Molluscan Research 28(3): 165–170
http://www.mapress.com/mr/

ISSN 1323-5818
Magnolia Press

Heleobia dobrogica (Grossu & Negrea, 1989)(Gastropoda: Rissooidea: Cochliopidae) and the estimated time of its isolation in a continental analogue of hydrothermal vents

ANDRZEJ FALNIOWSKI[1], MAGDALENA SZAROWSKA[1], IOAN SIRBU[2], ALEXANDRA HILLEBRAND[3] & MIHAI BACIU[4]

[1] *Department of Malacology, Institute of Zoology, Jagiellonian University, ul. Ingardena 6, 30-060 Kraków, Poland, faln@zuk.iz.uj.edu.pl*

[2] *Lucian Blaga University, Department of Ecology and Environmental Protection, 31 Oituz St., 550337 Sibiu, Romania*

[3] *"Emil Racovita" Institute of Speleology, 13 Septembrie Ave., no 13, sect. 1, 050711, Bucharest, Romania*

[4] *Group for Underwater and Speleological Exploration, Frumoasa St no 31, sect. 1, 010986, Bucharest, Romania*

Abstract

Heleobia dobrogica is the only gastropod species living in the Movile Cave in Dobrogea, Romania. In the cave there is little oxygen but large amounts of carbon dioxide and methane in the atmosphere, and a large amount of hydrogen sulphide in the water. All the non-predatory animals feed on the chemoautotrophic microorganisms that draw energy from the sulphide hot springs beneath the cave. Five COI mtDNA sequences were used for maximum likelihood phylogeny reconstruction together with eight sequences of cochliopids and two outgroup rissooids from GenBank., *Salenthydrobia ferrerii* and *Peringia ulvae* were used as an outgroup for the calibration of the molecular clock. The estimated time of divergence between the two species was 2.172±0.171 Mya. This coincides with the period marking the beginning of the fall in temperature and precipitation that initiated the glacial period in Europe, predating the Pleistocene. Most probably at that time *Heleobia dobrogica* found a safe shelter within a warm cave. Our results suggest that *H. dobrogica* is closely related to *H. dalmatica*, and both species may be congeneric with *Heleobops docimus*.

Key words: troglobiont, Cochliopidae, phylogeny, mtDNA, molecular clock, Romania

Introduction

In 1986, while excavating for a construction project, engineers found an unusual cave near Mangalia (south of Constanta in Dobrogea), a few kilometres from the Black Sea coast. The small cave (12,000 m^2), named Movile, is about 300 metres long and less than 3 metres high. It contains a small lake in its lower part. The physical and chemical conditions within the cave are unusual: the water is rich in hydrogen sulphide (8–12 mg/l); and the atmosphere is poor in oxygen (7–10%), rich in carbon dioxide (2–3.5%) and charged with a significant amount of methane (1–2%) (Marin and Nicolescu 1993; Sarbu *et al*. 1996). The cave has no natural entrance and the only man-made entrance is entirely sealed off (by two gates and an airtight lid) to prevent alteration of the natural conditions within the cave. Forty-six invertebrate species, including 33 endemics, have been found in the cave: 28 of these (22 endemic) are terrestrial and 18 (11 endemic) are aquatic, one gastropod included. All of the primary consumers in this ecosystem feed on the chemoautotrophic bacteria and fungi that draw energy from the sulphide hot springs beneath the cave (Sarbu 1991; Sarbu and Kane 1995; Sarbu *et al.* 1996; Lascu 2001). This is a clear analogue to the well-known hydrothermal vents ('hot vents') found in oceanic rift zones. Lascu (1989) hypothesized that this unusual fauna found a shelter in the cave some five million years ago, when the climate became colder.

In 1989 Grossu and Negrea described a new species of *Paladilhiopsis* Pavlovic, 1913 from the lake in Movile Cave. Bernasconi (1991) subsequently transferred this species to the genus *Heleobia* Stimpson, 1865 (=*Semisalsa* Radoman, 1974) and studied the shell variation and anatomy of this species (Bernasconi 1994, 1997), while Szarowska (2006) described and illustrated its protoconch and female reproductive anatomy. The shells of *Heleobia dobrogica* are illustrated in Fig. 1.

