Biological Journal of the Linnean Society, 2005, **85**, 501–512. With 3 figures

A molecular approach to the phylogenetic relationships of the western palaearctic Helicoidea (Gastropoda: Stylommatophora)

GIUSEPPE MANGANELLI[1], NICOLA SALOMONE[2] and FOLCO GIUSTI[1]*

[1]*Dipartimento di Scienze Ambientali, Università di Siena, Via P.A. Mattioli 4, 53100 Siena, Italy*
[2]*Dipartimento di Biologia Evolutiva, Università di Siena, Via A. Moro 2, 53100 Siena, Italy*

Received 10 October 2003; accepted for publication 15 October 2004

Molecular phylogenetic relationships among 45 members of the Helicoidea (Gastropoda: Stylommatophora) were examined using partial mitochondrial 16S rRNA sequences. Phylogenetic relationships were inferred using maximum parsimony, maximum likelihood and Bayesian methods. The reconstructed phylogenies showed a good degree of support for more recent branches, but gave little support to deeper nodes. Mitochondrial rDNA data further confirmed monophyletic status of helicids, recognized monachine hygromiid and bradybaenid clades and resolved a number of relationships in the helicelline hygromiids. With the respect to the latter assemblage, most of the anatomically based groups are confirmed, corroborating the diagnostic value of the dart-sac complex and a close affinity between *Ichnusomunda sacchii* and species of the genus *Cernuella*. Nevertheless, some well resolved branches challenge previous systematic arrangements, grouping species previously placed in different arrangements. In particular, support was not found for the monophyly of helicelline hygromiids with pedal penial innervation. Possible explanations for these incongruencies are suggested. 16S sequence data are appropriate for studies of relationships within the different species groups and less so for recovery of more ancient radiations in the Helicoidea. It will be valuable to combine the 16S data with other gene sequences to estimate basal relationships. *Biological Journal of the Linnean Society*, 2005, **85**, 501–512.

ADDITIONAL KEYWORDS: 16S rRNA – Bayesian inference – maximum likelihood – maximum parsimony – molecular phylogeny.

INTRODUCTION

Of the many morphological, evolutionary, phenetic and cladistic approaches to phylogenetic relationships among the Stylommatophora, none has achieved general consensus (see references in Emberton *et al*., 1990; Hausdorf, 1998; Muratov, 1999). Evidently, shell, excretory, locomotor and genital gross anatomy offer different solutions to the problem, solutions which are easily disproved when either a new set of characters or a different outgroup is considered. When morphological features fail to give clear information about relationships, molecular techniques have proved invaluable (Wade, Mordan & Clarke, 2001). We did research similar to that of Wade *et al*. (2001), but limited to one group of the Stylommatophora: the Helicoidea.

The systematics of the Helicoidea (as traditionally considered), one of the most studied stylommatophoran groups, reflects this general situation. Uncertainty exists not only with regard to position among the Stylommatophora, but also on how many families it includes. There is uncertainty about whether to keep the group as a vast inclusive superfamily or to divide it into separate superfamilies. Adopting an evolutionary approach based on gross anatomy, Schileyko (1979) divided it into four superfamilies: Sphincterochiloidea, Helicodontoidea, Helicoidea and Hygromioidea (later reduced to three: Helicoidea, Xanthonychoidea and Hygromioidea; Schileyko, 1991a, b). A spermatological approach which revealed substantial uniformity among many different

*Corresponding author. E-mail: giustif@unisi.it

families/superfamilies induced Giusti *et al.* (1992) to conclude that there had been excessive fragmentation into superfamilies and families.

The aim of the present study was to address phylogenetic relationships among representative taxa of Helicoidea using 16S sequence variation. The helicoidean taxa examined only partly match those examined by Wade *et al.* (2001), so the results are not easily compared. Nevertheless, significant concordance emerged between certain results, which appear to reinforce the phylogenetic hypotheses of Wade *et al.* (2001) as well as our own.

MATERIAL AND METHODS

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

In total, 45 specimens representing most major helicoidean lineages were sequenced for molecular analysis. Three additional sequences – *Euhadra herklotsi* (Helicoidea, Bradybaenidae), *Discus catskillensis* (Punctoidea, Discidae) and *Albinaria caerulea* (Clausiliidae, Alopiinae) – were retrieved from the GenBank database. *A. caerulea* and *D. catskillensis* were used as outgroups. Species, sampling sites, and GenBank accession numbers are given in Table 1, while the systematics of the genera to which the species examined belong appears in Table 2.

Genomic DNA was isolated from foot muscle of fresh, frozen or ethanol preserved specimens using C-TAB buffer (0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% 2-mercaptoethanol) and standard phenol-chloroform/ethanol extraction method. This protocol performed well in avoiding problems related to mucopolysaccharides in snail mucus (Sokolov, 2000; Wade & Mordan, 2000), while less tissue seemed to yield better quality DNA.

A fragment of the 16S rRNA gene, representing most of the 3′ half, was amplified by polymerase chain reaction using the primer pairs 5′–CGATTTGAACT CAGATCA–3′ (LR-J-12887, Simon *et al.*, 1994) and 5′–GTGCAAAGGTAGCATAATCA–3′ (designed for scorpions, Gantenbein *et al.*, 1999). Amplification was performed in 50 μL of reaction volume following a profile consisting of 25 cycles with temperatures of 92 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, plus a final extension step at 72 °C for 5 min. Reaction products were visualized on a 1.5% agarose gel and the appropriate band was cut and purified with 'Nucleospin extract' (Genenco) column kit following the manufacturer's instructions. The final products were used as template for sequencing reactions. Both strands of all specimens were sequenced at the core facility of MWG-Biotech (Ebersberg, Germany) using the appropriate amplification primers as sequencing primers. Sequences were submitted to GenBank.

PHYLOGENETIC ANALYSIS

Sequences were initially aligned using CLUSTAL W (Thompson, Higgins & Gibson, 1994). This matrix was then inspected and refined manually on the basis of the ribosomal secondary structure of *Cepaea nemoralis* and *Euhadra herklotsi* obtained from the comparative RNA website (CRW) (http://www.rna.icmb.utexas.edu; Cannone *et al.*, 2002). The molluscan 16S consensus secondary structure model was also used to identify gap positions and variable regions across all taxa (Lydeard *et al.*, 2000). In order to assess the amount of phylogenetic signal in our data set, we generated 10000 random trees and calculated the skewness (g_1) of the resulting tree distribution with PAUP* v.4.0b10 (Swofford, 2001).

