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VINODOLIA FIUMANA RADOMAN, 1973 (CAENOGASTROPODA: RISSOOIDEA): REDISCOVERY AND RELATIONSHIPS OF A SPECIES PRESUMED EXTINCT

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ABSTRACT: *Vinodolia fiumana* was described by Radoman from Glogi spring in 1973, and later not found, thus considered extinct. In the CLECOM list *Anagastina*, *Dalmatinella* and *Prespiana* were classified, as subgenera, within the genus *Vinodolia*. After visiting the Glogi spring on several occasions, we found four specimens of *V. fiumana*. Its shell, soft part external morphology, radula, penis and female reproductive organs are described. Partial cytochrome oxidase subunit I mitochondrial and 18S rRNA nuclear gene sequences were used to infer phylogenetic relationships of *Vinodolia*. *Sadleriana* was found to be the sister taxon of *Vinodolia*, but neither *Anagastina* (closely related with *Radomaniola*), nor *Dalmatinella* were closely related with it.

KEY WORDS: Rissooidea, COI, 18S rRNA, radula, anatomy, extinction, phylogeny

INTRODUCTION

The monotypic genus *Vinodolia*, with the type species *V. fiumana* Radoman, 1973, was described by RADOMAN (1973) from the Glogi spring at Bribir, northern Croatia. He reported this species also from the Drišt spring, close to Glogi and from the spring in the Javor village, N of Martinščica, E of Rijeka (RADOMAN 1973, 1974, 1983). To our knowledge, nobody has found this species since, and RÉGNIER et al. (2009) listed it among extinct species. Despite very fragmentary information and without any explanation, *Anagastina* Radoman, 1978, *Dalmatinella* Radoman, 1973, and *Prespiana* Radoman, 1973, are considered subgenera of *Vinodolia* in both the CLECOM list (FALKNER et al. 2001), and in Fauna Europaea (BANK 2012).

We visited the Glogi spring a few times, from 1999 to 2004, only to find the spring almost dry, and nothing more than a few corroded empty shells of an unidentified rissooid snail in the bottom sediments. Finally, in June of 2011, we found four specimens of *V. fiumana*. The aim of the present study was to check the morphology of the species, and to infer phylogenetic relationships with molecular data.

MATERIAL AND METHODS

Four specimens and a few corroded empty shells of *Vinodolia fiumana* were collected, using a sieve (two meshes per mm), from Glogi Spring at Bribir (Fig. 1): 45°10'02.1"N, 14°44'25.6"E, 154 m a.s.l., on June 15th, 2011. There was no outflow outside the spring, only a little water inside the small artificial pool.

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,

Fig. 1. Glogi Spring at Bribir, type locality of *Vinodolia fiumana*

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. One adult male and one female were dissected, using a NIKON SMZ-U stereoscope microscope. The penis and female genitalia (pallial oviduct) were examined using a MOTIC light microscope. The radula of the female was examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

DNA was extracted from foot tissue of the fourth specimen not presented in the photograph. 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'-TAAACTTCA GGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene and SWAM18SF1 (5'-GAATGGCTCAT TAAATCAGTCGAGGTTCCTTAGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCCTCGTTAAAGGGT TTAAAGTGTACTCATTCCAATTACGGAGC-3') for the 18S rRNA gene (PALUMBI 1996). 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 (HILLIS et al. 1996) 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 two sequences were deposited in GenBank (Table 1).

In the phylogeny reconstruction, we used 28 rissooid taxa sequences from GenBank (Table 1). Seven of them, used as an outgroup, represented the

