Resolving riddles and presenting new puzzles in Chonorinidae Phylogenetics

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The family Chondrinidae (Gastropoda, Pulmonata)

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INTRODUCTION

ORTHURETHRA

In molluscan taxonomy, the suborder Orthurethra Pilsbry, 1900 (Mollusca, Pulmonata) has been a relatively stable taxon as compared to other higher gastropod taxa. Several authors have addressed its interpretation (Pokryszko, 1994 and references therein) and it is morphologically well characterized. The main apomorphic character separating this group from the other Stylommatophora is the structure of the kidney. In the Orthurethra it consists of two parts, one of which is homologous to the kidney found in the other Stylommatophora.

CHONDRINIDAE

In the first large-scale molecular studies on land snails, including the Orthurethra, the monophyly of the suborder was confirmed (Wade et al., 2001, 2006). The Chondrinidae occupy a basal position in the orthuretran clade. The genetic distances between the Chondrinidae and the remaining orthuretran families are relatively large in comparison to distances within the latter group. All this suggests that we deal with a relatively ancient lineage. This is supported by the fossil record. The earliest fossils known (Wenz, 1923, p. 950) are from the Lutetian (Eocene), i.e. at least 40.4 ± 0.2 Myr old.

The Chondrinidae have previously been considered to encompass four subfamilies (Zilch, 1959), viz. Gastrocoptinae Pilsbry 1916, Hypselostomatinae Zilch, 1959, Aulacospirinae Zilch, 1959, and Chondrininae Steenberg, 1925. The first three consist of taxa that are represented outside Europe, although the Gastrocoptinae are well known from the European fossil record as well (Zilch, 1959). The Chondrininae, as interpreted by Zilch (1959) and Gittenberger (1973), consist of taxa from the western Palaearctic. Wade et al. (2006) have convincingly demonstrated that this taxon is not closely related to the alleged other chondrinid subfamilies and so confirmed earlier (Schileyko, 1998a, b) views that they should be considered separate families.

The family Chondrinidae, as interpreted here, consists of about 60 species, that are classified in six genera (Gittenberger, 1973), viz. *Abida* Leach, 1831, *Chondrina* Reichenbach, 1828, *Rupestrella* Monterosato, 1894, *Solatopupa* Pilsbry, 1917, *Granaria* Held, 1838, and the monotypic genus *Granopupa* Boettger, 1889 (fig. 1). All species are obligate limestone dwellers that occur on moist soils and vegetation (always on or near limestone rock) (*Abida*, *Granaria*), dry sheltered habitats (*Granopupa*) or more or less exposed, limestone cliffs (*Rupestrella*, *Chondrina* and *Solatopupa*). The genera differ conspicuously in species numbers and in their biogeography.

Figure **1**. Representatives of the land snail family Chondrinidae. **A**, *Solatopupa similis* (Bruguière, 1792), France, Alpes Marittimes, Mercantour. **B**, *Chondrina burtscheri* Falkner & Stummer, 1996, Italy, Mauls. **C**, *Granaria stabilei* (E. von Martens, 1865), Italy, Alpi Marittime, Palanfré. **D**, *Rupestrella rhodia* (Roth, 1839), Greece, Thasos. **E**, *Chondrina maginensis* Arrébola & Gómez, 1998, Spain, Andalucia, Sierra Magina. **F**, *Abida secale secale* (Draparnaud, 1801), Belgium, Limburg, Lanaye.

Granopupa granum (Draparnaud, 1801) has by far the largest range of all chondrinid species and by itself defines most of the distributional range of the family. It occurs from the Canary Islands in the west, circum-mediterranean to Pakistan and Afghanistan in the East. The occurrence on the remote, Atlantic, Ascension island, ".. may possibly have reached Ascension naturally on birds or by drifting in air currents." (Ashmole & Ashmole, 1997: 296), might not be natural after all. Several other small European snails occur on the island, viz. *Cochlicopa* spec., *Lauria cylindracea* (Da Costa, 1778), *Vallonia excentrica* Sterki, 1892, *V. pulchella* (Müller, 1774), *Vertigo pygmaea* (Draparnaud, 1801), Zonitidae spec. However, the adaptive radiation of *Balea* species in the Tristan-Gough archipelago, even more to the south (Preece & Gittenberger, 2003; Gittenberger et al., 2006), shows that aerial dispersal of small snails cannot be excluded.

The northern boundary of the distributional range of the Chondrinidae is marked by the Swedish islands of Öland and Gotland, where *Chondrina arcadica clienta* (Westerlund, 1883) is found. *Chondrina* ranges from there eastwards to the Caucasus and Iran and southwards to Greece, Italy, the Iberian peninsula and Morocco. The westernmost records for *Chondrina* are from the U.K.

