



LANZAIOPSIS BOLE, 1989 (CAENOGASTROPODA: TRUNCATELLOIDEA): ITS PHYLOGENETIC AND ZOOGEOGRAPHIC RELATIONSHIPS

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ABSTRACT: *Lanzaiopsis savinica* was collected from the type locality. The shell as well as the sculpture of the proto- and teleoconch are presented. Mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone 3 (H3) gene sequences were used to infer phylogeny. *Lanzaiopsis* clearly belongs to the Moitessieriidae but it is only distantly related to *Lanzaia*. Its sister clade is *Moitessieria*, while *Bythiospeum* is the sister clade of the one formed by *Lanzaiopsis* and *Moitessieria*. To our knowledge, *Lanzaiopsis* is the first representative of the moitessieriid non-Balkan clade whose range is so close to the Balkans.

KEY WORDS: genus distinctness, Moitessieriidae, COI, H3, Balkans, shell sculpture, molecular phylogeny

INTRODUCTION

Lanzaiopsis Bole, 1989 is a monotypic genus introduced for a species reported from Slovenia: *L. savinica* Bole, 1989. Earlier, BOLE & VELKOVHRH (1984) introduced *Lanzaia savinica* as nomen nudum. The type locality of *L. savinica* is: “Kraški izvir Pečovski studenec, 1.6 km severozahodno od Luč v Savinjski dolini (Savinjske Alpe)” [karstic spring Pečovski izvir north-west of Luče in the Savinja Valley] (Bole 1989: 67). Its empty shells were collected also in nearby springs: a permanent spring about 100 m southeast of the bridge across the river Savinja (on its left bank) and deposits of temporary springs located 100–200 m north of the bridge (also on the left bank), as well as sand deposits in the cave Zavratnikova zijalka, about 1 km north of Pečovski

studenec (BOLE 1989). The species was not found elsewhere (SLAPNIK 2011). As is the case with most stygobiotic snails, the number of empty shells found far exceeds the few finds of live specimens. In two summer seasons with extremely low water levels only three males and four females were found (BOLE 1989) alongside hundreds of empty shells. The species is included in the Hydrobiidae Troschel, 1857, Belgrandiellinae Radoman, 1983 (e.g. BOLE 1989, KABAT & HERSHLER 1993, BANK & NEUBERT 2017). According to BODON et al. (1996), *Lanzaiopsis* may become a junior synonym of *Lanzaia* Brusina, 1906. The aim of our paper was to establish phylogenetic relationships of *Lanzaiopsis*, applying molecular markers.

MATERIAL AND METHODS

Specimens of *Lanzaiopsis* were collected from the type locality: spring Pečovski izvir, Luče, Ljubno ob Savinji, Slovenia; 46°21'56"N, 14°43'55"E. Two live specimens and dozens of empty shells were found. The material was fixed in 96% ethanol changed twice, and later stored in 96% ethanol.

The shells were cleaned with an ultrasonic cleaner, and examined using a HITACHI S-4700 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

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 extracted DNA was stored at -80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland). Mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone 3 (H3) loci were sequenced. Details of PCR conditions, primers used and sequencing were given in SZAROWSKA et al. (2016).

Sequences were initially aligned in the MUSCLE (EDGAR 2004) program 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 Bayesian analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with the default priors. The Maximum Likelihood analysis was conducted in RAxML v. 8.2.12 (STAMATAKIS 2006) using the 'RAxML-HPC v.8 on XSEDE (8.2.12)' tool via the CIPRES Science Gateway (MILLER et al. 2010). We selected the GTRGAMMA nucleotide substitution model, empirical stationary base frequencies and proportion of invariant sites.