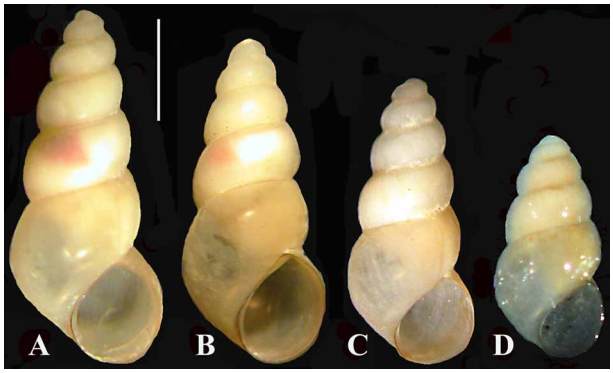

FIGURE 1: Shells of *Heleobia dobrogica*: A,B—female, C,D—male. Movile Cave, ZMUJ RO06M1, 2, 3, 4, respectively.

The aim of the present paper is to try to estimate the time of isolation of *Heleobia dobrogica* (Grossu & Negrea, 1989) in the cave, as a probable time of isolation of all the Movile Cave fauna, applying the molecular clock approach. We also intend to infer the relationships of *H. dobrogica* within the Cochliopidae.

Material and methods

Snails were collected by hand, fixed with 80% ethanol and stored in 96% ethanol. They were hydrated in TE buffer (3 x

10 min.), DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in 20 μm of TE buffer. The PCR reaction was performed with the following primers: LCOI490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') for the COI gene (Folmer *et al*. 1994). The PCR conditions were as follows: 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, with an additional elongation step of 4 min performed at 72 ˚C after all the cycles. The total volume of each PCR reaction mixture was 50 μl. 10 μl of the PCR product was run on 1 % agarose gel to check the quality of the PCR products. The PCR product was purified using Clean-Up columns (A & A Biotechnology). The purified PCR product was sequenced (Hillis *et al.* 1996a) using BigDye Terminator v3.1 (Applied Biosystems) and the protocols and primers described above. The sequencing reaction products were purified using ExTerminator Columns (A & A Biotechnology), and the sequences were read using the ABI Prism sequencer.

The sequences were aligned by eye, using BioEdit 5.0.0 (Hall 1999) and edited with MacClade 4.05 (Maddison and Maddison 2002).

The only sequence of *Heleobia* available was the *H. dalmatica* (Radoman, 1974) found in GenBank, thus we used it for the estimation of time of isolation. All the cochlipid sequences available from GenBank were used for the phylogenetic analysis. The sequences of *Radomaniola callosa* (Paulucci, 1881) (Hydrobiidae) and *Bithynia tentaculata* (Linnaeus, 1758) (Bithyniidae) were included, as a multiple outgroup. To test the molecular clock, the same set of cochliopid taxa was used, the outgroups were the hydrobiids *Peringia ulvae* (Pennant, 1777) and *Salenthydrobia ferrerii* Wilke, 2003 whose divergence time was used to calibrate the clock.

TABLE 1. GenBank Accession Numbers and references of the COI sequences of species considered (*Heleobia dobrogica*: specimens ZMUJ RO06M10- ZMUJ RO06M14, respectively).