Most of the 12 *Abida* species are restricted to parts of the Pyrenees or the Cantabrian mountains (Gittenberger, 1973; Kokshoorn & Gittenberger, [chapter 6]). Only *A. secale* (Draparnaud, 1801) is widespread and occurs additionally in large parts of western and central Europe, whereas *A. polyodon* reaches from the Pyrenees northwards as far as the southwesternmost Alps. The centers of diversity in *Rupestrella* lie in N-Africa, with a poorly known radiation in Algeria and Tunisia (Pilsbry, 1918) and especially Sicily (Beckmann, 2002). Two species, i.e. *R. philippii* (Cantraine, 1840) and *R. rhodia* (Roth, 1839), are widespread, occurring from southern Spain and the Balearic Islands in the west to Italy, Greece, Turkey, Libya, and Israel more to the east. *Rupestrella dupotetii* (Terver, 1839) has a smaller range, reaching from southern Spain (Arrebola Burgos & Gittenberger, 1994) to northern Africa (Pilsbry, 1918; Holyoak & Seddon, 1986).

Representatives of the genus *Solatopupa* occur in Corsica and Sardinia, and in a relatively narrow zone of the southwestern European mainland, from Italy to southern France and northeastern Spain (Gittenberger, 1973; Ketmaier et al., 2006). There is no obvious center of diversity in this genus.

The seven species of *Granaria* show a disjuct distribution. *Granaria brauni* (Rossmässler, 1842), *G. frumentum* (Draparnaud, 1801), *G. variabilis* (Draparnaud, 1801) and *G. stabilei* (Von Martens, 1865) occur in southern and central Europe, separated by a gap of approximately 4,000 km from *G. arabica* (Dohrn, 1860), *G. lapidaria* (Hutton, 1849) and *G. persica* Gittenberger, 1973, that are known from Oman, Iran, Pakistan, and Yemen (Gittenberger, 1973).

Gittenberger (1973) proposed a phylogeny reconstruction of the chondrinid genera on the basis of conchological and genital-morphological characters. In his clas-

sification *Granopupa* takes a basal position because of the symplesiomorphy in the structure of the male part of the genital tract, a character state shared with the 'Gastrocoptinae', which were taken as the outgroup of the 'Chondrininae'. However, the molecular data invalidated the gastrocoptids as sistergroup of the Chondrinidae, suggesting that the combined other Orthurethra should be considered as such.

Here we present a reconstruction of the phylogenetic relationships between the genera of the Chondrinidae at genus level on the basis of molecular data.

Material and methods

Samples and DNA extraction

For the six genera 12 species were selected (Table 1), including the type species of each genus. It is still unclear whether *Granaria frumentum* should be considered a polytypic species, with *G. f. frumentum*, *G. f. illyrica* (Rossmässler, 1835) and *G. f. appenina* (Küster, 1850) as subspecies, or a group of three closely related species. In the absence of new information, we here accept the former view, in accordance with Gittenberger (1973). Unfortunately we were unable to obtain sequence data for the nominal subspecies. Hence *G. f. illyrica* has been chosen to represent the (sub)species complex.

Ena montana (Draparnaud, 1801) (Orthurethra, Enidae) was selected as outgroup taxon. The material used in this study was already available in the collection of the National Museum of Natural History in Leiden, The Netherlands (= NNM), or was sampled during the period 2004-2006 and is now also kept in that institute (Table 1). Additional material was obtained from Messrs. H. Mienis (Hebrew University, Jerusalem, Israel) and the late K.H. Beckmann (Ascheberg-Herbern, Germany). Sequence data concerning *Solatopupa* species and *Chondrina avenacea*, were obtained from Genbank (after Ketmaier et al., 2006: Accession numbers DQ305042-DQ305101).

Snail shells were broken in two parts to extract the snail tissue. Shell remains, including the undamaged aperture, which may be diagnostic, were stored as vouchers. The snail tissue was then sliced in pieces and genomic DNA was extracted using the E.Z.N.A. Mollusc DNA kit (Omega Bio-tek) following the manufacturers protocol. Final elution of the DNA was done in 100 µl of provided buffer. Four markers were (partially) sequenced, i.e. two mitochondrial markers (Cytochrome Oxidase subunit 1 and 16s) and two nuclear markers (28s and Histone H3) (Table 2).

Table **1**. Samples used in this study. The sample of *R. rhodia* was kindly provided by Mr. H. Mienis. The other *Rupestrella* samples were kindly put at our disposal by the late Mr. K.-H. Beckmann.