To apply the molecular clock, we used the data from COI. Sequences of two hydrobiids, *Peringia ulvae* Pennant, 1777 and *Salenthydrobia ferreri* Wilke, 2003 deposited in GenBank by WILKE (2003) as AF478401 and AF478410, respectively, were used as outgroups (tree not shown). The divergence time between these two species was used to calibrate the molecular clock, with correction according to FALNIOWSKI et al. (2008). The likelihoods for trees with and without the molecular clock assumption for a likelihood ratio test (LRT) (NEI & KUMAR 2000) were calculated with PAUP (SWOFFORD 2002). The relative rate test (RRT) (TAJIMA 1993) was performed in MEGA. As Tajima's RRTs and the LRT test rejected the equal evolutionary rate throughout the tree, time estimates were calculated using a non-parametric rate smoothing (NPRS) analysis with the recommended Powell algorithm, in r8s v.1.7 for Linux (SANDERSON 1997, 2003).

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

Species	Gen Bank numbers		References
	COI	H3	
<i>Agrafia wiktoria</i> Szarowska et Falniowski, 2011	JF906762		SZAROWSKA & FALNIOWSKI 2011
		MG543158	GREGO et al. 2017
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367650		WILKE et al. 2001
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616		SZAROWSKA 2006
<i>Avenionia brevis berenguieri</i> (Draparnaud, 1805)	AF367638		WILKE et al. 2001
<i>Belgrandiella kusceri</i> (Wagner, 1914)	KT218511		FALNIOWSKI & BERAN 2015
		MG551366	OSIKOWSKI et al. 2018
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643		WILKE et al. 2001
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858		FALNIOWSKI et al. 2012b
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854		FALNIOWSKI et al. 2012b
<i>Bythiospeum acicula</i> (Hartmann, 1821)	KU341350		RICHLING et al. 2016
		MK609534	FALNIOWSKI et al. 2019
<i>Bythiospeum alzense</i> Boeters, 2001	KU341354		RICHLING et al. 2016
<i>Dalmatinella fluvialis</i> Radoman, 1973	KC344541		FALNIOWSKI & SZAROWSKA 2013
<i>Daphniola louisii</i> Falniowski et Szarowska, 2000	KM887915		SZAROWSKA et al. 2014a
<i>Ecrobia maritima</i> (Milaschewitsch, 1916)	KJ406200		SZAROWSKA & FALNIOWSKI 2014b
		MG551322	GREGO et al. 2017
<i>Emmericia expansilabris</i> Bourguignat, 1880	KC810060		SZAROWSKA & FALNIOWSKI 2013a