To address phylogenetic relationships among the included taxa, three different analytical methods were used: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP and ML reconstructions were performed with PAUP*. The MP analyses were performed by heuristic search with random sequence addition (TBR branch swapping; Multrees option in effect). Ten replicates were performed within each heuristic search. The ML analysis requires a specific model of evolution to be specified a priori. In order to select the substitution model that best describes our data, sequences were analysed with Modeltest v.3.04 (Posada & Crandall, 1998). This program allows comparison of different models of DNA substitution to be tested in a hierarchical hypothesis testing framework. The optimal model defined by Modeltest was then selected for ML analysis. The resulting best fit model was the general time-reversible model, with six rate classes, unequal base frequencies, a parameter for invariable sites and a gamma distributed rate heterogeneity parameter ($GTR + \Gamma + I$). This model of nucleotide evolution was incorporated in PAUP*.

To assess the robustness of relationships, 1000 bootstrap replications were performed for MP. Due to prohibitive computer time, the bootstrapping method could not be used in the ML analysis. Instead, for the ML tree we report bootstrap values for 1000 replicates obtained from a neighbour-joining analysis of ML distances obtained using the parameter estimates derived from Modeltest.

Bayesian analysis was performed with MrBayes v. 2.01 (Huelsenbeck & Ronquist, 2001) with $GTR + \Gamma + I$ parameters estimated during the run, and using the default value of four Markov chains. Each chain was started from a random tree. Multiple chains can assist in traversing tree space and help to avoid entrapment in local topological optima. The 'temperature' parameter was set to 0.2. The Monte Carlo Markov chain length was 2000 000 generations and trees were sampled every 100 generations.

RESULTS AND DISCUSSION

Sequence characteristics

Among the helicodean taxa, sequences ranged in length from 357 bp (*Cantareus apertus* and the two *Chilostoma planospira*) to 387 bp (*Xerotricha conspurcata* and *X. apicina*). Relatively longer sequences characterized the two outgroups, with values of 398 bp for *A. caerulea* and 412 bp for *D. catskillensis*. For some taxa, two conspecific specimens were sequenced in order to evaluate intraspecific variability in the amplified fragment. Uncorrected percentage sequence divergence (p-distances) ranged from zero (*C. planospira*) to 1.3% (*Cochlicella acuta*) and was correlated with geographical sampling.

After deletion of all ambiguities and gaps, a data matrix with 342 unambiguously aligned positions was available for phylogenetic reconstructions. Of these, 197 were variable and 171 were phylogenetically informative under parsimony criteria. Base changes and indels were observed almost exclusively in regions known to be variable in molluscs (Lydeard *et al*., 2000), making accidental amplification of nuclear copies of 16S target fragments unlikely.

Average composition of the fragments studied was A = 34.5%, C = 13.8%, G = 19.8% and T = 31.7%. Significant differences across taxa were not detected ($\chi^2 = 65.697$; d.f. = 141; $P = 0.999$), although there was a bias in segment composition toward A + T (average = 66.2%), a pattern consistent with the one previously observed in most invertebrate mitochondrial genes (Simon *et al*., 1994).

Excluded regions

The accuracy of phylogenies deduced from molecular data depends critically on the accuracy of sequence alignment, since the process of identifying areas of ambiguous alignment in order to include or exclude them is somewhat subjective. However, the use of ribosomal RNA secondary structure information in combination with a computer-assisted optimality approach generally produces an increase in the number of alignments that provide a topology congruent with a well-corroborated morphological hypothesis (Titus & Frost, 1996).

In our data set, most gaps were of 1–2 nucleotides and observed length polymorphism was mainly determined by a fragment corresponding to a loop in the secondary structure of 16S rRNA (Lydeard *et al*., 2000; Cannone *et al*., 2002). Comparing ten sequences belonging to taxa representing the main molluscan classes, Lydeard *et al*. (2000) described this loop region as the most variable of the entire 3′ half, ranging in size from 8 to 96 sites. In our data set, which only included stylommatophoran gastropods, this loop region varied in size from 2 to 28 sites. Although we did not use this data in phylogenetic analysis, it is interesting that observed length variation seemed to be correlated with the phylogenetic reconstructions.

Representatives of helicids consistently had the shortest loop region of all taxa examined, with values ranging from 2 (*C. planospira*) to 7 (*Marmorana saxetana*) sites. Among representatives of the genus *Monacha*, loop length varied from 8 (*M. cartusiana*, *M. parumcinta*) to 17 sites (*M. cantiana*). In the two bradybaenid taxa this region varied in size from 16 (*Euhadra herklotsi*) to 18 sites (*Bradybaena fruticum*). Specimens belonging to hygromiids had considerably longer loop regions. All these taxa had length variations ranging from 24 to 28 sites.

Phylogenetic analyses

The g_1 test statistic indicated that the observed tree length distribution was significantly more skewed than expected from random ($g_1 = -0.582$, $P < 0.01$), suggesting the presence of a good phylogenetic signal in our sequences (Hillis & Huelsenbeck, 1992). MP produced six equally parsimonious trees (tree length = 1574, CI = 0.269, RI = 0.503), differing in minor branch arrangements at internal nodes (Fig. 1).

The best-fit ML model contained the nucleotide substitution rate parameters: [A-C] = 1.24, [A-G] = 4.05, [A-T] = 1.00, [C-G] = 0.20, [C-T] = 5.39 and [G-T] = 1.00. The estimated nucleotide frequencies were A = 0.373, C = 0.106, G = 0.136 and T = 0.384. The proportion of invariable sites was estimated to be 0.365 and the shape of the gamma parameter was 0.495. This model of sequence evolution supported two best trees (– lnL = 6274.04) differing only in the position of *Helicella itala*. One of these trees is shown as a phylogram in Figure 2.

The Bayesian Markov chain reached stationary after approximately 70 000 generations. Therefore, only trees sampled after this burn-in period were used to determine posterior probabilities of model parameters, branch lengths and clades. The consensus tree from Bayesian analysis is shown in Figure 3.

Parsimony, likelihood and Bayesian methods produced phylogenetic hypotheses that were largely congruent, with a few differences in the positioning of certain internal nodes. Nevertheless, in all reconstructions, greater support was found in more recent branches than at deeper nodes.

