



TANOUSIA ZRMANJAE (BRUSINA, 1866) (CAENOGASTROPODA: TRUNCATELLOIDEA: HYDROBIIDAE): A LIVING FOSSIL

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ABSTRACT: A living population of *Tanousia zrmanjae* (Brusina, 1866) was found in the mid section of the Zrmanja River in Croatia. The species, found only in the freshwater part of the river, had been regarded as possibly extinct. A few collected specimens were used for this study. Morphological data confirm the previous descriptions and drawings while molecular data place *Tanousia* within the family Hydrobiidae, subfamily Sadlerianinae Szarowska, 2006. Two different sister-clade relationships were inferred from two molecular markers. Fossil *Tanousia*, represented probably by several species, are known from interglacial deposits of the late Early Pleistocene to the early Middle Pleistocene of several European countries. Thus *T. zrmanjae* may be regarded as a living fossil restricted to the short section of the Zrmanja River.

KEY WORDS: protoconch, radula, reproductive organs, DNA, *COI*, *18S*, phylogeny, Pleistocene

INTRODUCTION

The Zrmanja River (Figs 1–2) in Croatia, 69 km long, with the catchment area of 907 km², is one of the European hot spots of freshwater gastropod diversity. It is inhabited by 16 species, five of them endemic (STRONG et al. 2008), or – according to BERAN (2011) – even 22 species. Truncatelloidea are represented by three species found in the brackish section below the Jankovica Buk waterfalls, and by eight species in the freshwater part above Jankovica Buk. Four of the freshwater species: *Belgrandiella krupensis* Radoman, 1973, *B. zermanica* Radoman, 1973, *Islamia zermanica* Radoman, 1973, and *Tanousia zrmanjae* (Brusina, 1866) (= *Lithoglyphulus tedanicus* Schlickum et Schütt, 1971), were described from this part including its tributary, the Krupa River (RADOMAN 1985). However, the species distinctness of *Belgrandiella krupensis* and *B. zermanica* is doubtful (FALNIOWSKI & BERAN 2015).

BRUSINA (1866) described *Limnaea zrmanjae*, later included in the Hydrobiidae, and the genus *Tanousia* Bourguignat (in SERVAIN 1881, see: KABAT & HERSHLER 1993). In the same paper she described the genus *Sandria*, a junior objective synonym of *Tanousia* (KABAT & HERSHLER 1993). Later, SCHLICKUM & SCHÜTT (1971) described *Lithoglyphulus tedanicus*, identical with *Tanousia zrmanjae*, thus creating a junior synonym. RADOMAN (1973, 1983) described and illustrated the radula, penis and female reproductive organs of this species, classifying it, with reservations, into a distinct family Lithoglyphulidae.

According to the IUCN Red List, *T. zrmanjae* is Critically Endangered (Possibly Extinct), based on the habitat loss in the type locality (FALNIOWSKI 2011). It occurs exclusively in the freshwater parts of the Zrmanja River. The river was dammed in its lower part (which divides the site with *T. zrmanjae*)



Figs 1–2. Zrmanja River at its mid freshwater section

and in its upper course; due to its karstic character, during dry periods the river represents a system of stagnant pools (like that at Kaštel Zegarski) interspersed with dry river bed (like that at Ervenik): only few gastropod species live in its upper part. A small living population was found in the mid, freshwater

section of the river (BERAN 2011) and later also at a locality upstream of this site. The aim of the present paper was to check the morphological characters of the species and, applying molecular markers, to resolve its phylogenetic relationships.

MATERIAL AND METHODS

A few specimens of *Tanousia zrmanjae* were collected at Dramotići, Zrmanja River, Croatia at 44°11'31.6"N, 15°47'05"E on 29.06.2014. The snails were collected from the sediment using a metal sieve. Individuals for molecular analyses were washed in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice during 24 hours and, after a few days, 80% ethanol was replaced with 96% ethanol. The samples were then stored at –20°C prior to DNA extraction.

