FOLIA MALACOLOGICA
Folia Malacol. 27(2): 111–118
ISSN 1506-7629 (print) • ISSN 2300-7125 (on-line)
The Association of Polish Malacologists
Faculty of Biology, Adam Mickiewicz University
Bogucki Wydawnictwo Naukowe
Poznań, June 2019
https://doi.org/10.12657/folmal.027.013

# SPECIES DISTINCTNESS OF *BITHYNIA CETTINENSIS* CLESSIN, 1887 AND *B. ZETA* GLÖER ET PEŠIĆ, 2007 (CAENOGASTROPODA: TRUNCATELLOIDEA)

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ABSTRACT: Shells of *Bithynia*: widely distributed *B. tentaculata* (Linnaeus), Balkan *B. cettinensis* Clessin from Cetina River in Croatia, and *B. zeta* Glöer et Pešić from Vitoja spring at Lake Skadar, Montenegro, as well as male genitalia of *B. zeta* and *B. cettinensis* were examined. The shells of all three taxa are similar, also the penes differ only slightly. The mean value of the length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin, was 1.63 for *B. zeta*, and 1.33 for *B. cettinensis*. Those values differed slightly, especially compared to 5.0–5.9 for *B. tentaculata*. In the maximum likelihood (ML), as well as Bayesian (BI) trees, *B. cettinensis* and *B. tentaculata* were sister clades with p-distance of 0.007, and *B. zeta* was more distinct, with p-distance of 0.122 to *B. cettinensis* and 0.154 to *B. tentaculata*. The species distinctness of the three studied taxa was confirmed.

KEY WORDS: species distinctness, shell, penis, tubular penial gland, mtDNA, COI

## INTRODUCTION

The Bithyniidae Gray, 1857 inhabit fresh and brackish waters of Europe, Asia Minor, northern Asia, India, Indochina, China, part of Australia, western Africa, Lake Tanganyika, and some introduced localities in North America (FALNIOWSKI 1989). Apart from the "normal" feeding on detritus, they are also filter-feeders (LILLY 1953, FRETTER & GRAHAM 1962). *Bithynia tentaculata* (Linnaeus, 1758) is one of the most widely distributed and most common freshwater species. Some other nominal species of *Bithynia* Leach, 1818, whose distinctness remains unclear, are known from southern Europe. CLESSIN (1887) described *B. cettinensis* Clessin, 1887, from Cetina River in Croatia. GLÖER & PEŠIĆ (2007) described three new species of *Bithynia* from Lake Skadar in Montenegro: *B. zeta* Glöer et Pešić, 2007, *B. skadarskii* Glöer et Pešić, 2007, and *B. radomani* Glöer et Pešić, 2007. The penis and penial gland morphology presented by GLÖER & BERAN (2009) for *B. cettinensis* was based on only one penis, most probably somewhat damaged, and molecular data for the European *Bithynia* are known only for *B. tentaculata*. The aim of the present paper was to describe the penial morphology of *B. cettinensis* and to check the species distinctness of *B. tentaculata*, *B. cettinensis*, and *B. zeta*, applying molecular data, namely cytochrome oxidase subunit I (COI) sequences.

Ministry of Science and Higher Education
Republic of Poland

Folia Malacologica is funded by the Ministry of Science and Higher Education, Republic of Poland, under agreement no 534/P-DUN/2018 of April 4th, 2018 allocated to the activities for disseminating science:
Task 1: Preparation of English versions of publications (sum funded by DUN 12,000 PLN) and
Task 2: Digitalisation of publications and scientific monographs to enable their open access in the Internet (sum funded by DUN 11,070 PLN).

## MATERIAL AND METHODS

We collected specimens of the three *Bithynia* species: *B. zeta* from Vitoja spring (42°19'32"N, 19°21'46"E; Figs 1–2), Montenegro, *B. cettinensis* from Cetina River at Radmanove Mlinice (43°26'37"N, 16°45'02"E), Croatia, and *B. tentaculata* from pond in Spytkowice near Kraków (50°00'14"N, 19°29'10"E), Poland. The morphology of *B. tentaculata* was studied in many localities throughout Poland (FALNIOWSKI 1989); shells from Lake Gardno (N. Poland) are shown in the present paper.