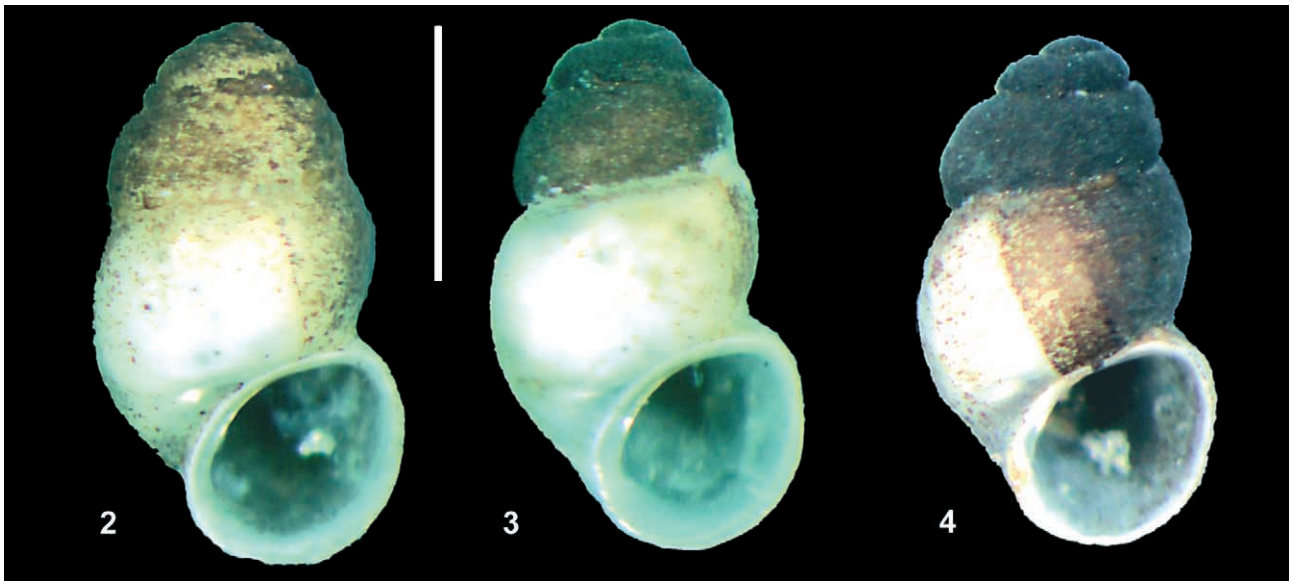
Table 1. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references

Species	18S GB#	COI GB#	References
Adriohydrobia gagatinella (Küster, 1852)	AF367657	AF317881	WILKE & FALNIOWSKI (2001)
Adrioinsulana conovula (Frauenfeld, 1863)	AF367656	AF367628	WILKE et al. (2001)
Agrafia wiktori Szarowska et Falniowski, 2011	JF906758	JF906762	SZAROWSKA & FALNIOWSKI (2011a)
Alzoniella finalina Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
Anagastina zetavalis (Radoman, 1973)	EF070622	EF070616	SZAROWSKA (2006)
Bithynia tentaculata (Linnaeus, 1758)	AF367675	AF367643	WILKE et al. (2001)
Boleana umbilicata (Kuščer, 1932)	JX982797	JX982795	FALNIOWSKI & SZAROWSKA (2012)
Bythinella austriaca (Frauenfeld, 1857)	AF212917	FJ545132	FALNIOWSKI et al. (2009)
Bythiospeum sp.	AF367664	AF367634	WILKE et al. (2001)
Dalmatinella fluviatilis Radoman, 1973	KC344539	KC344541	FALNIOWSKI & SZAROWSKA (2013)
Daphniola graeca Radoman, 1973	EF070624	EF070618	SZAROWSKA (2006)
Dianella thiesseana (Kobelt, 1878)	AY676125	AY676127	SZAROWSKA et al. (2005)
Graecoarganiella parnassiana Falniowski et Szarowska, 2011	JN202341	JN202348	FALNIOWSKI & SZAROWSKA (2011)
Graziana alpestris (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
Grossuana codreanui (Grossu, 1946)	EF061916	EF061919	SZAROWSKA et al. (2007)
Hauffenia tellinii (Pollonera, 1898)	AF367672	AF367640	WILKE et al. (2001)
Heleobia dalmatica (Radoman, 1974)	AF367661	AF367631	WILKE et al. (2001)
Hydrobia acuta (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
Islamia piristoma Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
Lithoglyphus naticoides (C. Pfeiffer, 1828)	AF367674	AF367642	WILKE et al. (2001)
Marstoniopsis insubrica (Küster, 1853)	AF367676	AY027813	FALNIOWSKI & WILKE (2001)
Pseudamnicola lucensis (Issel, 1866)	AF367687	AF367651	WILKE et al. (2001)
Pyrgula annulata (Linnaeus, 1767)	AY676124	AY341258	SZAROWSKA et al. (2005)
Radomaniola callosa (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
Rissoa labiosa (Montagu, 1803)	AY676126	AY676128	SZAROWSKA et al. (2005)
Sadleriana fluminensis (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
Trichonia kephalovrissonia Radoman, 1973	EF070630	EF070619	SZAROWSKA (2006)
Ventrosia ventrosa (Montagu, 1803)	AF367681	AF118335	WILKE & DAVIS (2000)
Vinodolia fiumana Radoman, 1973	KF359899	KF359900	present study