The snails were dissected using a NIKON SMZ18 stereo-microscope with dark field and phase contrast, and shells and penes photographed with CANON EOS 50D digital camera. Protoconchs and radulae were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

DNA was extracted from foot tissue of each snail. The tissue was hydrated in TE buffer (3 × 10 min.); 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'-GGTCAACAAATCATAAAG ATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (*COI*) mitochondrial gene and SWAM18SF1 (5'-GAATGGCTCATTAATCAGTCGAGGTTCT TAGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTAAAGTGTACTCAT TCCAATTACGGAGC-3') for the 18S ribosomal

DNA (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 a final extension step of 4 min at 72°C. 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 a 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 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.

Sequences were initially aligned with MUSCLE (EDGAR 2004) in MEGA 6 (TAMURA et al. 2013) and then checked manually in Bioedit 7.1.3.0 (HALL 1999). The saturation test of XIA et al. (2003) was performed using DAMBE (XIA 2013) and revealed no saturation in any of the sequences studied. The sequences obtained from *Tanousia* specimens in the present work were used in the phylogenetic analysis with other sequences obtained from GenBank (Table 1). A maximum likelihood (ML) approach was conducted in RAxML v8.0.24 (STAMATAKIS 2014). One thousand searches were initiated with starting

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

Species	18S GB#	COI GB#	References
<i>Agrafia wiktorei</i> Szarowska et Falniowski, 2011	JF906758	JF906762	SZAROWSKA & FALNIOWSKI (2011)
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070622	EF070616	SZAROWSKA (2006)
<i>Avenionia brevis</i> (Draparnaud, 1805)	AF367670	AF367638	WILKE et al. (2001)
<i>Belgrandiella kusceri</i> (Wagner, 1914)	JX970574	JX970610	WILKE et al. (2013)
<i>Dalmatinella fluviatilis</i> Radoman, 1973	KC344539	KC344541	FALNIOWSKI & SZAROWSKA (2013)
<i>Daphniola graeca</i> Radoman, 1973	EF070624	EF070618	SZAROWSKA (2006)
<i>Fissuria boui</i> Boeters, 1981	AF367690	AF367654	WILKE et al. (2001)
<i>Graecoarganiella parnassiana</i> Falniowski et Szarowska, 2011	JN202341	JN202348	FALNIOWSKI & SZAROWSKA (2011)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061916	EF061919	SZAROWSKA et al. (2007)
<i>Hauffenia tellinii</i> (Pollonera, 1898)	AF367672	AF367640	WILKE et al. (2001)
<i>Horatia klecakiana</i> Bourguignat, 1887	KJ159127	KJ159128	SZAROWSKA & FALNIOWSKI (2014)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
<i>Montenegrospeum bogici</i> (Pešić et Glöer, 2012)	KM875509	KM875510	FALNIOWSKI et al. (2014)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
<i>Tanousia zrmanjæ</i> (Brusina, 1866)	KU041813	KU041811	present study
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070630	EF070619	SZAROWSKA (2006)
<i>Truncatella scalaris</i> (Michaud, 1830)	JX970596	JX970621	WILKE et al. (2013)

trees obtained through randomised stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of the phylogeny. Bootstrap sup-

port was calculated with 1,000 replicates and summarised on the best ML tree. RAxML analyses were performed using free computational resources of the CIPRES Science Gateway (MILLER et al. 2010).