All the helicids analysed in this study (*Marmorana*, *Chilostoma*, *Eobania*, *Cantareus*, *Cepaea* and *Theba*) always formed a monophyletic group well supported by Bayesian analysis (bpp = 95%). Inside this clade, the two species of *Helix* (*H. lucorum* and *H. pomatia*) and the two species of *Cantareus* (*C. apertus* and

Table 1. List of the species examined (* spirit–preserved specimens), collecting data (country, locality, municipality and province names in parentheses, collector(s) and date) and accession numbers

Species	Localities/source	Accession numbers
Albinaria caerulea	Hatzoglou *et al.* (1995)	X83390
Bradybaena fruticum	Italy: Anzù di Feltre (Feltre, Belluno), C. Dalfreddo leg. 8.4.2001	AY741450
Candidula intersecta	Denmark: Mon Island, Tiornemarke, S. Cianfanelli and M. Calcagno leg. 1.8.2000	AY741437
Candidula spadae 1	Italy: Monte Nerone (Apecchio, Pesaro), G. Manganelli, L. Manganelli and A. Mazzeschi leg. 6.6.1999	AY741435
Candidula spadae 2*	Italy: Monte Cucco (Costacciaro, Perugia), S. Hallgass leg. 14.5.1989	AY741436
Candidula unifasciata	Italy: Parco La Tebaide, Cetinale (Sovicille, Siena), G. Manganelli leg. 6.1.2001	AY741438
Cantareus apertus	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 17.5.2000	AY741407
Cantareus aspersus	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 17.5.2000	AY741408
Cepaea nemoralis	Italy: Fosso delle Filicaie, San Giusto in Salcio (Radda in Chianti, Siena), G. Manganelli and L. Manganelli leg. 28.5.2000	AY741406
Cernuella cisalpina	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 22.9.1999	AY741423
Cernuella neglecta	Italy: Torrente Arbia, Vallina (Castelnuovo Berardenga, Siena), G. Manganelli leg. 12.7.1999	AY741426
Cernuella virgata	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 11.7.1999	AY741422
Cernuellopsis ghisottii	Italy: Monte Pollino, Cozzo Vardo (Morano Calabro, Cosenza), G. Manganelli leg. 25.4.1999	AY741429
Chilostoma planospira 1	Italy: Cappella Nagli (Colle di Val d'Elsa, Siena), S. Cianfanelli and G. Manganelli leg. 2.4.2000	AY741412
Chilostoma planospira 2	Italy: Capanno (Castelnuovo Berardenga, Siena), G. Manganelli and L. Manganelli 28.5.2000	AY741413
Cochlicella acuta 1	Italy: Lampedusa Island, Valle Imbriacole (Lampedusa e Linosa, Agrigento), S. Cianfanelli and E. Talenti leg. 14.5.2000	AY741442
Cochlicella acuta 2	Italy: Acquaviva (Rapolano Terme, Siena), G. Manganelli leg. 26.12.2000	AY741443
Discus catskillensis	Ross T.K. (unpublished)	AF063140
*Elona quimperiana**	Spain: Bendicion y Cueva de San Roman, B.G. Gomez and C.E. Prieto leg. 14.9.1988	AY741449
Eobania vermiculata	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 17.5.2000	AY741409
Euhadra herklotsi	Yamazaki *et al.* (1997)	Z71693
*Helicella itala**	Spain: Cornégula, C.E. Prieto leg. 13.9.1984	AY741441

Helicodonta obvoluta 1	Italy: Cappella Nagli (Colle di Val d'Elsa, Siena), 32TPP7602, S. Cianfanelli and G. Manganelli leg. 2.4.2000	AY741446
Helicodonta obvoluta 2	Italy: Capanno (Castelnuovo Berardenga, Siena), G. Manganelli and L. Manganelli 28.5.2000	AY741447
Helicotricha carusoi	Italy: Linosa Island, Monte Calcarella (Lampedusa e Linosa, Agrigento), S. Cianfanelli and E. Talenti leg. 17.5.2000	AY741434
Helix lucorum	Italy: Fortezza Medicea (Siena, Siena), M. Zatini leg. 10.3.2001.	AY741410
Helix pomatia	Italy: Lago di Cavazzo (Cavazzo Carnico, Udine), S. Cianfanelli and G. Manganelli leg.20.8.2001.	AY741411
Hygromia cinctella	Italy: Pian di Giuncheto (Cetona, Siena), M. Calcagno and S. Cianfanelli leg. 3.10.1999	AY741421
*Ichnusomunda sacchii**	Italy: Is Arenas, Cuccuru Pranu (Arbus, Oristano), G. Cappelli, G. Manganelli and L. Manganelli leg. 19.10.1995	AY741424
Lindholmiola girva	Greece: Igoumenitsa, G. Manganelli leg. 6.5.2000	AY741448
Marmorana saxetana	Italy: Roccalbegna (Roccalbegna, Grosseto), L. Favilli, G. Manganelli and S. Piazzini leg. 9.6.2000	AY741414
Microxeromagna armillata	Italy: Capraia Island: San Rocco (Capraia Isola, Livorno), F. Giusti leg. 15.8.2000	AY741433
Monacha cantiana	Italy: Podere Grania (Asciano, Siena), G. Manganelli and L. Manganelli leg. 15.10.2000	AY741419
Monacha cartusiana	Italy: Villa Medane (Siena, Siena), G. Manganelli leg. 11.9.1999	AY741416
Monacha martensiana	Italy: Piana di Colfiorito (Foligno, Perugia), F. Giusti leg. 28.09.2000	AY741420
Monacha parumcincta	Italy: Medane (Asciano, Siena), G. Manganelli leg. 8.10.2000	AY741418
Monacha sp.	Greece: Evros Delta, Lutrous, G. Manganelli leg. 10.5.2000	AY741417
Pollonériella contermina	Italy: Lago di Burano (Capalbio, Grosseto), G. Manganelli leg. 21.10.1999	AY741425
Sphincterochila candidissima	Maltese Islands: Qalet Marku, M. Sant leg. 08.06.2000	AY741445
Theba pisana	Italy: Lago di Burano (Capalbio, Grosseto), G. Manganelli leg. 21.10.1999	AY741415
Trochoidea pyramidata	Italy: San Giusto (Murlo, Siena), G. Manganelli and L. Manganelli leg. 1.4.1999	AY741444
Xerolenta obvia	Italy: Fiume Tagliamento, Lago di Cornino (Folgaria nel Friuli, Udine), Cianfanelli and G. Manganelli leg. 17.8.2000	AY741431
Xeromunda durieui	Italy: Marina di Pescoluse (Salve, Lecce), D. Ferreri leg. 12.09.1999	AY741432
Xeromunda sp.	Greece: Olympos, Monte Mitikos, G. Manganelli leg. 12.05.2000	AY741430
Xerosecta arigonis	Spain: Universidad de Lejona, C.E. Prieto leg. 15.07.1999	AY741428
*Xerosecta cespitum**	Italy: Monte Pietravecchia (Pigna, Imperia), A. Boato leg. 19.06.1979	AY741427
Xerotricha apicina	Italy: Lago di Burano (Capalbio, Grosseto), G. Manganelli leg. 21.10.1999	AY741440
Xerotricha conspurcata	Italy: Stazione di Castelnuovo Berardenga (Asciano, Siena), G. Manganelli leg. 11.07.1999	AY741439