Species	GenBankAN	references	extraction
Heleobia dobrogica (Grossu & Negrea, 1989)[1]	EU938128	present study	M5R
Heleobia dobrogica (Grossu & Negrea, 1989)[2]	EU938129	present study	E09
Heleobia dobrogica (Grossu & Negrea, 1989)[3]	EU938130	present study	AB11
Heleobia dobrogica (Grossu & Negrea, 1989)[4]	EU938131	present study	G115
Heleobia dobrogica (Grossu & Negrea, 1989)[5]	EU938132	present study	G116
Heleobia dalmatica (Radoman, 1974) 1	AF367631	Wilke *et al.* 2001	
Heleobia dalmatica (Radoman, 1974) 2	AF129321	Hershler *et al.* 1999	
Heleobops docimus Thompson, 1968	AF129322	Hershler *et al.* 1999	
Onobops jacksoni (Bartsch, 1953)	AF367645	Wilke *et al.* 2001	
Spurwinkia salsa (Pilsbry, 1905),	AF367633	Wilke *et al.* 2001	
Cochliopa sp.	AF354762	Liu *et al.* 2001	
Pyrgophorus platyrachis Thompson, 1968	AF367632	Wilke *et al.* 2001	
Littoridinops monroensis (Frauenfeld, 1863)	AF367644	Wilke *et al.* 2001	
Radomaniola callosa (Paulucci, 1881)	AF367649	Wilke *et al.* 2001	
Bithynia tentaculata (Linnaeus, 1758)	AF367643	Wilke *et al.* 2001	
Salenthydrobia ferrerii Wilke, 2003	AF449200	Wilke 2003	
Peringia ulvae (Pennant, 1777)	AF118288	Wilke 2003	

The maximum likelihood (ML) approach often tends to find the wrong reconstructions, especially in analyses involving a large number of taxa with short sequences (Nei *et al*. 1998; Nei and Kumar 2000). There is no parameter associated with a tree topology in the entire maximum likelihood theory: one must simply assume that the tree with the "truest" branch lengths is also the one with the best topology (Yang *et al.* 1995; Nei 1987, 1996). There is also strong evidence that the more complicated the model of evolution, the higher the variance of the resulting reconstructions (Nei and Kumar 2000). Our knowledge of the evolution of DNA is incomplete, thus all the available models are probably unrealistic. Thus, it may happen that the simplest models will result in phylogeny reconstructions which are closest to the real historical processes (Gaut and Lewis 1995; Yang 1997; Takahashi and Nei 2000, Falniowski 2003). On the other hand, similar remarks may be made about other phylogenetic techniques as well, and the ML approach is not sensitive to the violation of some of its assumptions (Swofford *et al.* 1996). Thus we decided to apply the maximum likelihood approach as implemented in PAUP*4.0b10 (Swofford 2002). PAUP together with Modeltest (Posada and Crandall 1998) was used to find the appropriate model of evolution, with the Akaike Information

Criterion (Posada and Buckley 2004). This model was also selected for the set of taxa with *Peringia* and *Salenhydrobia* as an outgroup, and the best ML trees were found to perform the Likelihood Ratio Test (LRT) (Nei and Kumar 2000; Posada 2003) with PAUP. MEGA4 (Tamura *et al.* 2007) was used to run the Relative Rate Tests (RRT) (Tajima 1993). The pairwise ML distances were calculated with PAUP. Wilke's (2003) data were used to calibrate the clock. Maximum Composite Likelihood (Γ) with standard errors (1000 bootstrap replicates) was calculated with MEGA4.

The specimens are lodged in the collection of the Department of Malacology of Jagiellonian University, Kraków (ZMUJ RO06M).

Results

Molecular distances and estimation of time of divergence

Five partial (638 bp) sequences of COI (Table 1) represented four haplotypes that differed in seven positions. For all the cochliopid taxa, with *Salenthydrobia ferrerii* and *Peringia ulvae* as outgroups, the Akaike Information Criterion (AIC) selected the model TIM+I+G, with base frequencies: A = 0.2678, C = 0.1459, G = 0.1627, T = 0.4236, substitution rate matrix: [A-C] = 1.0000, [A-G] = 537.9000, [A-T] = 59.6603, [C-G] = 59.6603, [C-T] = 285.8066, [G-T] = 1.0000, proportion of invariable sites: (I) = 0.6183, and Γ distribution with the shape parameter 1.5631. The LRT of this data set does not reject the molecular clock hypothesis (log L_0 = -2733.4536, log L_1 = -2719.8802, Δ = 27.1468, DF = 13, P>0.1239. Tajima's RRT for *Heleobia dobrogica* and *H. dalmatica* with *Salenthydrobia* and *Peringia* as an outgroup resulted in P>0.2230, and P>0.7237, respectively, thus not rejecting the molecular clock. RRTs for each pair of cochliopids with either *Salenthydrobia* or *Peringia* as an outgroup did not reject the molecular clock hypothesis either, except where *Onobops* was included and *Salenthydrobia* was used as an outgroup. Thus *Onobops jacksoni* (Bartsch, 1953) was excluded from the data set, for which the model GTR+I+G was selected, with base frequencies: A = 0.2682, C = 0.1526, G = 0.1627, T = 0.4166, substitution rate matrix: [A-C] = 0.0005, [A-G] = 20.4928, [A-T] = 1.6171, [C-G] = 2.1206, [C-T] = 9.0843, [G-T] = 1.0000, proportion of invariable sites: (I) = 0.6343, and Γ distribution with the shape parameter 2.5596. The LRT of this data set also does not reject the molecular clock hypothesis (log L_0 = -2546.0733, log L_1 = -2538.6285, Δ = 14.8874, DF = 12, P>0.2940.