Table 1. continued

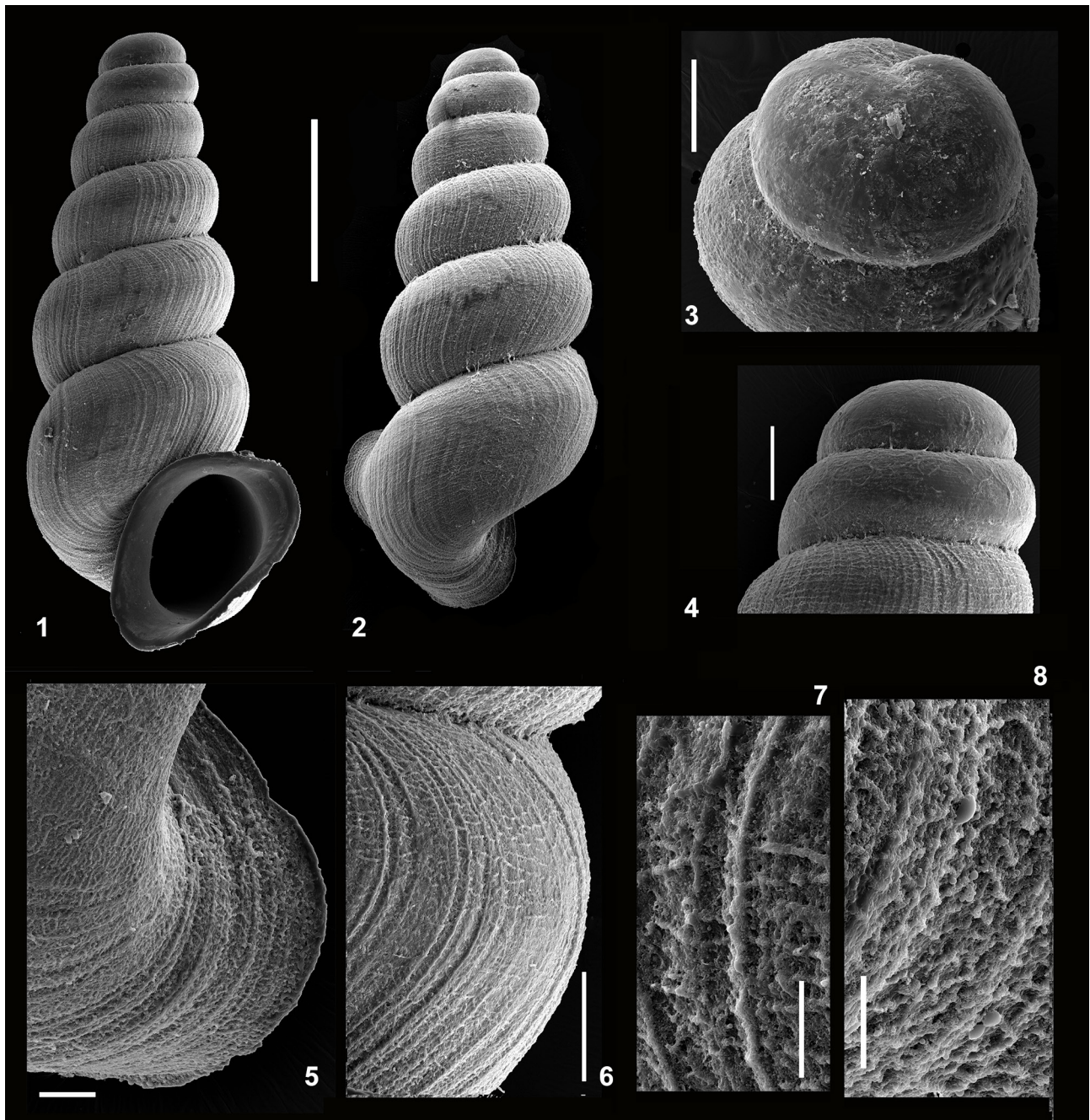
Species	Gen Bank numbers		References
	COI	H3	
<i>Fissuria boui</i> Boeters, 1981	AF367654		WILKE et al. 2001
<i>Graecoarganiella parnassiana</i> Falniowski et Szarowska, 2011	JN202352		FALNIOWSKI & SZAROWSKA 2011
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641		WILKE et al. 2001
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061919		SZAROWSKA et al. 2007
<i>Hauffenia tellinii</i> (Pollonera, 1898)	KY087861		RYSIEWSKA et al. 2017
<i>Hauffenia michleri</i> Kuščer, 1932	KY087865	KY087878	RYSIEWSKA et al. 2017
<i>Heleobia dalmatica</i> (Radoman, 1974)	AF129321		HERSHLER et al. 1999
<i>Heleobia dobrogica</i> (Grossu et Negrea, 1989)	EU938131		FALNIOWSKI et al. 2008
<i>Heleobia maltzani</i> (Westerlund, 1886)	KM213723		SZAROWSKA et al. 2014b
		MK629762	FALNIOWSKI et al. 2019
<i>Horatia klecakiana</i> Bourguignat 1887	KJ159128		SZAROWSKA & FALNIOWSKI 2014a
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808		WILKE et al. 2000
<i>Iglica</i> cf. <i>gracilis</i> (Clessin, 1882)	MH720985– MH720989	MH721002– MH721004	HOFMAN et al. 2018
<i>Iglica</i> cf. <i>hauffeni</i> (Brusina, 1886)		MH720995	HOFMAN et al. 2018
<i>Iglica</i> cf. <i>forumjuliana</i> (Pollonera, 1887)		MH721006	HOFMAN et al. 2018
<i>Iglica hellenica</i> Falniowski et Sarbu, 2015	KT825581		FALNIOWSKI & SARBU 2015
		MH721007	HOFMAN et al. 2018
<i>Islamia zermanica</i> (Radoman, 1973)	KU662362		BERAN et al. 2016
		MG551320	GREGO et al. 2017
<i>Lanzaia bosnica</i> Bole, 1970		MH721021	HOFMAN et al. 2018
<i>Lanzaiopsis savinica</i> Bole, 1989	MN272428– MN272429	MN272430– MN272431	This paper