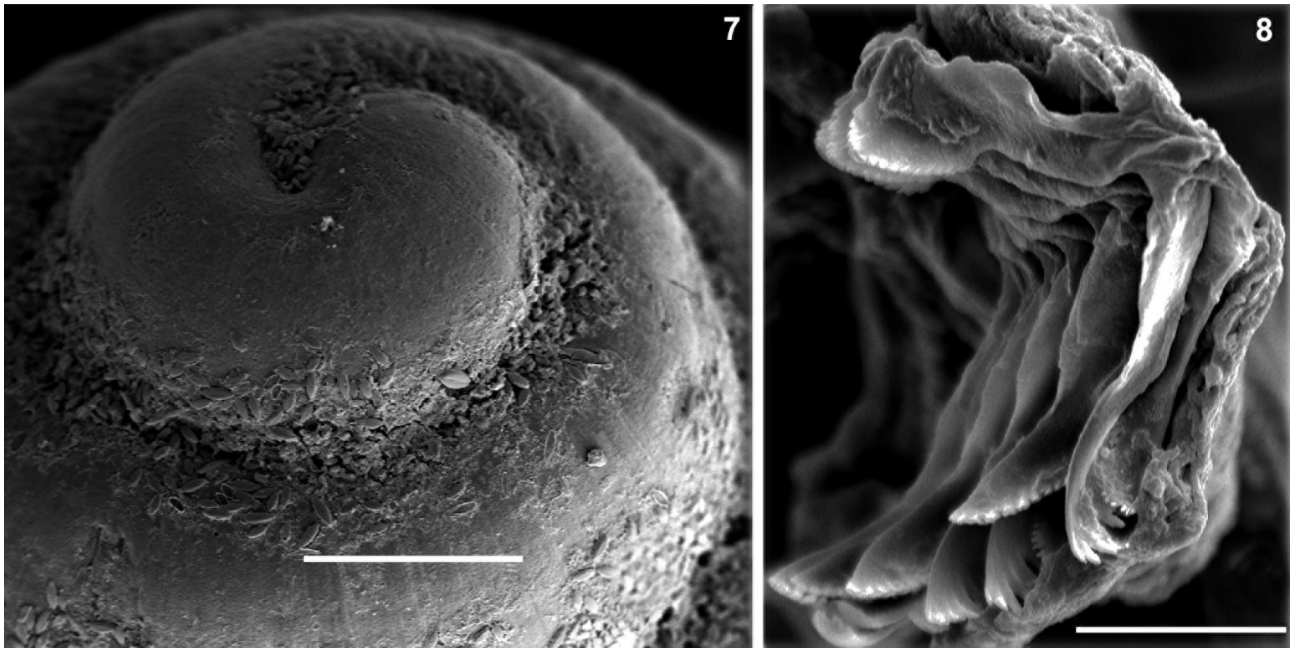
RESULTS

MORPHOLOGY

The shell (Figs 3–6) is characteristic, nearly neritiform, although with relatively high spire, unusually thick-walled (for a hydrobiid), light cream-coloured, slightly variable. The protoconch (Fig. 7) is smooth,

without any sculpture even under high magnification. The radula is similar to the one illustrated by RADOMAN (1983), with a very small central tooth lacking basal cusps, and lateral teeth (Fig. 8) with unusually massive bases (nomenclature after HERSHLER & PONDER 1998). The female reproductive organs

Figs 3–6. Shells of *Tanousia zrmanjæ*; scale bar 1 mm



Figs 7–8. *Tanousia zrmanjiae*: 7 – protoconch (scale bar 100 μm), 8 – lateral and marginal radular teeth (scale bar 20 μm)

(Fig. 9) are similar to those presented by RADOMAN (1983), with two proportionally large seminal receptacles (rs_1 and rs_2 per RADOMAN 1973, 1983), rs_2 much larger than rs_1 , and a moderately large bursa copulatrix. The penis is relatively large and massive (Fig. 10), simple, without any outgrowths, terminally with a long filament (Fig. 11), similar to the one drawn by RADOMAN (1983).