The pairwise ML distances calculated for both models are given in Table 2. The distances calculated for the model without *Heleobops* (0.05774-0.07135, mean 0.0636±0.005) were used to estimate the time of divergence. The value 0.15605 between *Salenthydrobia* and *Peringia* was calibrated for 5.33 Mya by Wilke (2003). Thus, applying this calibration, the mean divergence time between the two species of *Heleobia* was 2.172±0.171 Mya (2.139±0.167 Mya for the model calculated including *Onobops jacksoni*). The Maximum Composite Likelihood distances (Γ distribution, α=1.5631) gave estimates within the range 2.390±0.665 Mya – 2.870±0.733Mya.

TABLE 2. Pairwise distances: below diagonal: for the model selected for the cochliopids with *Salenthydrobia ferrerii* and *Peringia ulvae* as outgroup; above diagonal: for the model selected for the same set of taxa, but without *Onobops jacksoni*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Heleobia dobrogica* 1	-.-----	0.00464	0.00943	0.00621	0.00621	0.07135	0.07135	0.08285	-.-----	0.45423	0.57045	0.57001	0.45233	1.06288	0.93075
2. *Heleobia dobrogica* 2	0.00463	-.-----	0.00463	0.00463	0.00463	0.06431	0.06431	0.08023	-.-----	0.45157	0.55256	0.54563	0.43379	1.03172	0.91376
3. *Heleobia dobrogica* 3	0.00941	0.00462	-.-----	0.00621	0.00621	0.05774	0.05774	0.07794	-.-----	0.42699	0.53051	0.51499	0.40015	1.06421	0.94848
4. *Heleobia dobrogica* 4	0.00619	0.00462	0.00621	-.-----	0.00000	0.06230	0.06230	0.07740	-.-----	0.42817	0.52970	0.51073	0.40537	1.05255	0.92934
5. *Heleobia dobrogica* 5	0.00619	0.00462	0.00621	0.00000	-.-----	0.06230	0.06230	0.07740	-.-----	0.42817	0.52970	0.51073	0.40537	1.05255	0.92934
6. *Heleobia dalmatica* 1	0.07183	0.06479	0.05821	0.06276	0.06276	-.-----	0.00000	0.07135	-.-----	0.40260	0.52397	0.53707	0.40380	1.05776	0.98889
7. *Heleobia dalmatica* 2	0.07183	0.06479	0.05821	0.06276	0.06276	0.00000	-.-----	0.07135	-.-----	0.40670	0.52397	0.45594	0.42889	1.05776	0.94705
8. *Heleobops docimus*	0.08523	0.08259	0.08027	0.07987	0.07987	0.07541	0.07541	-.-----	-.-----	0.40260	0.54283	0.53707	0.40380	1.06301	0.98889
9. *Onobops jacksoni*	0.29101	0.28042	0.26942	0.26972	0.26972	0.34971	0.34971	0.28906	-.-----	-.-----	-.-----	-.-----	-.-----	-.-----	-.-----
10. *Spurwinkia salsa*	0.40012	0.39843	0.38311	0.38033	0.38033	0.39270	0.39270	0.38580	0.26464	-.-----	0.28329	0.13245	0.06589	1.07729	1.32330
11. *Cochliopa sp.*	0.60086	0.58128	0.56351	0.55892	0.55892	0.57809	0.57809	0.60960	0.38109	0.31285	-.-----	0.32152	0.26291	1.07483	1.13441
12. *Pyrgophorus platyrachis*	0.53188	0.50922	0.48418	0.47586	0.47586	0.54765	0.54765	0.44527	0.35341	0.13930	0.35811	-.-----	0.12377	1.32797	1.30126
13. *Littoridinops monroensis*	0.44204	0.42437	0.39528	0.39667	0.39667	0.42292	0.42292	0.44038	0.30130	0.07007	0.28840	0.13025	-.-----	1.11022	1.30494
14. *Salenthydrobia ferrerii*	0.91425	0.89576	0.93218	0.91853	0.91853	0.96271	0.96622	0.96271	0.89361	1.28863	1.20540	1.39863	1.36799	-.-----	0.15605
15. *Peringia ulvae*	1.03326	0.99630	1.03949	1.02609	1.02609	1.06461	1.07114	1.06461	0.88446	1.03335	1.14962	1.33896	1.15377	0.15968	-.-----