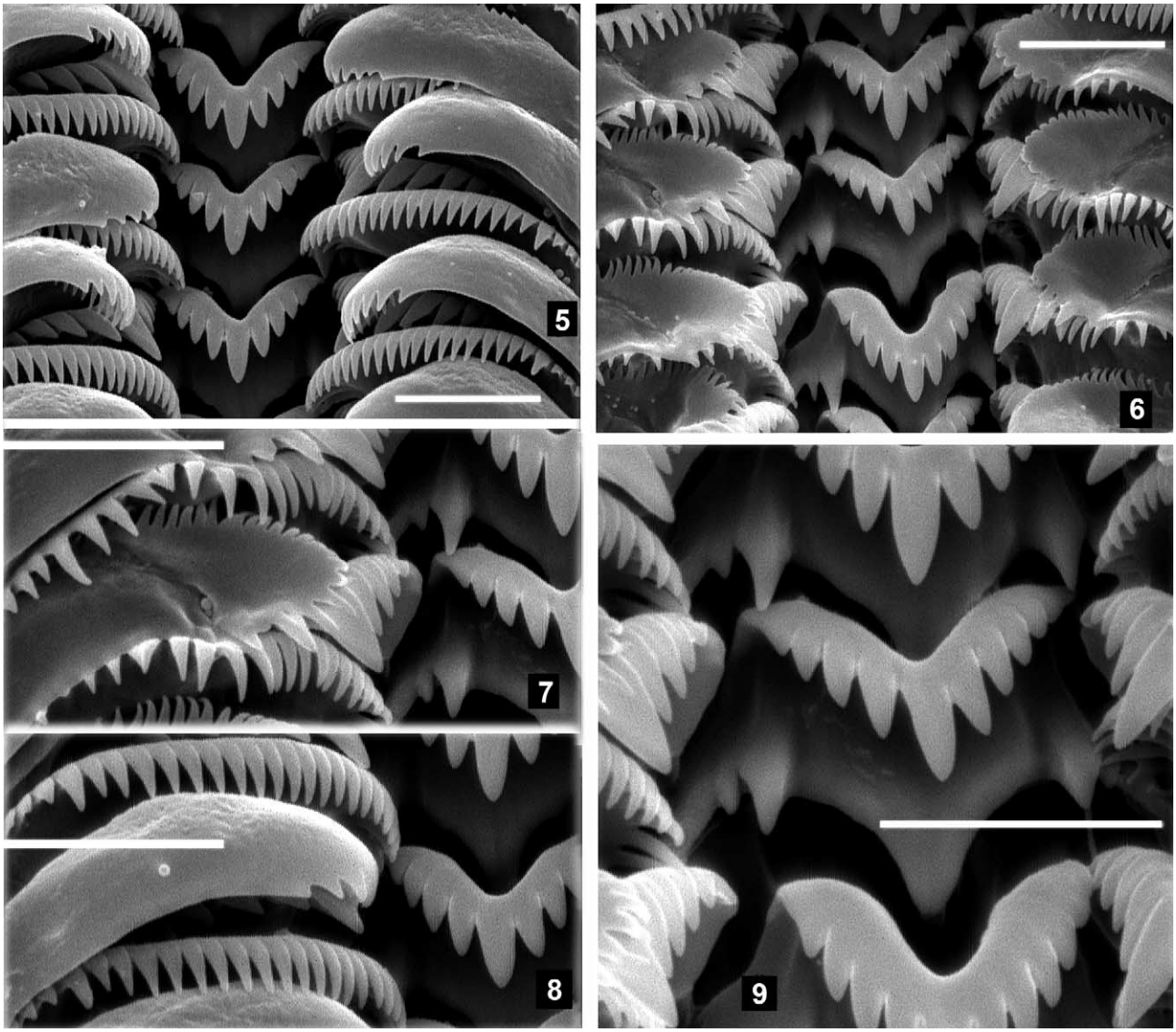
main non-hydrobiid lineages within the Rissooidea (WILKE et al. 2001); the other seven taxa represented the Hydrobiinae (including "Pyrgulinae": SZAROWSKA et al. 2005). The remaining taxa were chosen to represent all the main lineages within the European Sadlerianinae (SZAROWSKA 2006).

The COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999) and edited with MACCLADE 4.05 (MADDISON & MADDISON 2002). For 18S, an initial alignment was performed using CLUSTALX 1.82 (THOMPSON et al. 1997) and edited with MACCLADE. 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). It revealed a significant degree of saturation in the third position of the sequences. In 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 and it was used for the analysis. In fact, the analysis carried out on the 2nd and 3rd position only resulted in similar deep phylogeny, but with several polytomies within more terminal nodes.