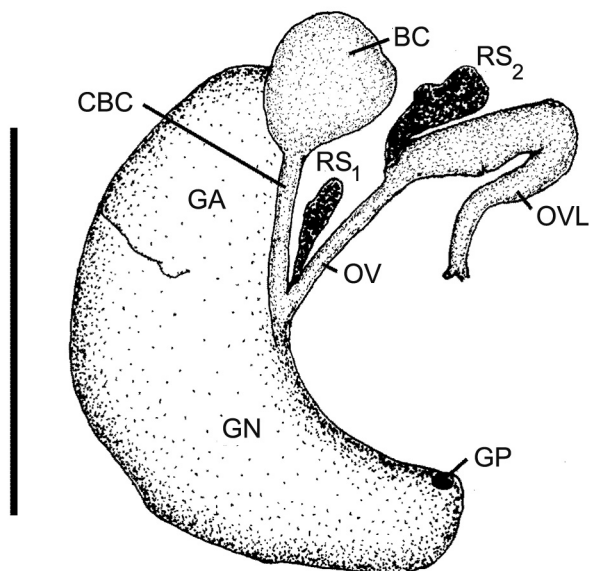
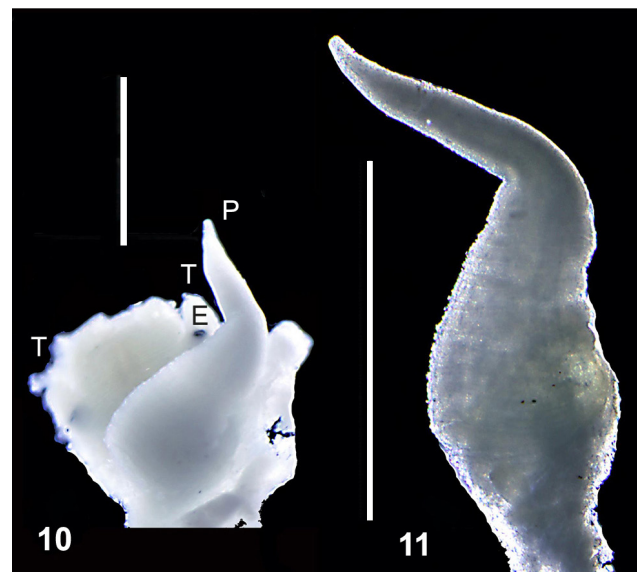


Fig. 9. Renal and pallial section of female reproductive organs of *Tanousia zrmanjiae* (BC – bursa copulatrix, CBC – duct of bursa copulatrix, GA – albumen gland, GN – nidamental gland, GP – gonoporus, OV – oviduct, OVL – loop of (renal) oviduct, RS_1 , RS_2 – receptaculum seminis, symbols after RADOMAN 1973, 1983; scale bar 1 mm)

MOLECULAR PHYLOGENY

We obtained for *Tanousia zrmanjiae* two sequences for the COI gene (552 bp, GenBank Accession numbers KU041811–KU041812) and two for the 18S (414 bp, GenBank Accession numbers KU041813–KU041814).

In the COI analyses (197 polymorphic sites) (Fig. 12) our two identical sequences of *Tanousia* were sister to *Dalmatinella fluviatilis* and *Montenegrospeum bogici*, with p-distances of 0.158 and 0.160, respectively (bootstrap support 79%). The divergence level



Figs 10–11. *Tanousia zrmanjiae*: 10 – head with penis (E – eye, P – penis, T – cephalic tentacle), 11 – penis; scale bars 1 mm