For molecular study, the snails were washed twice in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice in 24 hours and finally, after a few days, the 80% solution was exchanged for a 96% one, stored at −20 °C.

The shells were photographed with a CANON EOS 50D digital camera, under a NIKON SMZ18 microscope with dark field. Dissection was done under NIKON SMZ18 microscope, and photographed with dark field. The measurements of the penis and tubular penial gland were taken on the photographs, with SILVA Map Measurer Plus curvimeter. The morphological terminology follows HERSHLER & PONDER (1998).

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 $\mu$l TE buffer. The extracted DNA was stored at −80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland). Details of PCR conditions, primers used and sequencing were given in SZAROWSKA et al. (2014). Cytochrome oxidase subunit I (COI) mtDNA


Figs 1–2. Vitoja Spring

Table 1. Taxa used for phylogenetic analyses with their GenBank accession numbers and references

| Species | COI GB numbers | References |
|---|---|---|
| *Bithynia tentaculata* (Linnaeus, 1758) | MN013036-MN013037 | present study |
| *Bithynia zeta* Glöer et Pešić, 2007 | MN013038-MN013041 | present study |
| *Bithynia cettinensis* Clessin, 1887 | MN013042 | present study |
| *Bithynia* sp. Leach, 1818 – Egridir Lake, Turkey | FJ160291 | BENKE et al. 2009 |
| *Bithynia* sp. Leach, 1818 – Poso Lake, Indonesia | KY574006 | VAN BOCXLAER et al. 2017 |
| *Bithynia funiculata* Walker, 1927 | KY118598 | KULSANTIWONG et al. 2013 |
| | KY118601 | KULSANTIWONG et al. 2013 |
| *Bithynia siamensis goniomphalos* (Morelet, 1866) | KY118603 | KULSANTIWONG et al. 2013 |
| | KY118610 | KULSANTIWONG et al. 2013 |
| | KY118619 | KULSANTIWONG et al. 2013 |
| *Bithynia siamensis siamensis* (Lea, 1856) | KY118658 | KULSANTIWONG et al. 2013 |
| *Bythinella austriaca* (von Frauenfeld, 1857) | JQ639858 | FALNIOWSKI et al. 2012 |
| *Bythinella micherdzinskii* Falniowski, 1980 | JQ639854 | FALNIOWSKI et al. 2012 |
| *Gabbia pygmaea* (Preston, 1908) | KY118682 | KULSANTIWONG et al. 2013 |
| *Gabbia wykoffi* (Brandt, 1968) | KY118724 | KULSANTIWONG et al. 2013 |
| *Hydrobioides nassa* (Theobald, 1865) | KY118756 | KULSANTIWONG et al. 2013 |
| | KY118765 | KULSANTIWONG et al. 2013 |
| *Wattebledia baschi* Brandt, 1968 | KY118769 | KULSANTIWONG et al. 2013 |
| *Wattebledia crosseana* (Wattebled, 1884) | KY118793 | KULSANTIWONG et al. 2013 |


Figs 3–15. Shells of the studied *Bithynia*: 3–6 – *B. tentaculata*, Lake Gardno, Poland; 7–11 – *B. cettinensis*, Radmanove Mlinice, Cetina River, Croatia; 12–15 – *B. zeta*, Vitoja Spring, Montenegro. Scale bar 2 mm