Table 2. Systematics of the genera to which the helicoid species examined belong, according to Schileyko (1991b), Schileyko & Menkhorst (1997), and Nordsieck (1987, 1993)

Genus	Schileyko (1991b) and Schileyko & Menkhorst (1997)	Nordsieck (1987, 1993)
Bradybaena	Xanthonychoidea, Bradybaenidae, Bradybaeninae	Helicoidea, Bradybaenidae, Bradybaeninae
Candidula	Hygromioidea, Hygromiidae, Hygromiinae, Cernuellini	Helicoidea, Hygromiidae, Hygromiinae, *Helicella–Candidula* group
Cantareus	Helicoidea, Helicidae, Helicinae	Helicoidea, Helicidae, Helicinae, Helicini
Cepaea	Helicoidea, Helicidae, Helicinae	Helicoidea, Helicidae, Helicinae, Helicini
Cernuella	Hygromioidea, Hygromiidae, Hygromiinae, Cernuellini	Helicoidea, Hygromiidae, Hygromiinae, *Hygromia–Cernuella* group
Cernuellopsis	Hygromioidea, Hygromiidae, Trichinae, Helicellini	Helicoidea, Hygromiidae, Hygromiinae, *Hygromia–Cernuella* group
Chilostoma	Helicoidea, Helicidae, Ariantinae	Helicoidea, Helicidae, Ariantinae
Cochlicella	Xanthonychoidea, Cochlicellidae	Helicoidea, Hygromiidae, Monachinae, Cochlicellini
Elona	Helicoidea, Elonidae	Helicoidea, Xanthonychidae, Elonidae
Eobania	Helicoidea, Helicidae, Helicinae	Helicoidea, Helicidae, Helicinae, Helicini
Euhadra	Xanthonychoidea, Bradybaenidae, Bradybaeninae	Helicoidea, Bradybaenidae, Bradybaeninae
Helicella	Hygromioidea, Hygromiidae, Trichinae, Helicellini	Helicoidea, Hygromiidae, Hygromiinae, *Helicella–Candidula* group
Helicodonta	Hygromioidea, Helicodontidae, Helicodontinae	Helicoidea, Hygromiidae, Helicodontinae, Helicodontini
Helicotricha		
Helix	Helicoidea, Helicidae, Helicinae	Helicoidea, Helicidae, Helicinae, Helicini
Hygromia	Hygromioidea, Hygromiidae, Hygromiinae, Hygromiini	Helicoidea, Hygromiidae, Hygromiinae, *Hygromia–Cernuella* group
Ichnusomunda		
Lindholmiola	Hygromioidea, Helicodontidae, Lindholmiolinae	Helicoidea, Hygromiidae, Helicodontinae, Lindholmiolini
Marmorana	Helicoidea, Helicidae, Helicinae [as *Murella*]	Helicoidea, Helicidae, Helicinae, Murellini
Microxeromagna	Hygromioidea, Hygromiidae, Hygromiinae, Cernuellini	Helicoidea, Hygromiidae, Hygromiinae, *Pyrenaearia–Xerosecta* group
Monacha	Hygromioidea, Hygromiidae, Euomphalinae, Euomphalini	Helicoidea, Hygromiidae, Monachinae, Monachini
Polloneriella		Helicoidea, Hygromiidae, *Pyrenaearia–Xerosecta* group
Sphincterochila	Sphincterochiloidea, Sphincterochilidae	Helicoidea, Sphincterochilidae
Theba	Helicoidea, Helicidae, Euparyphinae	Helicoidea, Helicidae, Helicinae, Euparyphini
Trochoidea	Hygromioidea, Hygromiidae, Euomphalinae, Trochoideini	Helicoidea, Hygromiidae, Monachinae, Trochoideini
Xerolenta	Hygromioidea, Hygromiidae, Trichinae, Helicellini	Helicoidea, Hygromiidae, Hygromiinae, *Xerolenta–Xeromunda* group
Xeromunda	Hygromioidea, Hygromiidae, Hygromiinae, Cernuellini	Helicoidea, Hygromiidae, Hygromiinae, *Xerolenta–Xeromunda* group
Xerosecta	Hygromioidea, Hygromiidae, Hygromiinae, Cernuellini	Helicoidea, Hygromiidae, Hygromiinae, *Pyrenaearia–Xerosecta* group
Xerotricha	Hygromioidea, Hygromiidae, Trichinae, Helicellini	Helicoidea, Hygromiidae, Hygromiinae, *Helicella–Candidula* group

C. aspersus) were very close to each other and formed two monophyletic groups, although support for the latter clade was less robust. Interesting but poorly supported nodes, suggesting close relationships between *Eobania vermiculata* and the two *Cantareus* (already suggested by Giusti, Manganelli & Schembri, 1995, on a morphological basis) and between *Marmorana saxetana* and the two populations of *Chilostoma plano-*