<i>Lithoglyphus prasinus</i> (Küster, 1852)	JX073651		FALNIOWSKI & SZAROWSKA 2012
<i>Littorina littorea</i> (Linnaeus, 1758)	KF644330		LAYTON et al. 2014
		KP113574	NERETINA 2014, unpublished
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF322408		FALNIOWSKI & WILKE 2001
<i>Moitessieria</i> cf. <i>puteana</i> Coutagne, 1883	AF367635		WILKE et al. 2001
		MH721012	HOFMAN et al. 2018
<i>Montenegrospeum bogici</i> (Pešić et Glöer, 2012)	KM875510		FALNIOWSKI et al. 2014
		MG880218	GREGO et al. 2018
<i>Paladilhiopsis</i> cf. <i>absoloni</i> (A. J. Wagner, 1914)		MH721021	HOFMAN et al. 2018
<i>Paladilhiopsis blihensis</i> (Glöer et Grego, 2015)		MH721015	HOFMAN et al. 2018
<i>Paladilhiopsis bosniaca</i> (Clessin, 1910)		MH721020	HOFMAN et al. 2018
<i>Paladilhiopsis grobbeni</i> Kuščer, 1928	MH720991	MH721014	HOFMAN et al. 2018
<i>Paladilhiopsis turruta</i> (Kuščer, 1933)	MH720992	MH721015	HOFMAN et al. 2018
<i>Paladilhiopsis gittenbergeri</i> (A. Reischutz et P. L. Reischutz, 2008)	MH720993	MH721025	HOFMAN et al. 2018
<i>Paladilhiopsis maroskoi</i> (Glöer et Grego, 2015)		MH721017	HOFMAN et al. 2018
<i>Peringia ulvae</i> (Pennant, 1777)	AF118302		WILKE & DAVIS 2000
<i>Pontobelgrandiella</i> sp. Radoman, 1978	KU497024		RYSIEWSKA et al. 2016
		MG551321	GREGO et al. 2017
<i>Pseudamnicola</i> sp. Paulucci, 1878		KT710579	SZAROWSKA et al. 2016
<i>Radomaniola curta</i> (Küster, 1853)	KC011814		FALNIOWSKI et al. 2012a
<i>Sadleriana fluminensis</i> (Küster, 1853)	KF193067		SZAROWSKA & FALNIOWSKI 2013b
<i>Salenthydrobia ferrerii</i> Wilke, 2003	AF449213		WILKE 2003
<i>Tanousia zрманjae</i> (Brusina, 1866)	KU041812		BERAN et al. 2015