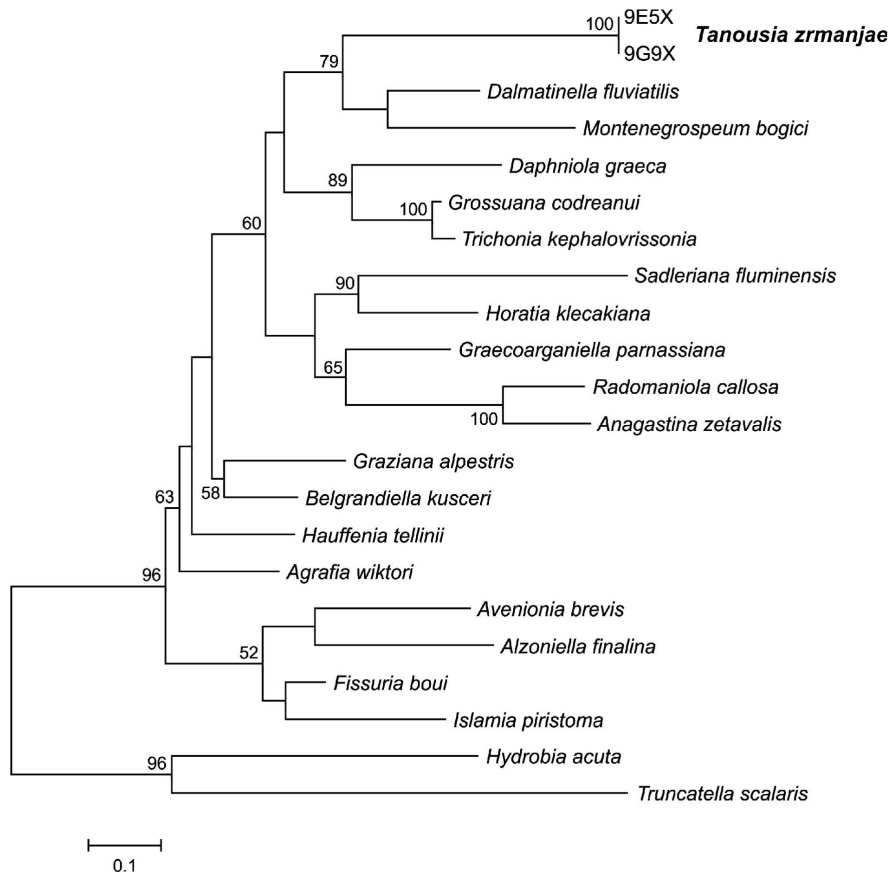


Fig. 12. Maximum likelihood tree of COI sequences, 10,000 replicates; bootstrap supports given if >50%



Fig. 13. Maximum likelihood tree of 18S with 10,000 replicates; bootstrap supports given if >50%

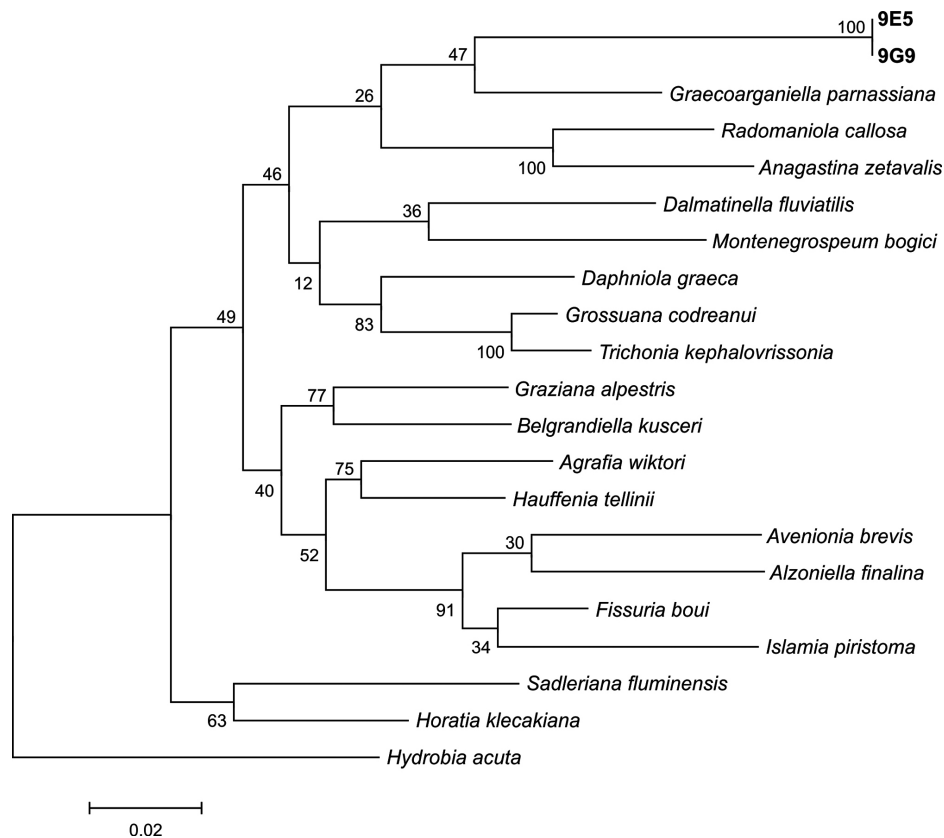


Fig. 14. Maximum likelihood tree of the two concatenated sequences (18S and COI), bootstrap supports (10,000 replicates)

between *Tanousia* and the other Sadlerianinae species varied from 16% (for *Dalmatinella fluviatilis*) to 19.3% (for *Sadleriana fluminensis*) and exceeded 20% for the outgroups.