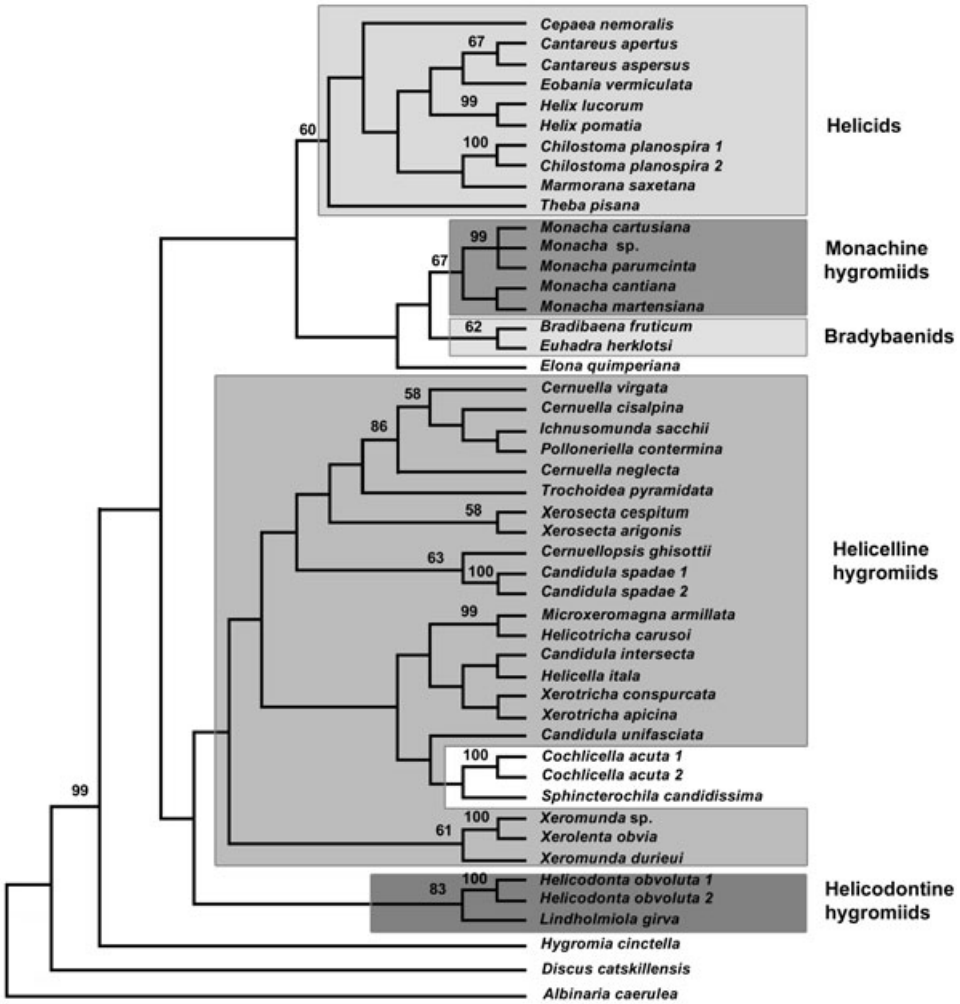

Figure 1. Consensus tree (50% majority rule) from six most parsimonious reconstructions. Bootstrap values >50% are shown above the branches (1000 replications).

spira were also obtained. The last two groups are often assigned to distinct subfamilies. At deeper nodes, *Theba pisana* was always in basal position.

This result perfectly matches that of Wade *et al*. (2001) who showed the monophyletic nature of a rather similar group of helicids (*Arianta*, *Marmorana*, *Helicigona*, *Cantareus*, *Cepaea* and *Theba*) on the basis of different nuclear rDNA gene sequences (5.8S, ITS-2, 28S).

Phylogenetic reconstructions recognized the five *Monacha* species as a monophyletic clade with MP bootstrap support of 67% and posterior probability of 86% in the Bayesian tree. Inside this assemblage, *M. cartusiana*, *Monacha* sp. and *M. parumcinta* were consistently grouped together (MP = 99%, ML = 95%, bpp = 100%). The two species of the Bradybaenidae, although genetically and geographically distant, formed a monophyletic assemblage strongly supported by Bayesian topology (bpp = 100%). These two clades indicated a good phylogenetic resolution for the amplified fragment, at least to this taxonomic rank. On the other hand, the assemblage grouping of the monachine hygromiids with bradybaenids is completely new and requires further verification because it suggests relationships between two morphologically different groups of helicoids. Considering the lack of statistical supports for this node, these relationships are best viewed with caution.

Remarkably, all the phylogenetic analyses consistently suggested paraphyly of the helicelline hygromiids examined in this study (*Candidula*, *Cernuella*, *Cernuellopsis*, *Helicella*, *Helicotricha*, *Ichnusomunda*, *Microxeromagna*, *Polloneriella*, *Trochoidea*, *Xerolenta*, *Xeromunda*, *Xerosecta*, and *Xerotricha*), with significant differences in the three reconstructions. However, lack of support for the internal nodes connecting heli-

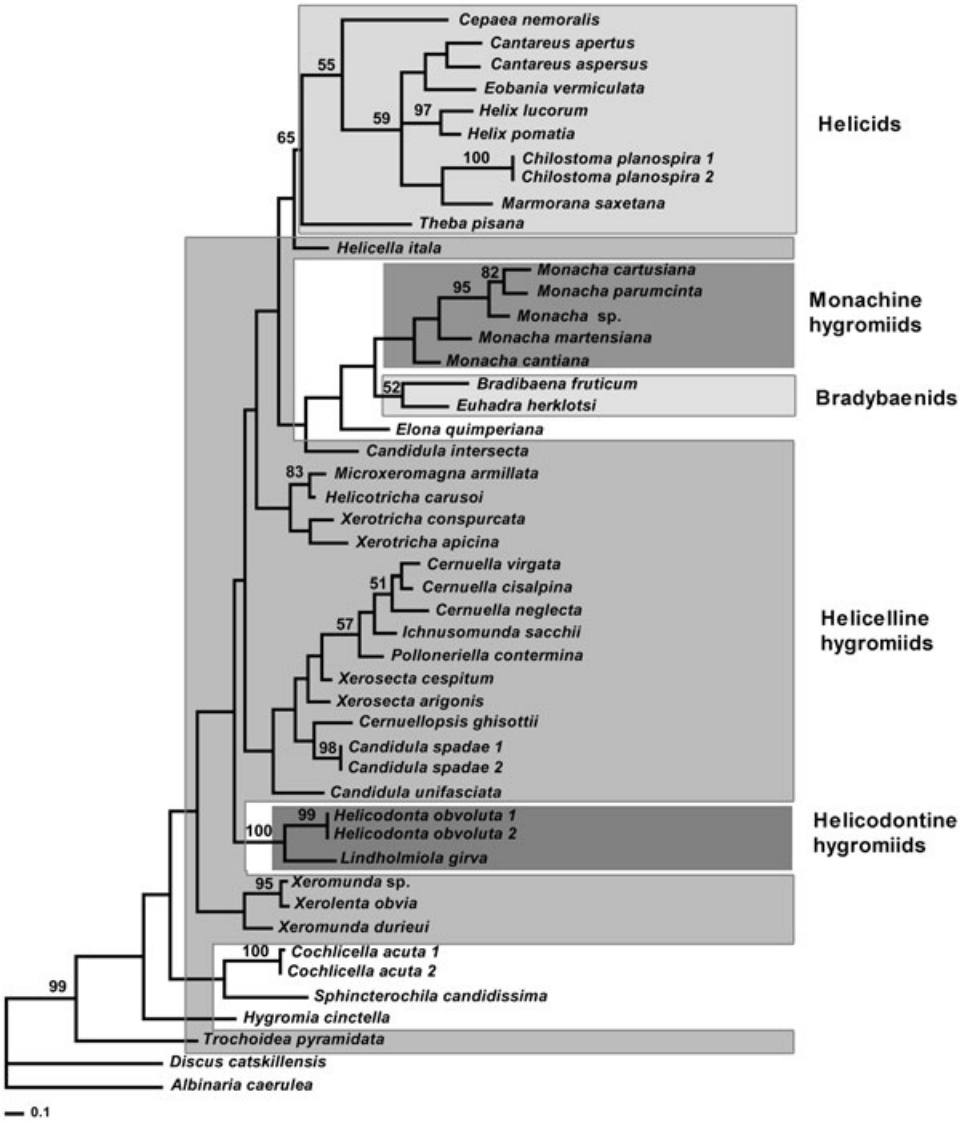

Figure 2. Phylogram from best-fit maximum likelihood analysis using general time reversible model. Bootstrap values obtained via NJ (using ML distance setting) are indicated at nodes with more than 50% support (1000 replications).

