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SPECIES DISTINCTNESS OF *HAUFFENIA MICHLERI* (KUŠČER, 1932) (CAENOGASTROPODA: TRUNCATELLOIDEA: HYDROBIIDAE)

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ABSTRACT: *Hauffenia michleri* (Kuščer, 1932) was described from Močilnik spring in Slovenia. Later it was regarded as conspecific with *H. tellinii* Pollonera, 1898, since no differences in the soft parts morphology and anatomy were found. In the present paper, applying molecular markers (mitochondrial cytochrome oxidase subunit I and nuclear 18S ribosomal RNA partial gene sequences), the genetic differences typical of congeneric distinct species (p distances 0.067, and 0.005, respectively) were found, proving the species distinctness of *H. michleri*.

KEY WORDS: mtDNA, *COI*, *18SrRNA*, p-distance, Slovenia

INTRODUCTION

Močilnik, the main source of the Ljubljanica River, Slovenia is inhabited by interesting representatives of the Hydrobiidae (BOLE 1967, 1985, RADOMAN 1983). KUŠČER (1932) described a minute valvatiform hydrobiid snail *Hauffenia michleri* (Fig. 1) from this locality. Later, considering the shell and anatomy, BODON et al. (2001) synonymised *H. michleri* (Kuščer, 1932) with *H. tellinii* Pollonera, 1898 from Italy. However, in the truncatelloidean gastropods, morphology alone is often insufficient for species delimitation (e.g. SZAROWSKA & FALNIOWSKI 2008). Thus, we have tried to evaluate the species distinctness of *H. michleri* applying molecular markers (mitochondrial cytochrome oxidase subunit I and nuclear 18S ribosomal RNA partial gene sequences).

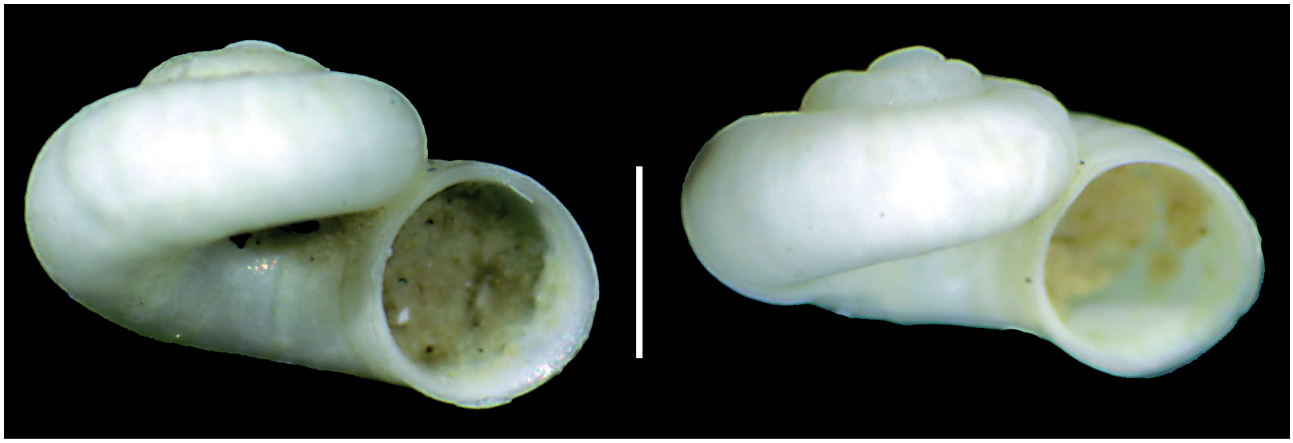

Fig. 1. Shells of *Hauffenia michleri* (Kuščer, 1932) from Močilnik spring. Bar equals 0.5 mm

MATERIAL AND METHODS

Five live specimens of *Hauffenia michleri* were collected using a sieve (two meshes per mm), from Močilnik, the type locality of this species, the huge spring of the Ljubljanica River (45°57'15"N, 14°17'33"E, 313 m a.s.l.). The 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. The shells were photographed with a CANON EOS 50D digital camera attached to NIKON SMZ-18 stereoscopic microscope with the dark field.

DNA was extracted from foot tissue of two specimens. 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'-GGTCAACAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the mitochondrial cytochrome oxidase subunit I (*COI*) gene and SWAM18SF1 (5'-GAATGGCTCATTAAATCAGTCGAGGTTCCTTAGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTTAAATTAAAGTGGTGTACTCATTCCAATTACGGAGC-3') for the nuclear 18S ribosomal RNA (*18S*) gene (ATTWOOD et al. 2003). 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 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 ran on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and were then 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. The *COI* sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999); alignment for *18S* was performed using CLUSTALX 1.82 (THOMPSON et al. 1997), and p-distances computed with MEGA5.10 (TAMURA et al. 2011).

RESULTS AND DISCUSSION

Two sequences of nuclear 18S ribosomal RNA (*18S*) gene, 404 bp long (GenBank number KT236155), were identical, and two sequences of mitochondrial cytochrome oxidase subunit I (*COI*) gene, 641 bp long (GenBank number KT236156) were identical as well. They were compared with the sequences of *Hauffenia tellinii* (Pollonera, 1898) (GenBank numbers AF367672, and AF367640: WILKE et al. 2001), collected at a spring near the Isonzo River near the dam of Sagrado, Friuli-Venetia-Julia, Gorizia, Italy. The p-distance for *18S* was 0.005 (two point mutations), and the p-distance for *COI* was 0.067 (43 substitutions). The two substitutions in *18S* are noteworthy, since there are often no differences in *18S* between closely related species. The distance calculated for *COI* between *H. michleri* and *H. tellinii* is typical – in the Truncatelloidea – of distinct species, belonging to the same genus (e.g. FALNIOWSKI et al. 2007, 2009, SZAROWSKA et al. 2007, FALNIOWSKI & SZAROWSKA 2011). Thus, the species distinctness of *H. michleri* seems evident, and synonymisation introduced by BODON et al. (2001) not justified.

Certainly, the genetic distances alone, especially calculated for a few short sequences only, may not always be decisive. There are some cases which need further explanation, for example morphologically distinct, sympatric taxa with slight differences in *COI* sequences in *Bythinella* (HAASE et al. 2007, our observation on the Polish *Bythinella*) on the one hand, and about 5% differences within the same nominal species of *Bythinella* (FEHÉR et al. 2013) on the other. Morphological differences may not be reflected molecularly (WILKE & FALNIOWSKI 2001), or molecularly distinct species may not be morphologically distinguishable (e.g. SZAROWSKA & FALNIOWSKI 2014). Genetic data, however, may be the only key to discover cryptic species, the latter especially frequent among minute gastropods, whose morphology is simplified because of miniaturisation, and unified because of the necessary adaptation to live and reproduce in freshwater.

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