Initially, we performed phylogeny reconstruction for 18S and COI data separately, using the maximum likelihood (ML) technique. Next, the partition homogeneity test (FARRIS et al. 1995) was performed (1,000 replicates) with PAUP*4.0b10 (SWOFFORD 2002), to



Figs 2–4. Shells of *Vinodolia fiumana*: 2–3 – female, 4 – male; scale bar equals 1 mm



Figs 5–9. Radula of *Vinodolia fiumana*, scale bars equal 5 µm

check whether the two genes could be analysed together. Since p>0.723, the maximum likelihood heuristic search was then run for the combined molecular data. Following the recommendations of SOBER (2002) and POSADA & BUCKLEY (2004), the best model for each dataset was chosen using the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC); both chose the same model. We performed ML analyses in PAUP* 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), and with MEGA5.10 (TAMURA et al. 2011). Nodal support was estimated using the bootstrap (BS) approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 10,000 bootstrap replicates, with MEGA5.10 and the same model parameters as for ML analysis.

RESULTS AND DISCUSSION

Shell (Figs 2–4) ovate-conical, thin-walled and translucent, with weakly marked possible dimorphism (compare Figs 2–3 – female, and 4 – male: the male only somewhat smaller). Operculum orange. Head, foot, and visceral sac unpigmented, with the exception of small, very delicate light grey spots behind the eyes. Ctenidium absent.

Radula (Figs 5–9) taenioglossate, with many cusps on each tooth; central tooth formula:

$$\frac{4-5(6)-4-5(6)}{2-2}$$

(sixth cusp rudimentary, if present), basal cusps prominent, all cusps long and sharp (Figs 5–6 and 9); lateral tooth (Figs 5 and 7–8) formula: 4 – 1 – 4(5), biggest cusp less than twice as long as adjacent cusps; inner marginal tooth (Figs 5–6 and 7–8) with 23–25 long, slender and sharp cusps; outer marginal tooth (Figs 5–7) with 24–27 cusps (small to medium-sized within a tooth).

Penis (Fig. 10) big, long and slender, terminally broadened, with slightly marked double lobe on the left side at half length, and two small lobes proximally at base, penial tip rather blunt, vas deferens visible inside terminal half.

Fig. 10. Penis of *Vinodolia fiumana*, ventrally, scale bar equals 100 μm

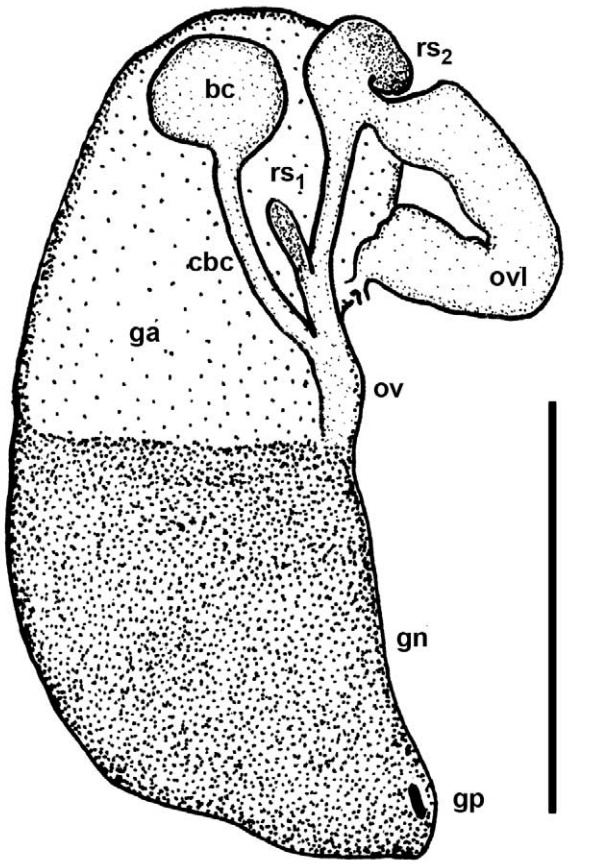


Fig. 11. Female reproductive organs of *Vinodolia fiumana* (bc – bursa copulatrix, cbc – duct of bursa copulatrix, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of oviduct, rs_1, rs_2 – receptaculum seminis 1 and 2, respectively), scale bar equals 250 μm