celline taxa, along with the short branches connecting these nodes, suggest that our data do not provide a clear resolution for resolving such ancient divergences. To further test helicelline monophyly, we constrained the helicellines to be monophyletic and performed another ML heuristic search. The –lnL of the resulting optimal tree was 6287.12, which was not significantly less likely than the best fit ML tree when tested by Shimodaira–Hasegawa ($P > 0.05$) (Shimodaira & Hasegawa, 1999). Thus, our estimated phylogenies suggest the existence of a heterogeneous assemblage of species within the helicellines, but we cannot confidently reject the possibility that all these taxa have a monophyletic origin.

Nevertheless, among representatives of the helicelline hygromiids, our phylogenetic analyses suggest several interesting clades, though some nodes lack statistical support. Examples include: (1) the assemblage grouping *Xerolenta obvia, Xeromunda* sp. and *Xeromunda durieui*; (2) the three species of *Cernuella*, *Ichnusomunda sacchii* and *Polloneriella contermina*; (3) the two populations of *Candidula spadae* and *Cernuellopsis ghisottii*; (4) the assemblage grouping *Helicotricha carusoi* and *Microxeromagna armillata*.

These results are of particular interest because they confirm most of the anatomically based groups. It is particularly significant that the affinities between certain groups of the helicelline hygromiids with 0 + 2

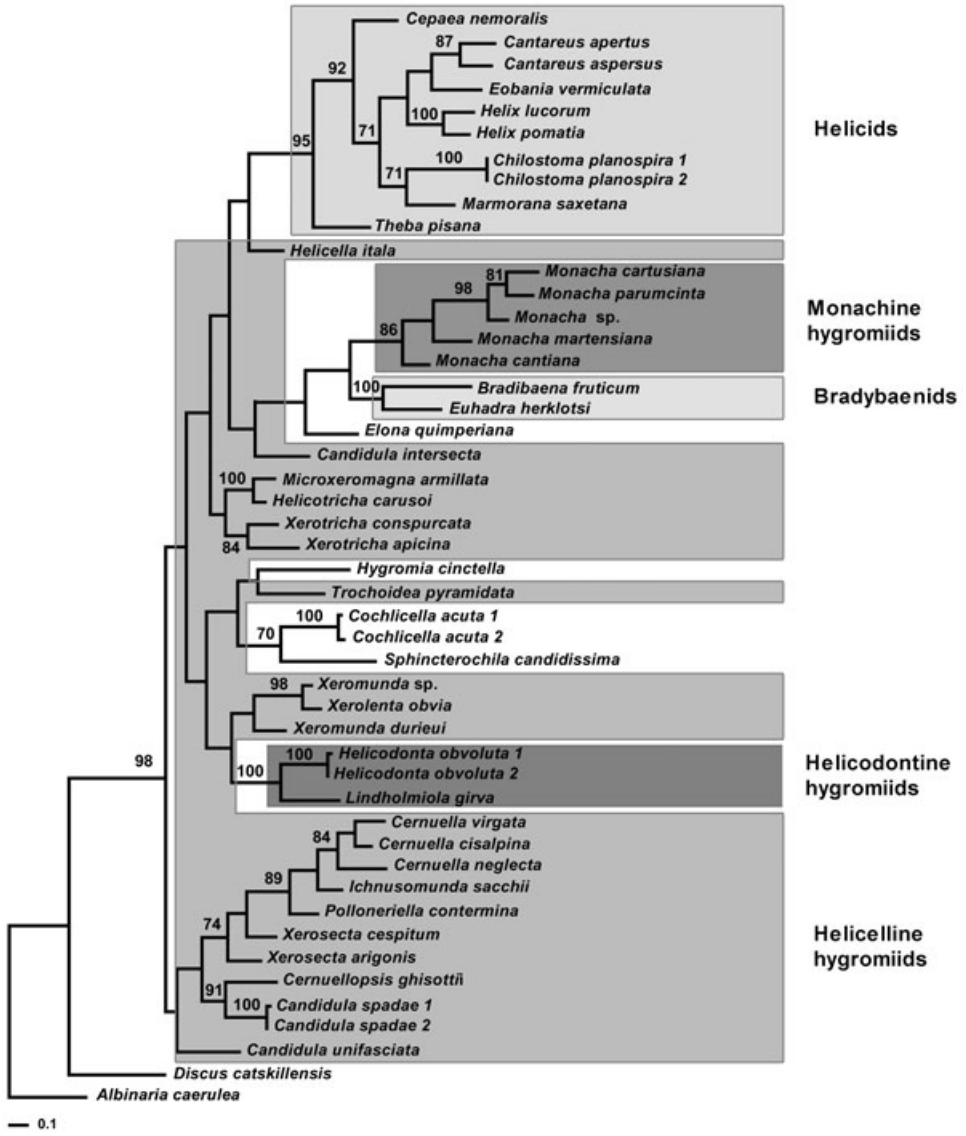

Figure 3. Consensus tree (50% majority rule) from Bayesian analysis. Values at nodes represent the marginal posterior probability of each clade.

dart-sac complex (DSC consisting of two sacs, one inner dartless and one outer dart-secreting, on only one side of the vagina) and certain of those with 2 + 2 dart-sac complex (DSC consisting of four sacs, one inner dartless and one outer dart secreting, on opposite sides of the vagina) have been confirmed. This is true of *Xeromunda* sp. (0 + 2) and *Xerolenta obvia* (2 + 2) and *Microxeromagna armillata* (0 + 2) and *Helicotricha carusoi* (2 + 2). These relationships, postulated by us (Giusti & Manganelli, 1987; Manganelli & Giusti, 1988; Giusti, Manganelli & Crisci, 1992), were disregarded by Schileyko (1991b).

Another interesting result is the fact that a problematic taxon, *Cernuellopsis ghisottii*, characterized by autapomorphic genital features (see Manganelli & Giusti, 1988), received an allocation which could never have been hypothesized on the basis of morphological data. The internal structure of the distal vagina in *C. ghisottii* is quite different from that of the *Candidula* species with which it clusters in our molecular trees and more similar to that of species of *Cernuella*. By contrast, close relationships between *Ichnusomunda sacchii* (another taxon characterized by autapomorphic genital features) and species of *Cernuella* were hypothesized by us on a morphological basis (Giusti & Manganelli, 1998).