In 18S analyses (32 polymorphic sites) two sequences of *Tanousia* were also identical (Fig. 13) and sister to *Graecoarganiella parnassiana* (with p-distance 0.019, bootstrap support 86%). The ML tree for combined COI and 18S was characterised by very

low bootstrap supports, and thus numerous polytomies (Fig. 14). This confirmed that the histories of the two genes were different. Despite different sister-species relationships of *Tanousia*, both studied loci clearly confirm that the genus belongs to the Hydrobiidae Troschel, 1857, subfamily Sadlerianinae Szarowska, 2006. Thus, the monogeneric family Lithoglyphulidae Radoman, 1973 should be rejected.

DISCUSSION

Our morphological data are compatible with the descriptions and illustrations of RADOMAN (1973, 1983). According to RADOMAN (1973, 1983) *Tanousia* belong to a distinct family Lithoglyphulidae, although in his opinion the relationships of the genus remained unclear. Morphological data, however, suggested a rather close relationship with *Dalmatinella* Radoman, 1973. Our molecular data without doubt placed *Tanousia* within the family Hydrobiidae, subfamily Sadlerianinae Szarowska, 2006. Thus, the monogeneric family Lithoglyphulidae Radoman, 1973 is no longer valid.

Unfortunately, the two molecular markers indicate two different sister-clade relationships (Figs 12, 13), each well supported. This is not unusual, since the evolution of different genes – especially a mitochon-

drial vs. a nuclear gene – need neither be identical, nor reflect the evolution of the organism (e.g. AVISE 2000, NEI & KUMAR 2000). Considering the level of distinctness (p-distances) for each gene, long-branch-attraction may be involved as well. In 18S the alignment is not certain. The tree inferred with COI (Fig. 12), with *Dalmatinella*/*Montenegrospeum* as the sister clade, is much more congruent with the morphological data.

According to WILKE (2003), an estimate of $1.83 \pm 0.21\%$ population divergence per million years could be applied for COI in the Hydrobiidae, as long as the data are ultrametric. With all the necessary reservations, the estimated time of divergence between *Tanousia* and its sister taxa is about 9 Mya, thus Tortonian in the Miocene, although the beginning of the Messinian is also within the estimated



range. This illustrates the level of longevity of this distinct lineage. It should be noted, however, that the presented tree is not ultrametric, and that different methods of relaxation of the ultrametricity resulted in estimates of about 2 or 18 Mya, respectively.

In the truncatelloidean gastropods, the shell is usually not very useful for genus determination. However, because the shell of *Tanousia* is rather characteristic, the fossil record could be considered in this case. Fossil *Tanousia*, represented by several species, are known from Denmark (MADSEN & NORDMANN 1901), England (SCHLICKUM 1974, PREECE 1990, 2001), the Netherlands (MEIJER 1989, GITTENBERGER et al. 1998, GLÖER 2002), France (SCHLICKUM 1974), Hungary (ROTH 1881, BRUSINA 1902), Ukraine (ANDRUSOV 1890, KONDRASHOV 2007), Romania (HERBICH & NEUMAYR 1875, JEKELIUS 1932, 1944), Italy (ESU & KOTSAKIS 1996, ESU & GIANOLLA 2007, ESU 2008), and Greece (SCHÜTT 1976). The

stratigraphic range of the genus is the late Early Pleistocene to the early Middle Pleistocene, thus till about 0.7 Mya, all the records coming from interglacial deposits (references cited above), and from lowland river deposits. Thus, the only extant species – *T. zrmanjæ* – may be regarded as a living fossil, and its present restricted distribution (a short section of one river) most probably reflects the existence of a glacial refugium at this place. However, there are no data on the real existence and size of the refugium, most probably not restricted to this short section of the Zrmanja River, and transport (by birds?) of *Tanousia* from another area cannot be excluded.

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