Phylogenetic analysis

The Akaike Information Criterion (AIC) selected the model K81uf+I+Γ, with base frequencies: A = 0.2972, C = 0.1472, G = 0.1551, T = 0.4005, substitution rate matrix: [A-C] = 1.0000, [A-G] = 201.3902, [A-T] = 35.5380, [C-G] = 35.5380, [C-T] = 201.3902, [G-T] = 1.0000, proportion of invariable sites: (I) = 0.5186, and Γ distribution with the shape parameter 0.7457. The resulting maximum likelihood phylogram (Fig. 2) confirmed placement of *H. dobrogica* within the Cochliopidae, and the close relationships between this species and *H. dalmatica*. *Heleobops docimus* was placed within the *Heleobia* clade, between *H. dalmatica* and *H. dobrogica*.

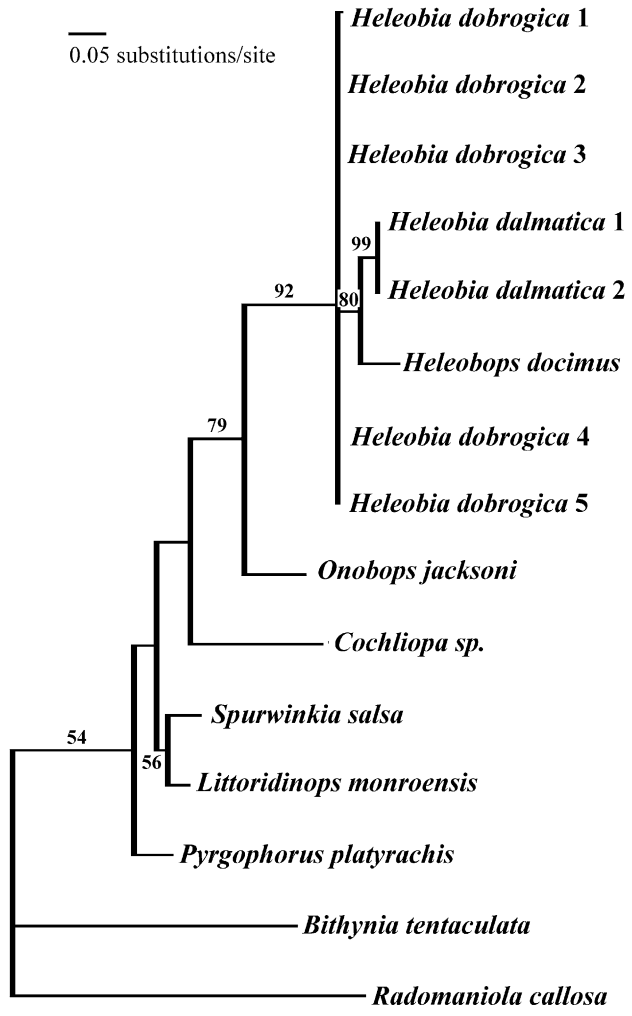

FIGURE 2. Maximum likelihood phylogram (see text for details). Bootstrap support indicated (10,000 replicates) when > 50%.