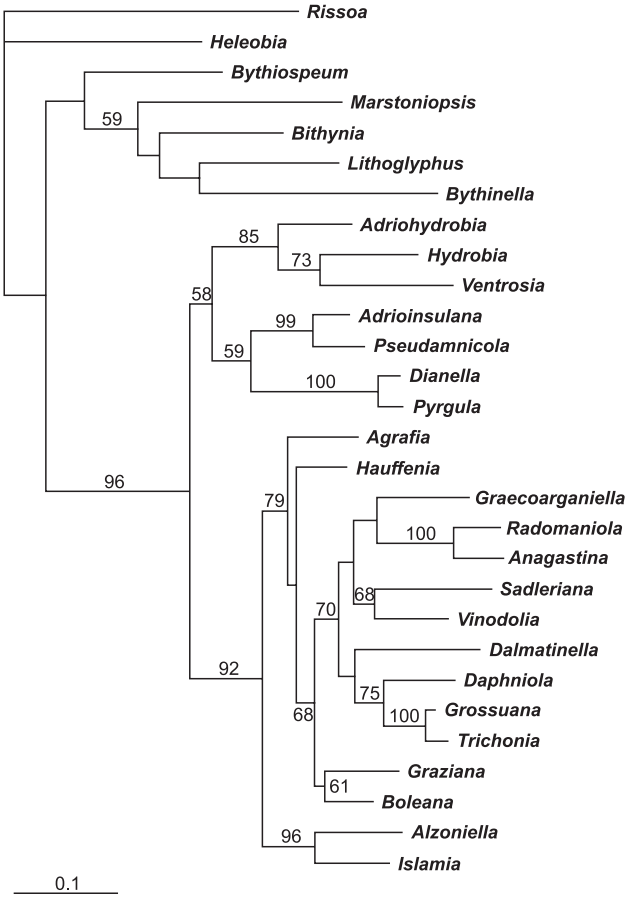


Fig. 12. Maximum likelihood tree of the two concatenated sequences (18S and COI), bootstrap supports (10,000 replicates) given if >50%

Female reproductive organs (Fig. 11), with two seminal receptacles, rs_2 bigger than rs_1, bursa copulatrix moderately big and spherical, coil of oviduct massive.

The studied *V. fiumana* shells are similar to the one described and figured by RADOMAN (1983: Plate III, fig. 47). This is also the case for the female genital system, which resembles the one described and drawn by RADOMAN (1973, 1974, 1983: fig. 23). This cannot be said about the penis. The penis figured by RADOMAN (1983: fig. 23) is short and broad; it is most probably contracted (due to fixation?), without basal lobes and with a bigger, proximal, double lobe. Neither the penis of *Anagastina* (FALNIOWSKI et al. 2012), nor the penis of *Dalmatinella* (FALNIOWSKI & SZAROWSKA 2013) resemble that of *Vinodolia*.

For the combined data set the Bayesian Information Criterion (BIC) and corrected Akaike Information Criterion (AICc) with MEGA5 found model TN93 (Tamura-Nei) + I + Γ, with base frequencies: A = 0.262, C = 0.212, G = 0.220, T = 0.306; substitution rate matrix: [A–C] = 0.030, [A–G] = 0.138, [A–T] = 0.037, [C–G] = 0.031, [C–T] = 0.245, [G–T] = 0.043; proportion of invariable sites: (I) = 0.49, and Γ distribution with the shape parameter = 0.33, and transition/transversion bias R = 2.49.

In the inferred phylogeny (Fig. 12) *Vinodolia* is the sister taxon of *Sadleriana* Clessin, 1890 (bootstrap support 68), not of *Dalmatinella*. Surely, the support is no high and *Sadleriana* cannot be more than a hypothetical sister taxon of *Vinodolia*, but there is enough evidence that it is closely related neither with *Dalmatinella* nor with *Anagastina*. It has to be stressed, that *Anagastina* is the sister taxon of *Radomaniola* Szarowska, 2006 (bootstrap support 100). Thus it is clear that the systematics proposed by FALKNER et al. (2001), as well as BANK (2012), or PEŠIĆ & GLÖER (2013), cannot be accepted, being contradicted by both morphological and molecular data.

Rissooid gastropods inhabiting springs are more or less troglobiontic, depending on species, and many of them could be found at the surface only as few specimens periodically washed out from the subterranean waters during the periods of intensive outflow. Thus, *Vinodolia* possibly has survived in subterranean waters. SZAROWSKA (2000) describes similar reappearance in a spring after a few years of absence, for *Bythinella*. Indeed, as long as a spring still exists, the gastropods inhabiting it may survive, but there are many totally destroyed springs in the Balkans (SZAROWSKA & FALNIOWSKI 2004, 2011b). Thus, *V. fiumana* may still exist in the other two localities listed by RADOMAN 1973, 1974, 1983) as well.

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