Species	RMNH	Locality	year	alc/ dry	COI	16s	H3	28s
Granopupa granum	99034	Spain, Cataluña, Escalo	2004	A	EU857460	EU857435	EU857470	EU857447
Granaria brauni	99035	Spain, Cataluña, Llavorsi	2004	A	EU857461	EU857436	EU857471	EU857448
Granaria variabilis	99074	France, Drôme, Andancette	2004	A	-	EU857437	EU857472	EU857449
Granaria frumentum illyrica	102867	Italy, Brescia, Toscolano valley	2006	A	EU857462	EU857438	EU857473	EU857450
Granaria stabilei	105232	France, Drôme, Rosans	2006	A	EU857463	EU857439	EU857474	EU857451
Solatopupa similis	105229	France, Drôme, St. May	2006	A	EU857464	EU857440	EU857475	EU857452
Chondrina maginensis	103205	Spain, Andalucia, Jean, Sierra de Magina	2006	A	EU857465	EU857441	EU857476	EU857453
Chondrina tenuimarginata	102433	Spain, Cataluña, Castellar de N'uch	2005	A	EU857466	EU857442	EU857477	EU857454
Abida secale secale	105219	France, Jura, Poligny	2005	A	EU395404	EU857443	EU857478	EU857455
Rupestrella rhodia	99583	Israel, Bet Ariah	2004	A	EU857467	EU857444	EU857479	EU857456
Rupestrella occulta gibilfunnensis	102324	Sicily, Bellocampo – San Calogero	2003	D	EU857468	EU857445	EU857480	EU857457
Rupestrella rupestris	102321	Sicily, Monte Pellegrino	2003	D	EU857469	EU857446	EU857481	EU857458
Ena montana (outgroup)	105215	France, Haute-Saône, Gy	2006	A	-	-	EU857482	EU857459

Marker	Fragment length	Primer pair and PCR program	PCR program
Cytochrome Oxidase subunit I	660 bp (40 bp excluded)	H2198-Alb (Uit de Weerd et al., 2004) 5′-ACT CAA CGA ATC ATA AAG ATA TTG G-3′ L1490-Alb (Uit de Weerd et al., 2004) 5′-TAT ACT TCA GGA TGA CCA AAA AAT CA-3′	94°-5′, 40x(94°-30″, 49°-30″, 72°-1′), 72°-5′
16s	331 bp	16s-109 (Ketmaier et al., 2006) 5′-CCT TGA CTG TGC AAA GGT AGC-3′ 16s-421 (Ketmaier et al., 2006) 5′-TAG GCC CTA ATC CAA CAT CG-3′	94°-20″, 40x(94°-20″, 50°-30″, 72°-30″), 72°-5′
28s	287 bp	GRA.4338.F (newly developed) 5'-GGT CCG CGG AAT TCA GCG CGG-3' GRA.4615.R (newly developed) 5'-GGT CGG GAG ACA CGG TTG CCC-3'	94°-2′, 35x(95°-1′, 50°-30″, 72°-1′), 72°-2′
Histone H3	329 bp (40 bp excluded)	H3F (Colgan et al., 2000) 5′-ATG GCT CGT ACC AAG CAG ACV GC-3′ H3R (Colgan et al., 2000) 5′-ATA TCC TTR GGC ATR ATR GTG AC-3′	94°-2′, 35x(95°-1′, 50°-30″, 72°-1′), 72°-2′

PCR products were cleaned using the Promega Wizard PCR cleaning kit and subsequently sequenced on a Megabace 1000, 96 capillary sequencer.

Phylogenetic analyses

Forward and reverse chromatograms were aligned using Chromas Pro (Technelysium, Australia). In most cases ambiguous positions could easily be resolved. If not, IUPAC ambiguity codes were used. Consensus sequences were exported as FASTA files and subsequently imported in BioEdit v7.0.0 (Hall, 1999). The Clustal W plugin was then used to calculate the optimal alignment, using the default settings. The alignment was exported in NEXUS file format. This file was then opened in MacClade v4.04 (Maddison & Maddison, 2002). This program was used to manually check the alignment for possible editing errors which were compared to the chromatogram files. Primer sites were identified and excluded from the analyses. For COI, Histone H3 and 28s, 20 basepairs of the sequences directly next to the primer sites were also excluded. This was done to increase the reliability of the dataset, since the terminal parts of the chromatograms were of low quality in many cases. Sequences were deposited at NCBI GenBank (for accession numbers see table 1).

For the protein coding sequences (COI and Histone H3) the codon positions were calculated by minimizing stop codons using the Drosophila mitochondrial code (COI) and the Universal genetic code (Histone H3).