Discussion

Despite all the precautions concerning the molecular clock concept, as well as its scaling (Hillis *et al*. 1996b; Avise 2000; Nei and Kumar 2000; Posada 2003), there are many examples of its usage, also for rissooid snails (Wilke 2003, 2004; Haase *et al*. 2007; Falniowski *et al*. 2007). As a cochliopid, *Heleobia* is phylogenetically not too far from the Hydrobiidae (Wilke *et al.* 2001). The clock was calibrated for two representatives of this family (Wilke 2003). Also the estimated time (2.172±0.171 Mya) is not far from the 5.33 Mya used as the calibration value. The distances are within the range that is considered not to be affected by saturation (Wilke *et al*. 2001), and one-point calibration should not give rise to a significant error in this case. However, with one-point calibration it is not possible to obtain reasonable estimates of confidence intervals (Hillis *et al.* 1996b). Another problem with calibration was pointed out by Haase *et al*. (2007). 5.33 Mya is the time of the end of the Messinian Salinity Crisis (Pliocene Flooding). In fact, the isolation of the Atlantic *Peringia* from the Mediterranean *Salenthydrobia* must have begun earlier – when the Mediterranean Basin started to separate from the Atlantic, 5.96 Mya (Krijgsman *et al*. 1999; Falniowski *et al*. 2007). 5.33 Mya *Salenthydrobia* became isolated in a freshwater habitat from the other *Hydrobia* Hartmann, 1821 in the Mediterranean, but its isolation from *Peringia* Paladilhe, 1874 began earlier. If a time of 5.96 is applied instead of 5.33, the estimates for *Heleobia dobrogica* are higher: 2.429±0.191 Mya (for the Maximum Composite Likelihood distances: 2.669±0.744 Mya – 3.209±0.820 Mya.

Another problem concerns the species we used to estimate the time of divergence. Unfortunately, *Heleobia dalmatica* is the only European cochliopid whose sequence is available. The European *Heleobia* (=*Semisalsa*) is known from the Netherlands, Italy, Croatia, Greece, Romania, Ukraine, Israel, Turkey and Jordan (Kabat and Hershler 1993). Eleven species of this genus have been described, but their status and relationships have not yet been resolved. *Heleobia* (=*Semisalsa*) is probably the only cochliopid representative in the Palearctic, thus its zoogeographic relationships remain enigmatic. According to some authors, *H. dalmatica* is found from Dalmatia to the Black Sea. In any case, both *H. dalmatica* and *H. dobrogica* occur in the Balkans.

Before 3 Mya there was a sharp decrease in temperature and in precipitation. Later, the temperature and humidity became higher, but there were several fluctuations, with alternate periods of cold and warm conditions, and the glacial period in Europe began at about 2.5 Mya (Stanley 1999), predating the Pleistocene. At that time subtropical vegetation definitively disappeared from Europe. The estimated divergence time between these two species, irrespective of the distance used and calibration point assumed, coincides with either the period of climate fluctuation that predated the glaciation period, or the beginning of the glaciation period. It was most probably then that *Heleobia dobrogica* found a safe shelter within a warm cave.

Heleobia dobrogica is closely related to *H. dalmatica*, and the K2P distances between these two species suggest that they are congeners (e.g. Wilke *et al*. 2001). Davis *et al.* (1982) synonymized *Semisalsa* Radoman, 1974, with the American *Heleobia* Stimpson, 1865. That was questioned by Bank and Butot (1984). Unfortunately, no COI sequences of any *Heleobia* from South America are available. On the other hand, the American *Heleobops* seems to be very closely related to the European *Heleobia* (=*Semisalsa*).

Acknowledgments

The study was supported by a grant from the Polish Ministry of Science and Higher Education (PB 2443/P01/2006/31) to Andrzej Falniowski. We are very grateful to the two anonymous reviewers, whose comments greatly improved an earlier version of the manuscript.

References

Avise, J. C. (2000) *Phylogeography. The history and formation of species*. Harvard University Press, Cambridge, Massachusetts and London, England.

Bank, R. A. & Butot, L. J. M. (1984) Some more data on *Hydrobia ventrosa* (Montagu, 1803) and *"Hydrobia" stagnorum* (Gmelin, 1791) with remarks on the genus *Semisalsa* Radoman, 1974 (Gastropoda, Prosobranchia, Hydrobiidae). *Malakologische Abhandlungen* 10, 5–15.