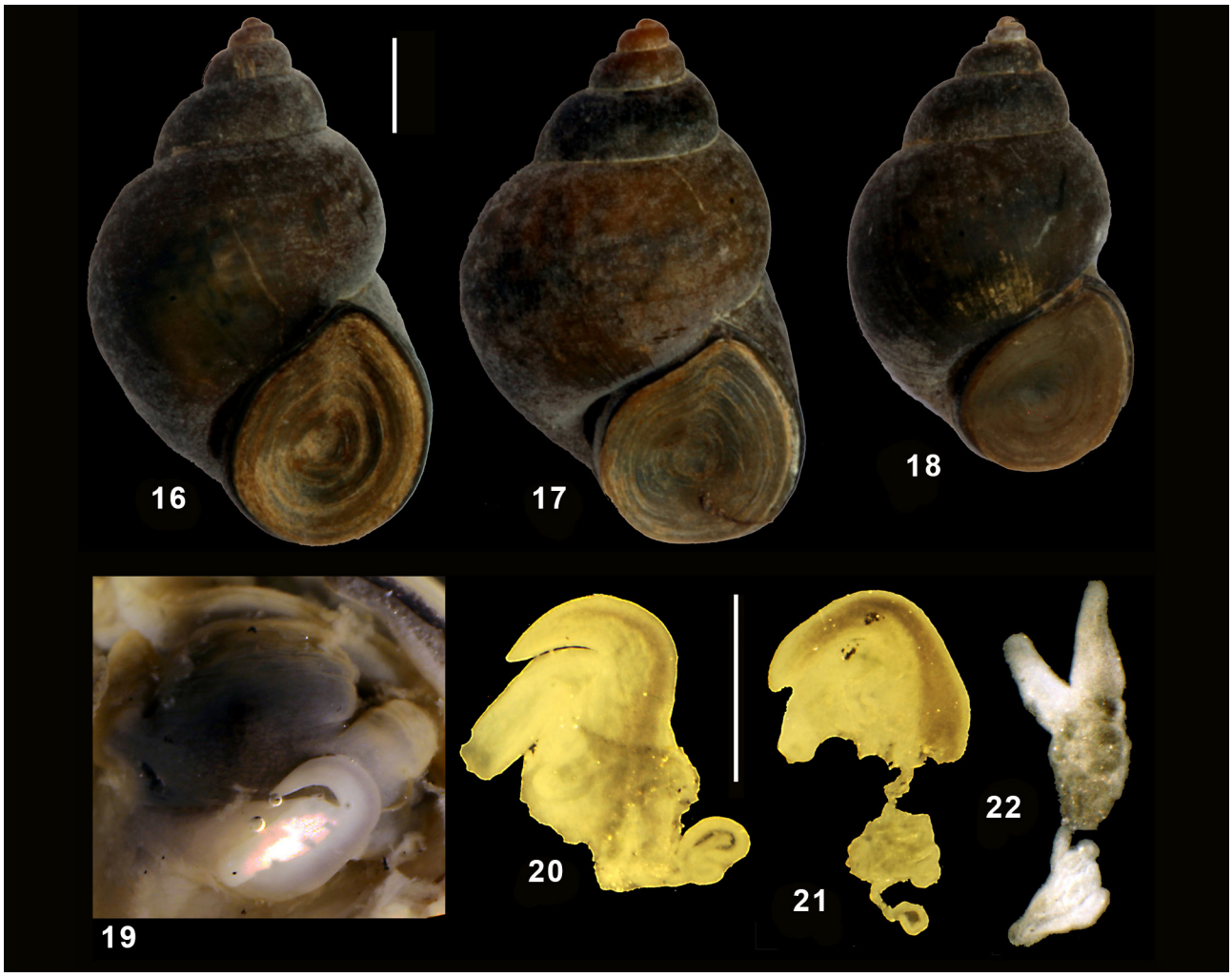
was amplified with the following primers: LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (FOLMER et al. 1994), and COR722b 5'-TAAACTTCAG GGTGACCAAAAAATYA-3' (WILKE & DAVIS 2000). PCR conditions for the COI were as follows: initial denaturation step of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension of 4 min at 72 °C. Sequencing methods are described in SZAROWSKA et al. (2014).

Sequences were initially aligned in the MUSCLE (EDGAR 2004) programme in MEGA 6 (TAMURA et al. 2013) and then checked in Bioedit 7.1.3.0 (HALL 1999). The saturation test (XIA 2000, XIA et al. 2003) was performed using DAMBE (XIA 2013). In the phylogenetic analysis additional sequences from GenBank were used (Table 1). The data were analysed using approaches based on Bayesian inference (BI) and maximum likelihood (ML). We applied the GTR model, whose parameters were estimated by the RaxML (STAMATAKIS 2014). The BI analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with the default priors. The ML approach was applied with RAxML v. 8.0.24 (STAMATAKIS 2014). RAxML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER et al. 2010).