Phylogenetic reconstruction failed to detect clear morphologically based relationships in at least two

cases: (1) the three species of *Candidula* that turned out to be grouped separately, and (2) the helicelline hygromiids with pedal penial innervation (*Cernuella*, *Ichnusomunda*, *Cernuellopsis*, *Candidula* and *Helicella*). The latter were grouped with taxa having cerebral penial innervation (*Polloneriella contermina* clustering with *Cernuella* and *Ichnusomunda*, *Candidula intersecta* and *Helicella itala* with *Xerotricha*, etc.).

With respect to the *Candidula* specimens, since morphological affinities among these three species are indisputable (body and genital scheme are identical), an explanation is that molecular results do not reflect the true taxonomic relationships. This absence of resolution was an unexpected result. In fact, 16S sequence data from this study appear, in general, to provide a good phylogenetic resolution within helicoids at the conspecific-congeneric level. On the other hand, our results indicated that the three *Candidula* species are genetically well differentiated, with an observed pattern that correlates with the proposed secondary structure (suggesting that accidental amplification of non-functional nuclear copies did not occur). A possible explanation for this high genetic divergence is that the 16S gene could be evolving faster in the *Candidula* lineage than in the other helicoid species analysed. Although an increased mutation rate seems a speculative explanation, it is interesting to note that a recent research based on 16S data of phenotypes belonging to *C. unifasciata* (presumably representing two different species) showed high genetic divergence, of 'the order of magnitude of those reported between genera of other gastropod species' (Pfenninger & Magnin, 2001). More detailed analyses on this genus are necessary to clarify this possibility.

With respect to helicelline hygromiids with pedal penial innervation, we tested whether this group could be held as a monophyletic clade without resulting in a topology significantly different from the best unconstrained solution. The resulting ML tree was 56.3 log units worse than the best unconstrained topology and was clearly rejected by Shimodaira–Hasegawa ($P < 0.05$). Two different hypotheses can be invoked to explain any discrepancy between molecular phylogeny and morphology-based reconstruction. Assuming that morphological systematics gives a true representation of phylogenetic relationships among these taxa, one may argue that the lack of correspondence for mtDNA phylogeny is due to stochastic lineage sorting. However, assuming that mtDNA reconstruction accurately represents phylogenetic relationships, the observed-non-monophyly would indicate that different innervation of the penis (from right pedal or cerebral ganglion) is irrelevant for phylogenetic reconstructions. These two explanations are interesting alternatives for future investigations.

All reconstructions strongly suggest monophyly for the clade grouping the two populations of *Helicodonta obvoluta* with *Lindholmiola girva* (both helicodontine hygromiids), although this assemblage is placed differently in the three topologies.

Finally, our analysis failed to resolve the affinities of *Elona quimperiana* and *Cochlicella acuta*. The former is an enigmatic helicoid of uncertain relationship (Zilch, 1960; Gittenberger, 1979; Nordsieck, 1987; Schileyko, 1991b; Puente & Altonaga, 1995). The latter is usually considered to have relationships with the monachine hygromiids (Zilch, 1960; Nordsieck, 1993), but recently Schileyko & Menkhorst (1997) claimed that cochlicellids must be assigned to a distinct family (Cochlicellidae) belonging to the superfamily Xanthonichoidea (to which *Sphincterochila* might also be related, according to Muratov, 1999). The present analysis placed *Cochlicella acuta* close to *Sphincterochila*, but without statistical support. This allocation is in agreement with Schileyko & Menkhorst (1997).

Absence of support for internal nodes

Even though the BI, and to a lesser extent the ML and MP trees recognized most currently accepted higher helicoid taxa, basal relationships are not sufficiently resolved in any of the analyses, suggesting that the amplified fragments do not provide enough information to detect relationships at this level.

Such problems may be alleviated by increasing the number of characters, under the assumption that additional nucleotides follow the same evolutionary patterns (Berbee, Carmean & Winka, 2000). A possible solution might therefore be to increase our data set by examining the complete 16S gene, but the 5′ half of the 16S rRNA gene is presumably too variable for our purposes (Gutell, Schnare & Gray, 1992; Simon *et al.*, 1994; Lydeard *et al.*, 1998). Alternatively, data from more conservative genes are needed to increase the support of deeper nodes. On the other hand, if the observed lack of phylogenetic resolution reflects a rapid helicoidean radiation, probably no amount of data would improve statistical support at deeper nodes.

To date, partial sequences from the mitochondrial 16S rRNA gene provide insights into the evolutionary history of several stylommatophoran species group. Resolution of relationships among different lineages is useful to test morphological hypotheses and to formulate new hypotheses for further morphological studies.

ACKNOWLEDGEMENTS

We thank A. Daviddi for technical assistance, H. Ampt revised the English, A. Boato, M. Calcagno, G. Cappelli, S. Cianfanelli, C. Dalfreddo, L. Favilli, D. Ferreri, B. G. Gomez, S. Hallgass, L. Manganelli, A.

Mazzeschi, S. Piazzini, C. E. Prieto, M. Sant, E. Talenti and M. Zatini for field collection, and T. Wilke for numerous helpful comments which greatly improved the accuracy and quality of this paper. This research was funded by grants from University of Siena, PAR 2001, project 'I Molluschi-non-marini della fauna italiana: filogenesi, sistematica, faunistica, zoogeografia, conservazione'.

REFERENCES

Berbee ML, Carmean DA, Winka K. 2000. Ribosomal DNA and resolution of branching order among the Ascomycota: how many nucleotides are enough? *Molecular Phylogenetics and Evolution* **17:** 337–344.

Cannone JJ, Subramanian S, Schnare MN, Collett JR, D'Souza LM, Du Y, Feng B, Lin N, Madabusi LV, Muller KM, Pande N, Shang Z, Yu N, Gutell RR. 2002. The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *Bioinformatics* **3:** 2.

Emberton KC, Kuncio GS, Davis GM, Phillips SM, Monderewicz KM, Guo YH. 1990. Comparison of recent classifications of stylommatophoran land-snail families, and evaluation of large-ribosomal-RNA sequencing for their phylogenetics. *Malacologia* **31:** 327–352.

Gantenbein B, Fet V, Largiadèr CR, Scholl A. 1999. First DNA phylogeny of *Euscorpius* Thorrell, 1876 (Scorpiones: Euscorpiidae) and its bearing on taxonomy and biogeography of this genus. *Biogeographica (Paris)* **75:** 49–65.