Bernasconi, R. (1991) Sur un gasteropode prosobranche de la grotte Pestera Movile, Mangalia Roumanie: *Semisalsa dobrogica* (Grossu, Negrea, 1989) comb. nov. (Hydrobiidae, Littoridininae, Heleobiini). *Mémoires de Biospéologie* 18, 237–243.

Bernasconi, R. (1994) Mollusca. In: Juberthie, C.& Decu, V. (eds.) *Encyclopaedia Biospeologica*. CNRS, Moulis, pp. 53–58.

Bernasconi, R. (1997) Conchological variability of *Heleobia [Semisalsa] dobrogica* (Mollusca Prosobranchia Hydrobiidae Cochliopinae) from subterranean waters of Mangalia, Dobrogea, Romania. *Proceedings of the 12th International Congress of Speleology, Switzerland* 3, 333–336.

Falniowski, A. (2003) *Metody numeryczne w taksonomii* [Numerical techniques in taxonomy]. Wydawnictwo Uniwersytetu Jagiellońskiego, Kraków.

Falniowski, A., Szarowska, M. & Grzmil, P. (2007) *Daphniola* Radoman, 1973 (Gastropoda: Hydrobiidae): shell biometry, mtDNA, and the Pliocene flooding. *Journal of Natural History* 41, 2301–2311.

Folmer, O., Black, M., Hoeh, W., Lutz, R. A. & Vrijenhoek, R. C. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3, 294–299.

Gaut, B. S. & Lewis, P. O. (1995) Success of maximum likelihood phylogeny inference in the four-taxon case. *Molecular Biology and Evolution* 12, 152–162.

Grossu, A.V. & Negrea, A. (1989) *Paladilhia (Paladilhiopsis) dobrogica* - une nouvelle espèce de la famille Moitessieriidae (Gastropoda, Prosobranchia). *Miscellanea speologica Romaniae, Bucharest* 1, 33–37.

Haase, M., Marshall, B. & Hogg, I. (2007) Disentangling causes of disjunction on the South Island of New Zealand: the Alpine fault hypothesis of vicariance revisited. *Biological Journal of the Linnean Society* 91, 361–374.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.

Hershler, R., Liu, H.P. & Mulvey, M. (1999) Phylogenetic relationships within the aquatic snail genus *Tryonia*: implications for biogeography of the North American Southwest. *Mollecular Phylogenetics and Evolution* 13, 377–391.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K. & Zimmer, E. A. (1996a) Nucleic Acids IV: Sequencing and Cloning. In: Hillis, D. M., Moritz, C. & Mable, B. K. (Eds.) *Molecular Systematics.* Second edition. Sinauer Associates, Inc., Sunderland, Massachusetts, pp. 321–381.

Hillis, D. M., Mable, B. K. & Moritz, C. (1996b) Applications of molecular systematics: The state of the field and a look to the future. In: Hillis, D. M., Moritz, C. & Mable, B. K. (Eds.) *Molecular Systematics*. Second edition. Sinauer Associates, Inc., Sunderland, Massachusetts, pp. 515–543

Kabat A. R. & Hershler, R. 1993. The prosobranch snail family Hydrobiidae (Gastropoda: Rissooidea): Review of classification and supraspecific taxa. *Smithsonian Contributions to Zoology* 547, 1–94.

Krijgsman. W., Hilgen, F. J., Raffi, I., Sierro, F. J. & Wilson, D. S. (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400, 652–655.

Lascu, C. (1989) Paleogeographical and hydrogeological hypothesis regarding the origin of a peculiar cave fauna. *Miscellanea speologica Romaniae* 1, 13–18.

Lascu, C. (2001) *Caves beyond time*. Group of Underwater and Speleological Exploration, Bucharest.

Liu, H.P., Hershler, R. & Thompson, F.G. (2001) Phylogenetic relationships of the Cochliopinae (Rissooidea: Hydrobiidae): an enigmatic group of aquatic gastropods. *Molecular Phylogenetics and Evolution* 21, 17–25.