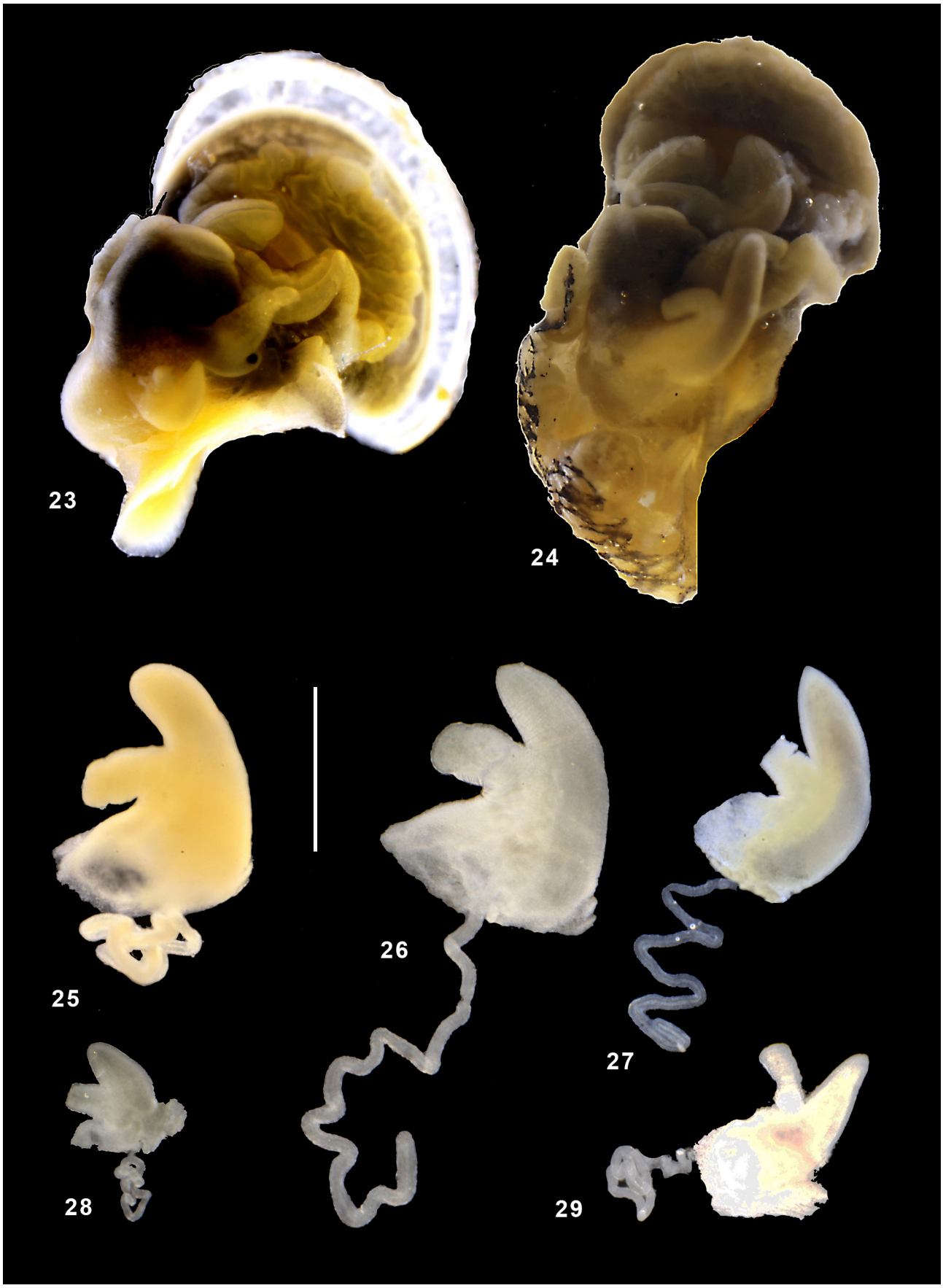
## RESULTS

### MORPHOLOGY

The shells of *Bithynia tentaculata* (Figs 3–6) and *B. cettinensis* (Figs 7–11) are slightly variable and similar: only smaller dimensions and somewhat lower spire distinguish *B. cettinensis*. The shells of *B. zeta* (Figs 12–18) are more variable, some of them with high spire (Fig. 15), some other resembling the ones of *B. tentaculata* (Figs 3–6).




