SF1 (5’-GGTCAACAAATCATAAAGATATTGG-3’) and SWAM18SR1 (5’-ATCCTCGTGAAGGTTCCATTCCATTACCGAGC-3’) for the 18S ribosomal RNA gene (PALUMBI 1996). The PCR conditions were as follows: COI – ini-
tial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°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 run on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&ABiotechnology) and the purified PCR products were amplified in both directions 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).

The COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999). For 18S, an initial alignment was performed using CLUSTALX 2.012 (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). Initially, we performed phylogeny reconstruction for 18S and COI data separately, using the maximum likelihood (ML) technique.

For each 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*4.0b10 (SWOFFORD 2002) 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 1000 bootstrap replicates, the “fast” heuristic

In the phylogeny reconstruction, we used sequences of 27 rissooid taxa from GenBank (Table 1). Seven of them, used as outgroup, represented the main non-hydrobid lineages within the Rissooidea (WILKE et al. 2001); another seven taxa represented the Hydrobiinae (including “Pyrgulinae”: SZAROWSKA et al. 2005). The remaining taxa were chosen to represent all the main lineages within the European Sadlerianinae (SZAROWSKA 2006).

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search algorithm, and the same model parameters as for each ML analysis. Next, the partition homogeneity test (FARRIS et al. 1995) was performed (1000 replicates) with PAUP*, to check whether the two genes could be analysed together. The maximum likelihood heuristic search was then run for the combined molecular data. MEGA4 (KUMAR et al. 2004) was used to estimate nodal support with BS and minimum-evolution (ME) and neighbor-joining (NJ) approach (SWOFFORD et al. 1996, NEI & KUMAR 2000), applying composite-likelihood distances with gamma distribution parameter estimated with MODELTEST.

RESULTS AND DISCUSSION

The shell (Figs 2–7) and soft parts of B. umbilicata resembled the ones shown in RADOMAN (1983: fig. 53, pl. VI, fig. 108) and SZAROWSKA (2006: fig. 26).

Two sequences of COI (each 653 bp long) and two of 18S (each 419 bp long) of B. umbilicata were analysed (Table 1). DAMBE 5.2.9 saturation test revealed a significant degree of saturation in the third position of the sequences. In the rissooids, COI approaches saturation with about 18.6% or 120 nucleotide differences (DAVIS et al. 1998), 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 but was used for the analysis. For the COI sequences the Akaike Information Criterion (AIC) with ModelTest found model TVM+I+Γ, with base frequencies: $A=0.3500$, $C=0.1330$, $G=0.1097$, $T=0.4073$; substitution rate matrix: $[A–C]=0.2566$, $[A–G]=10.7137$, $[A–T]=0.0709$, $[C–G]=1.1198$, $[C–T]=10.7137$, $[G–T]=1.0000$, proportion of invari-

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

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<th>Species</th>
<th>18S GB#</th>
<th>COI GB#</th>
<th>References</th>
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<tr>
<td>Adriodoria gogatina (Küster, 1852)</td>
<td>AF367657</td>
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<td>WILKE &amp; DAVIS (2000)</td>
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Figs 2–7. Shells of *Boleana umbilicata*, bar represents 1 mm

Fig. 8. ML phylogram based on COI, bootstrap supports given where >50

Fig. 9. ML phylogram based on COI and 18S, bootstrap supports given where >50
able sites: (I) = 0.4054, and $\Gamma$ distribution with the shape parameter $= 0.2528$. For the combined data set the Akaike Information Criterion (AIC) with ModelTest found model GTR+I+$\Gamma$, with base frequencies: A=0.3834, C=0.1506, G=0.1406, T=0.3486; substitution rate matrix: [A–C]=0.8317, [A–G]=7.6497, [A–T]=0.2018, [C–G]=2.7010, [C–T]=12.9307, [G–T]=1.0000, proportion of invariable sites: (I)=0.6017, and $\Gamma$ distribution with the shape parameter $= 0.3486$.

The ML tree computed for the COI sequences (Fig. 8), like the corresponding tree in SZAROWSKA (2006: fig. 239), shows Boleana in the Hydrobiidae, Sadlerianinae. However, in Fig. 8, unlike in SZAROWSKA (2006: fig. 239), the sister taxon of Graziana is not Daphniola Radoman, 1973, but Graziana Radoman, 1973. Moreover, in Fig. 8 the Boleana/ Graziana clade is close to Agrafia Szarowska et Falniowski, 2011, Hauffenia Pollonera, 1898, Islamia Radoman, 1973, and Alzoniella Giusti et Bodon, 1984, not to Grossiana Radoman, 1973 and Trichonia Radoman, 1973 (SZAROWSKA 2006: fig. 239). Interestingly, in the tree based on 18S in SZAROWSKA (2006: fig. 237), Boleana forms a trichotomy with Graziana and Trichonia, and we found the same in the present study (the tree not shown). In the ML tree computed for the two sequences together (Fig. 9), as in the COI tree (Fig. 8), Graziana is the sister taxon of Boleana while Daphniola is not close to the latter. Unlike in the COI tree, only the genera Hauffenia and Agrafia (but not Alzoniella nor Islamia) are closely related to Boleana and Graziana (Fig. 9).

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