RESULTS AND DISCUSSION

The shell of *Lanzaiopsis savinica* (Figs 1–2) is turritiform, with characteristically shaped aperture surrounded by a broad, continuous and folded lip. The protoconch (Figs 3–4) is smooth, all the teleoconch covered with characteristic macrosculpture (Figs 5–6), composed of prominent radial growth lines, and spiral cords. This macrosculpture resembles the one visible in *Costellina turrita* Kuščer, 1933, *Lanzaia*

bosnica Bole, 1970, or *Paladilhiopsis bosniaca* (Clessin, 1910), but not the one characteristic of *Moitessieria* Bourguignat, 1863 (HOFMAN et al. 2018). The microsculpture of the teleoconch (Figs 7–8) is composed of irregularly distributed small pores, resembling the ones found in *Marstoniopsis* van Regteren Altena, 1936, and *Dianella* Gude, 1913 (FALNIOWSKI & SZAROWSKA 1995).



Figs 1–8: Shell of *Lanzaiopsis savinica*: 1–2 – shell habitus (scale bar 500 μm), 3–4 – protoconch (scale bars 100 μm), 5 – lip from the outer side (scale bar 50 μm), 6 – body whorl macrosculpture (scale bar 100 μm), 7–8 – body whorl microsculpture (scale bar 25 μm)



The anatomy of the genus *Lanzaia* was described by BOLE (1970) on the basis of *L. vjetrenicae* Kuščer, 1933 and *L. bosnica* Bole, 1970. *Lanzaioopsis* was distinguished from *Lanzaia* by several traits: in *Lanzaioopsis* the bursa copulatrix is proportionally bigger, the receptaculum (rs_1 , distal one) smaller, the accessory gland complex of the female reproductive organs divided in two sections, but not folded, and the penis triangular and relatively short (BOLE 1989). The shell of *Lanzaioopsis*, conical and elongated, with strongly expanded, peristome and very oblique aperture axis resembles the shell of *Lanzaia*, except for the less prominent radial sculpture and spiral microsculpture (BOLE 1989).

Our COI tree (Fig. 9) (GenBank Accession numbers MN272428–MN272429) clearly confirmed the

systematic position of *Lanzaioopsis* within the Moitessieriidae Bourguignat, 1863, not within the Hydrobiidae, Belgrandiellinae, as postulated by numerous authors (e.g. BOLE 1989, KABAT & HERSHLER 1993, BANK & NEUBERT 2017). The sister clade of *Lanzaioopsis* is *Moitessieria*. Both genera form a well supported (bootstrap support 75%, Bayesian probability 1.0) clade, close to *Bythiospeum* Bourguignat, 1882. The COI-based tree does not confirm the monophyly of the Moitessieriidae, with the Cochliopidae Tryon, 1866 falling between the two main moitessieriid clades (although bootstrap supports were low), as already reported by HOFMAN et al. (2018) and FALNIOWSKI et al. (2019). The inclusion of the nuclear histone H3 sequences (Fig. 10) (GenBank Accession numbers MN272430–MN272431), how-