Figs 16–22. *Bithynia zeta*: 16–18 – shell, bar equals 2 mm for shells; 19 – male head with penis; 20-22 – penis with tubular penial gland. Scale bar 1 mm for penes




Figs 23–29. *Bithynia cettinensis*: 23–24 – male head with penis; 25–29 – penis with tubular penial gland. Scale bar 1 mm for penes

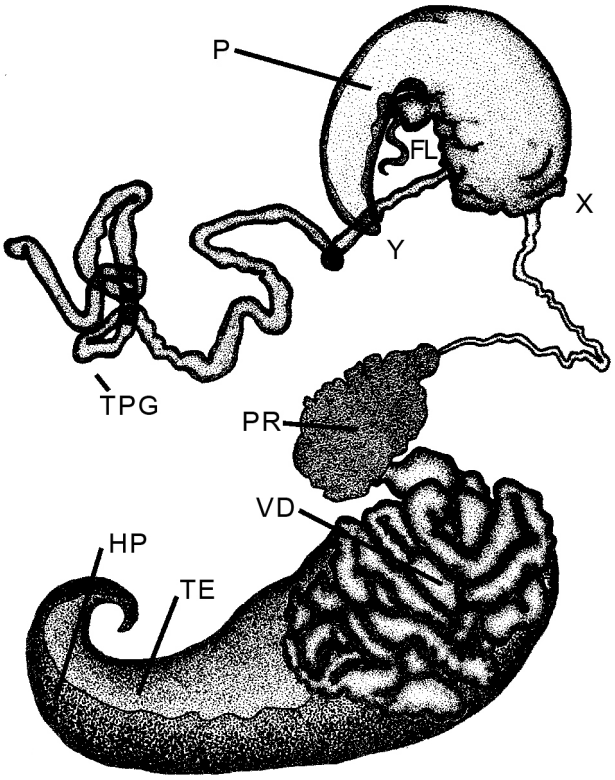



Table 2. Tubular penial gland: penis length ratio in *Bithynia cettinensis* and *Bithynia zeta*

| | *B. cettinensis* | *B. zeta* |
|---|---|---|
| AV | 1.33 | 1.63 |
| SD | 0.191 | 0.244 |
| MAX | 1.58 | 1.95 |
| MIN | 1.08 | 1.36 |
| n | 5 | 5 |

Fig. 30. Male reproductive organs of *Bithynia tentaculata* (FL – flagellum, left arm of penis harbouring outlet of tubular penial gland, HP – hepatopancreas, digestive gland; P – penis, right arm harbouring vas deferens; PR – prostate; TE – testis; TPG – tubular penial gland; VD – vas deferens; X, Y – points between which the length of right arm, along its right margin, is measured)

The penis is bifid in *B. zeta* (Figs 19–22), *B. cettinensis* (Figs 23–29) and *B. tentaculata* (Fig. 30). Its right arm includes the terminal section of vas deferens. The left arm is known as flagellum, containing the outlet of tubular penial gland situated apically. The penial gland is long and coiled, proximally extending into the cephalic haemocoel. The mean length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin (from point X to point Y in Fig. 30), is 1.63 for *B. zeta*, and 1.33 for *B. cettinensis* (Table 2).