Gittenberger E. 1979. On *Elona* (Pulmonata, Elonidae Fam. Nov). *Malacologia* **18:** 139–145.

Giusti F, Manganelli M. 1987. Notulae Malacologicae XXXVI. On some Hygromiidae (Gastropoda: Helicoidea) living in Sardinia and in Corsica. (Studies on the Sardinian and Corsican Malacofauna VI). *Bollettino Malacologico* **23:** 123–206.

Giusti F, Manganelli M. 1998. *Ichnusomunda sacchii,* a new hygromiid snail from Sardinia Island (Western Mediterranean): an intriguing case of homoplasy in the anatomical organization (Pulmonata, Hygromiidae). *Veliger* **41:** 319–332.

Giusti F, Manganelli M, Crisci JV. 1992. A new problematical Hygromiidae from the Aeolian Islands (Italy) (Pulmonata: Helicoidea). *Malacologia* **34:** 107–128.

Giusti F, Manganelli G, Schembri PJ. 1995. The nonmarine molluscs of the Maltese Islands. *Monografie Museo Regionale di Scienze Naturali* (Torino), 15.

Giusti F, Manganelli G, Selmi MG. 1992. Spermatozoon fine structure in the phylogenetic study of the Helicoidea (Gastropoda, Pulmonata). In: Meier-Brook C, ed. *Proceedings of the Tenth International Malacological Congress. (Tübingen, 1989).* Baja, Hungary: Unitas Malacologica, 611–616.

Gutell RR, Schnare MN, Gray MW. 1992. A compilation of large subunit RNA sequences (23S and 23S-like) ribosomal RNA structures. *Nucleic Acids Research* **20:** 2095–2109.

Hatzoglou E, Rodakis GC, Lecanidou R. 1995. Complete sequence and gene organization of the mitochondrial genome of the land snail *Albinaria coerulea*. *Genetics* **140:** 1353–1366.

Hausdorf B. 1998. Phylogeny of the Limacoidea *sensu lato* (Gastropoda: Stylommatophora). *Journal of Molluscan Studies* **64:** 35–66.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83:** 189–195.

Huelsenbeck JP, Ronquist FR. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754–755.

Lydeard C, Holznagel WE, Schnare MN, Gutell RR. 2000. Phylogenetic analysis of molluscan mitochondrial LSU rDNA sequences and secondary structures. *Molecular Phylogenetics and Evolution* **15:** 83–102.

Lydeard C, Yode JH, Holznagel WE, Thompson FG, Hartfield P. 1998. Phylogenetic utility of the 5′ half of the mitochondrial 16S rDNA gene sequences for inferring relationships of *Elimia* (Cerithioidea: Pleuroceridae). *Malacologia* **39:** 183–193.

Manganelli G, Giusti F. 1988. Notulae Malacologicae, XXXVIII. A new Hygromiidae from the Italian Apennines and notes on the genus *Cernuella* and related taxa (Pulmonata: Helicoidea). *Bollettino Malacologico* **23:** 327–379.

Muratov IV. 1999. Analysis of the phylogenetic relationships and their systematic implications in the Limacoinei (= Zonitinia) infraorder (Gastropoda, Pulmonata, Geophila). *Ruthenica* **9:** 5–26.

Nordsieck H. 1987. Revision des System der Helicoidea (Gastropoda: Stylommatophora). *Archiv für Molluskenkunde* **118:** 9–50.

Nordsieck H. 1993. Das System der paläarktischen Hygromiidae (Gastropoda: Stylommatophora: Helicoidea). *Archiv für Molluskenkunde* **122:** 1–23.

Pfenninger M, Magnin F. 2001. Phenotypic evolution and hidden speciation in *Candidula unifasciata* spp. (Helicellinae, Gastropoda) inferred by 16S variation and quantitative shell traits. *Molecular Ecology* **10:** 2541–2554.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Puente AI, Altonaga K. 1995. Revisión de las especies ibéricas de la familia Xanthonychidae (Gastropoda: Pulmonata: Helicoidea). *Butlletí de la Institució Catalana d'Historia Natural* **63:** 85–101.

Schileyko AA. 1979. Sistema otrjada Geophila (= Helicida) (Gastropoda Pulmonata) [The system of the order Geophila (= Helicida) (Gastropoda Pulmonata)]. *Trudy Zoologicheskogo Instituta* **80:** 44–69 [in Russian].

Schileyko AA. 1991a. Vodrosy filogenii vysšich Pulmonata [problems of the phylogeny of higher Pulmonata]. *Ruthenica* **1:** 3–16 [in Russian].

Schileyko AA. 1991b. Taxonomic status, phylogenetic relations and system of the Helicoidea sensu lato (Pulmonata). *Archiv für Molluskenkunde* **120:** 187–236.

Schileyko AA, Menkhorst HPMG. 1997. Composition and phylogenetic relations of the Cochlicellidae (Gastropoda, Pulmonata). *Ruthenica* **7:** 51–60.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular and Biological Evolution* **16:** 1114–1116.

Simon C, Frati F, Beckenbach AT, Crespi B, Liu H, Flook P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87:** 651–701.

Sokolov EP. 2000. An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues. *Journal of Molluscan Studies* **66:** 573–575.

Swofford DL. 2001. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, vers. 4. Sunderland, MA: Sinauer Associates.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Titus TA, Frost DR. 1996. Molecular homology assessment and phylogeny in the lizard family Opluridae (Squamata: Iguania). *Molecular and Biological Evolution.* **6:** 49–62.

Wade CM, Mordan PB. 2000. Evolution within the gastropod molluscs: using the ribosomal RNA gene cluster as an indicator of phylogenetic relationships. *Journal of Molluscan Studies* **66:** 565–570.

Wade CM, Mordan PB, Clarke B. 2001. A phylogeny of the land snails. *Proceedings of the Royal Society of London B* **268:** 413–422.

Yamazaki N, Ueshima R, Terrett JA, Yokobori S, Kaifu M, Segawa R, Kobayashi T, Numachi K, Ueda T, Nishikawa K, Watanabe K, Thomas RH. 1997. Evolution of pulmonate gastropod mitochondrial genomes: comparisons of complete gene organization of *Euhadra*, *Cepaea* and *Albinaria* and implications of unusual tRNA secondary structures. *Genetics* **145:** 749–758.

Zilch A. 1960. Euthyneura. In: Schindewolf OH, ed. *Handbuch der Paläozoologie.* Band 6, Teil 2 (Lieferung 4). Leipzig: Quelle & Meyer, i–xii + 601–835.