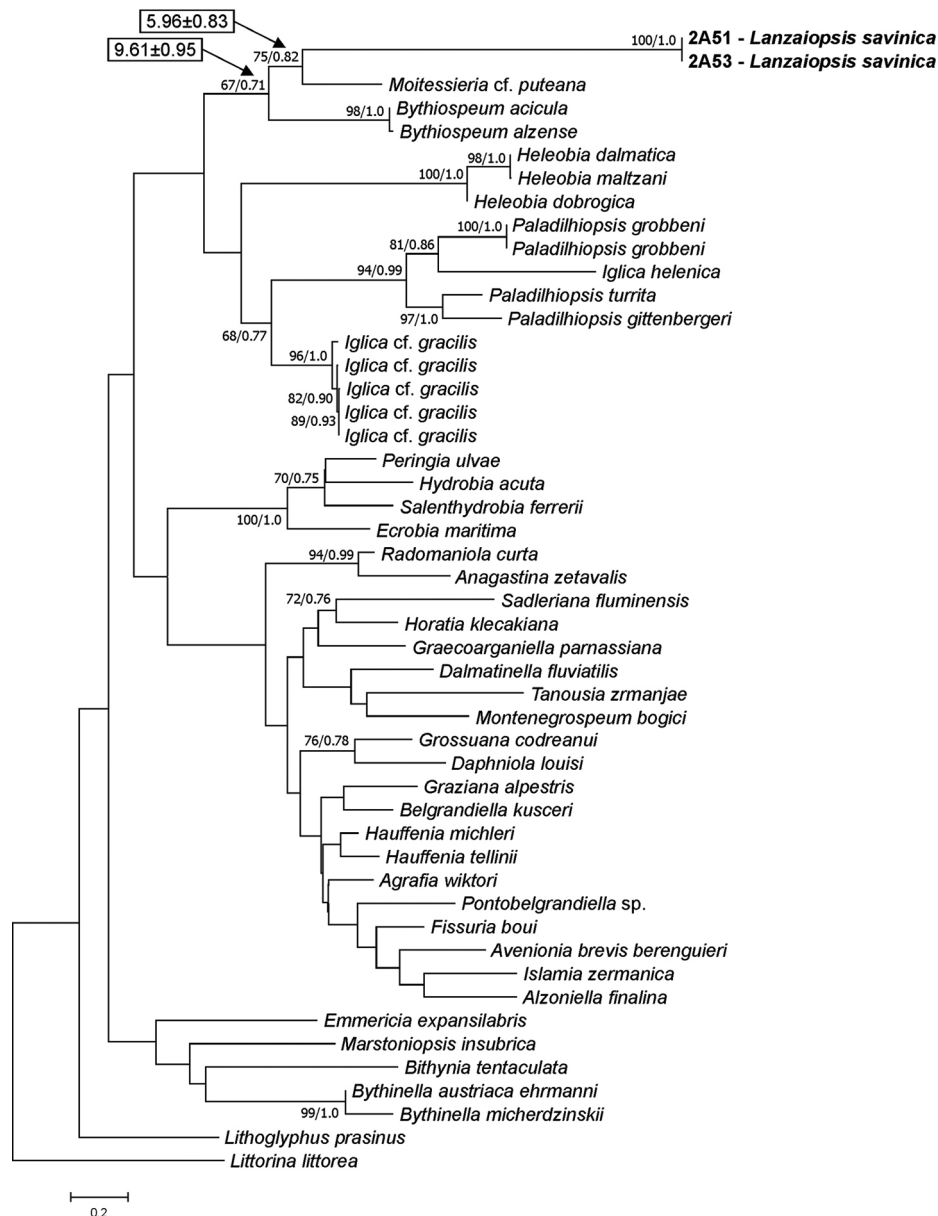


Fig. 9. Maximum Likelihood tree inferred from mitochondrial cytochrome oxidase subunit I (COI). Bootstrap supports and Bayesian probabilities given



ever, resulted in confirmation of the moitessieriid monophyly in both the H3 tree (bootstrap support 75%, Fig. 10) and the tree computed for the two concatenated sequences (bootstrap support 75%, Fig. 11), similarly as demonstrated in FALNIOWSKI et al. (2019). In all three trees, the sister clade of *Lanzaiopsis* is *Moitessieria*, with p-distances 0.225 for COI and 0.065 for H3, which are typical of the truncatelloid genus-level. The p-distance in H3 between

Lanzaiopsis and *Lanzaia* (represented in our phylogeny as *Paladilhiopsis (Lanzaia) bosnica*) is 0.106, which refutes the suggestion of BODON et al. (1996) that both taxa represent the same genus.

The reconstructed phylogeny presents also an interesting zoogeographic aspect. Within the European Moitessieriidae, two markedly distinct clades could be distinguished: the one consisting of *Bythiospeum* and *Moitessieria*, and the second including *Iglica* Wagner,