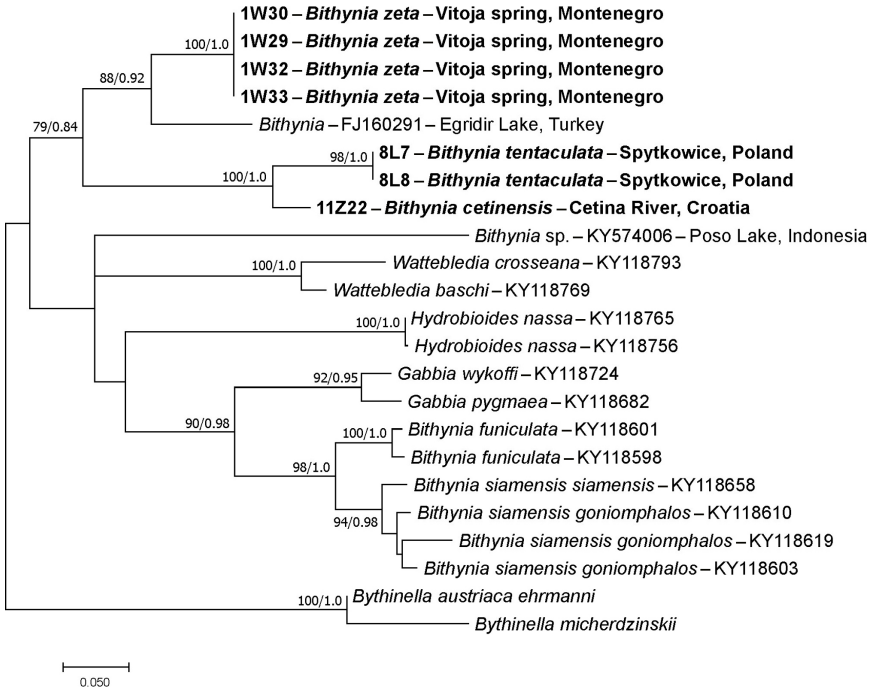



Fig. 31. Maximum likelihood tree based on cytochrome oxidase subunit I (COI); bootstrap supports and Bayesian probabilities given

## MOLECULAR ANALYSIS

In total we analysed seven new sequences of cytochrome oxidase subunit I (COI) (552 bp, GenBank Accession Numbers see Table 1). The tests of Xia et al. (2003) revealed no saturation. The topology of the trees obtained from BI and ML analyses was identical. The *Bithynia* sequences formed a distinct lineage with four clades (Fig. 31). *B. cettinensis* and *B. tentaculata* were sister clades with p-distance of 0.007. *B. zeta* was more distinct, with p-distance of 0.122 to *B. cettinensis* and 0.154 to *B. tentaculata*. The distances between the three studied species were not smaller than those between the other bithyniid species.

# DISCUSSION

The anatomy of the reproductive organs of *Bithynia* was described by Lilly (1953), Fretter & Graham (1962), Falniowski (1989, 1990), and Szarowska (2006). The female reproductive organs are characteristic of the Bithyniidae, but can hardly be used in species distinction (Falniowski 1990). However, in some publications (e.g. Falniowski 1989, 1990, Glöer & Pešić 2007, Glöer & Beran 2009) the term flagellum is used not for the left arm of the penis, but for the tubular penial gland.

Falniowski (1989, 1990) found that in the Polish *Bithynia* the length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin (from point X to point Y in Fig. 30), was the only anatomical character to allow for species discrimination. The value was 5.0–5.9 for *B. tentaculata* (Linnaeus, 1758), 2.5–3.25 for *B. leachi* (Sheppard, 1823), and ca. 4.3 for *B. troscheli* (Paasch, 1842). Later, this length ratio was considered in species delimitation in *Bithynia* (e.g. Glöer & Yildirim 2006, Glöer & Pešić 2007, Glöer & Rolán 2007, Glöer et al. 2009). For the *Bithynia* from Lake Skadar, the ratio was: 1.5 for *B. zeta*, 2 for *B. skadarskii*, and 4 for *B. radomani*. Considering the photographs in the present paper, as well as the drawings of Falniowski (1989, 1990), compared with the ones of Glöer & Yildirim (2006), Glöer & Pešić (2007), and Glöer & Rolán (2007), it is clearly visible that the shape and proportions of the penis are more variable, and interspecific differences less marked. The same concerns the tubular gland/penis proportion, less constant in our measurements, but still confirming the determination of our Vitoja spring material as *B. zeta*.

Molecularly *Bithynia zeta* is markedly distinct from the other studied species, and – especially compared with the other sequences of the Bithyniidae from the GenBank – the molecular data confirm its distinctness as a species. On the other hand, the differences between *B. cettinensis* and *B. tentaculata* are not necessarily large enough to confirm the distinctness of those two species. Only somewhat smaller distances can be seen within *B. siamensis* (Lea, 1856), but also between the two nominal species of *Gabbia* Tryon, 1856.

## ACKNOWLEDGMENTS

The study was supported by a grant from the National Science Centre 2017/25/B/NZ8/01372 to Andrzej Falniowski.

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*Received: March 8th, 2019*
*Revised: May 7th, 2019*
*Accepted: May 23rd, 2019*
*Published on-line: June 12th, 2019*