Maddison, D. R. & Maddison, W. P. (2002) *MACCLADE 4.05*. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, USA.

Marin, C. & Nicolescu, T. (1993) The geochemistry of groundwater from southeastern Dobrogea, Romania. *Trav. Inst. Spèleol. "Emile Racovitza". Bucharest* 32, 229–247.

Nei, M. (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei, M. (1996) Phylogenetic analysis in molecular evolutionary genetics. *Annual Review of Genetics* 30, 371–403.

Nei, M. & Kumar, S. (2000) *Molecular Evolution and Phylogenetics*. Oxford University press, Oxford, UK and New York.

Nei, M., Kumar, S. & Takahashi, K. (1998) The optimization principle in phylogenetic analysis tends to give incorrect topologies when the number of nucleotides or amino acids used is small. *Proceedings of the National Academy of Sciences of the U.S.A.* 76, 5269–5273.

Posada, D. (2003) Selecting models of evolution. In: Salemi M. & Vandamme A.-M. (Eds.) *The Phylogenetic Handbook. A Practical Approach to DNA and Protein Phylogeny.* Cambridge University Press, Cambridge, UK, pp. 256–282.

Posada, D. & Crandall, K .A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.

Posada, D. & Buckley, T. R. (2004) Model selection and model averaging in phylogenetics: Advantages of akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53, 793–808.

Sarbu, S. (1991) Contribution to the biological investigation of the "Movile cave": the species composition and trophic structure of the cave community and the origin of the fauna. *Mémoires Biospéologiques* 18, 193–197.

Sarbu, S.M. & Kane, T.C. (1995) A subterranean chemoautotrophically based ecosystem. *NSS Bulletin* 57, 91–98.

Sarbu, S. M., Kane, T. C. & Kinkle, B. K. (1996) A chemoautotrophically based groundwater ecosystem. *Science* 272, 1953–1955.

Stanley, S. M. (1999) *Earth System History.* W. H. Freeman & Co., New York – Basingstoke.

Swofford, D.L. (2002*) PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4*. Sinauer Associates Inc., Sunderland, Massachusetts.

Swofford, D. L., Olsen, G. J., Waddell, P. J. & Hillis, D. M. (1996) Phylogenetic inference. In: Hillis D. M., Moritz, C. & Mable, B. K. (Eds.) *Molecular Systematics.* Second edition. Sinauer Associates, Inc., Sunderland, Massachusetts, pp. 407–514.

Szarowska, M. (2006) Molecular phylogeny, systematics and

morphological character evolution in the Balkan Rissooidea (Caenogastropoda). *Folia Malacologica* 14, 99–168.

Tajima, F. (1993) Simple methods for testing molecular clock hypothesis. *Genetics* 135, 599–607.

Takahashi, K. & Nei, M. (2000) Efficiencies of fast algorithms of phylogenetic inference under the criteria of maximum parsimony, minimum evolution and maximum likelihood when a large number of sequences are used. *Molecular Biology and Evolution* 17, 1251–1258.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular rvolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.

Wilke, T. (2003) *Salenthydrobia* gen. nov. (Rissooidea: Hydrobiidae): a potential relict of the Messinian salinity crisis. *Zoological Journal of the Linnean Society* 137, 319–336.

Wilke, T. (2004) How dependable is a non-local molecular clock? A reply to Hausdorf et al. (2003). *Molecular Phylogenetics and Evolution* 30, 835–840.

Wilke, T., Davis, G. M., Falniowski, A., Giusti, F., Bodon, M. & Szarowska, M. (2001) Molecular systematics of Hydrobiidae (Mollusca: Gastropoda: Rissooidea): testing monophyly and phylogenetic relationships. *Proceedings of the Academy of Natural Sciences of Philadelphia* 151, 1–21.

Yang, Z. (1997) How often do wrong models produce better phylogenies? *Molecular Biology and Evolution* 14, 105–108.

Yang, Z., Goldman, N. & Friday, A. (1995) Maximum likelihood trees from DNA sequences: A peculiar statistical estimation problem. *Systematic Biology* 44, 384–399.