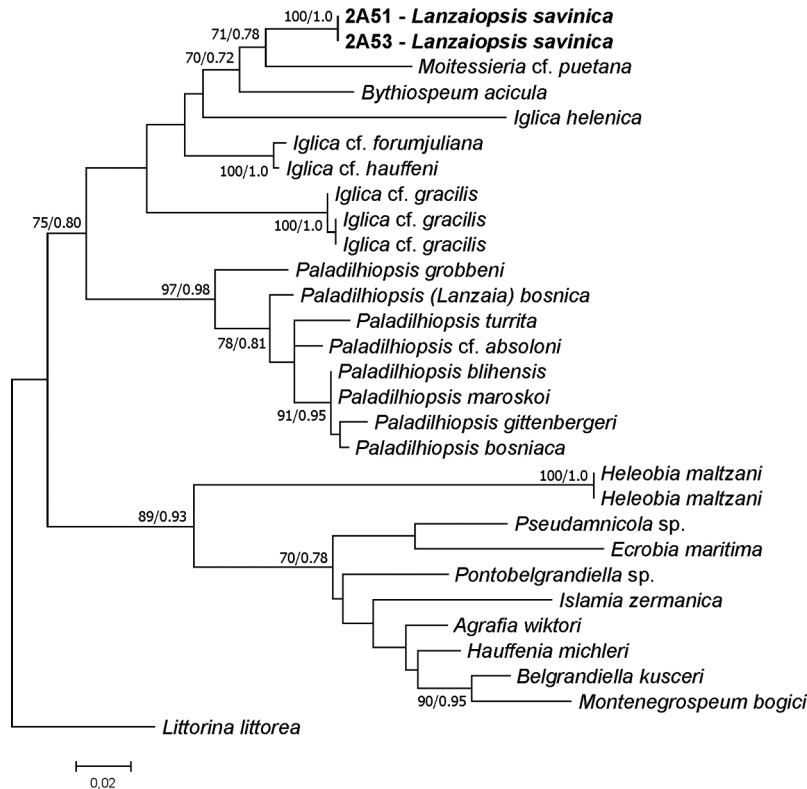


Fig. 10. Maximum Likelihood tree inferred from nuclear histone H3. Bootstrap supports and Bayesian probabilities given (if above 70%/0.7)

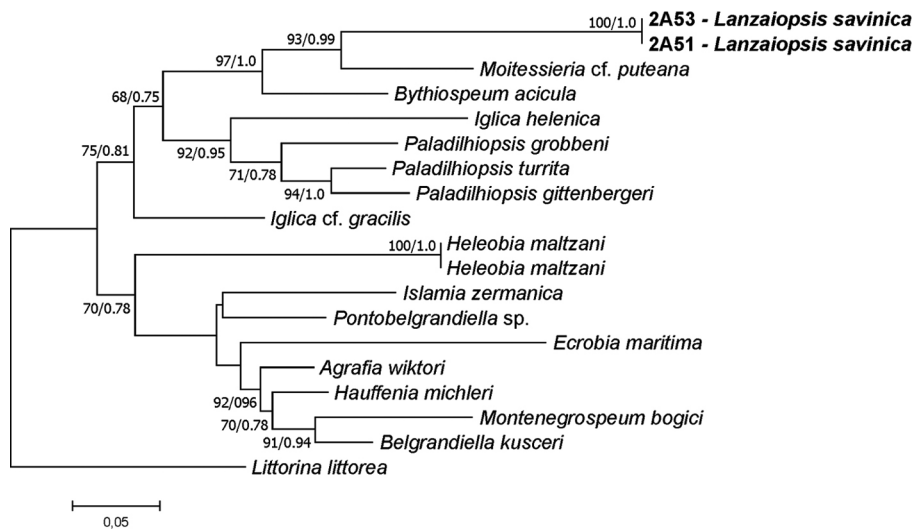


Fig. 11. Maximum Likelihood tree inferred from concatenated COI and H3 sequence. Bootstrap supports and Bayesian probabilities given (if above 70%/0.7)



1927, *Paladilhiopsis* Pavlovic, 1913 and *Lanzaia*. While representatives of the former clade were found only in subterranean habitats in central and southern Europe (Germany, Switzerland, Austria, Italy, France) so far, i.e. outside the Balkans, the latter inhabit the Balkans, but also Hungary and Romania. The single known locality of *Lanzaiaopsis*, although outside the Balkans, is within the range of this second, Balkan clade, and geographically is close to the Balkans. On the other hand, its locality belongs to a young Alpine arch named Southern Calcareous Alps, not to the Dinarides.

Despite the sister-clade relationships between *Lanzaiaopsis* and *Moitessieria*, the divergence between those two taxa took place rather long time ago. The application of molecular clock resulted in estima-

tion of the time of divergence as 5.96 ± 0.83 Mya (Fig. 11). This is the latest Miocene, around the time of the Messinian Salinity Crisis. The closure of the present Strait of Gibraltar resulted in desiccation of the Mediterranean Sea, thus the climate became drier and hotter (HSÜ 1983, KRIJGSMAN et al. 1999, GARCIA-CASTELLANOS et al. 2009). This probably resulted in extinction of the cold water fauna within a large area, possibly isolating the ancestors of the two genera in distant refuges.

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