A partition homogeneity test was performed as implemented in PAUP* (Swofford, 2002). The four markers were pairwise compared for compatible phylogenetic signal.

Several different combinations of the data were then analysed. The first was an alignment of 13 taxa (12 taxa plus the outgroup, *E. montana*) with the Histone H3 and 28s markers. This was done to determine the root of the tree. Secondly, an alignment was made of this same set of taxa with all four markers, excluding the outgroup *E. montana*. An alignment of 27 taxa for the four markers was constructed, including the data of Ketmaier et al. (2006). Their 12s data were added to this datamatrix. This dataset was also analysed without *Chondrina avenacea* and the 12s data.

A G1-skewness test (Hillis & Huelsenbeck, 1992) based on 1,000 random trees was used to test for phylogenetic signal. Saturation of the datasets was checked for all datasets, as well as the three codon positions separately in Histone H3 and COI. Transitions were plotted against transversions both including and excluding the outgroups. For the fast evolving genes COI and 16s the saturation was also checked within and between genera. Additionally, Nei's test for saturation was performed using the program DAMBE (Xia & Xie, 2001). Base frequencies were checked for the individual codon positions and for the entire dataset using chi-square statistics

Table **2**. Technical data for each marker used.

implemented in Paup*.

For the datasets the same phylogenetic and phenetic analyses were performed using PAUP*. The optimal model for nucleotide evolution was calculated for each partition using MrModeltest v2.2 (Nylander, 2004). A Neighbor Joining tree was calculated and subsequently bootstrapped with 1,000 replicates using uncorrected p-distances. A parsimony bootstrap was performed using 1,000 replicates and full heuristic search.

The optimal models for character evolution were applied with Bayesian analysis in MrBayes v3.01 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Here 5 incrementally heated chains were used next to a cold one. The program was run until the average standard deviation between the two simultane runs was below 0.01 for a minimum of 1,000,000 generations. An additional 1 million generations were then run with a sample frequency of 1000. The initial trees were discarded as burnin and a 50% majority rule consensus of the 2,000 remaining trees (from both runs) was created using the sumt option in MrBayes.

The resulting trees from all analyses were combined into a single consensus tree. Maximum Parsimony Reconstruction (MPR) methodology was used to estimate the consensus. Binary coding of the topologies was perfomed using Baum-Ragan coding (Baum, 1992; Ragan, 1992). Binary characters were weighted using decimal clade support values derived from the neighbor joining and parsimony bootstrap and Bayesian analyses (Ronquist, 1996). Although not completely comparable due to reported over estimation of support by Bayesian analyses (Erixon et al., 2003; Simmons et al., 2004; but see Wilcox et al, 2002; Taylor & Piel, 2004), clade probabilities were considered equal to those obtained from bootstrap. This will therefore result in slight overweighting of the Bayesian topology.

The topology of the consensus tree was set as a constraint in MrBayes. An identical run as described above (but with 3 million generations) was then perfomed. The last 100 saved trees were used from both simultane runs, resulting in a dataset of 200 likelihood values. The resulting likelihood values of this set were compared with those of a constrained MrBayes run using the topology from the unconstrained Bayesian analysis. A paired t-test was used to test for differences between both sets of likelihood values.

A constraint was also set up for the topology of *Solatopupa* as presented by Ketmaier et al. (2006).

The molecular clock hypothesis was tested using the Likelihood Ratio Test as implemented in DAMBE. The overall consensus tree was used as the input topology and the General Time Reversable (GTR) model was selected.

Molecular clock analyses were performed using BEAST v1.4 (Drummond & Rambaut, 2007). The dataset was partitioned and a specific model was assigned to each partition. The uncorrelated relaxed lognormal clock model (Drummond et al., 2006) was selected. Two pre-runs were performed with 1 million generations to optimize priors. The final run was performed with 10 million generations.

The tree with the highest likelihood score was selected using TreeAnnotator v1.4 (Drummond & Rambaut, program available from the authors), which was subsequently edited using FigTree v1.2 (Drummond & Rambaut, program available from the authors).

Two alternative hypotheses were tested. Several fossil taxa are known that are considered to be representatives of the Chondrinidae. Most of these are provisionally assigned to *Granaria* (Wenz, 1923; Gittenberger, 1973). The oldest fossils known are those of *G. bythiniformis* (Miller, 1907) from the Eocene, Lutetian (40.4±0.2 - 48.6±0.2 Mya); Bachhagel, Bayern, Gemany) and *G. multicostulata* (Gutzwiller, 1905) from the upper Lutetian (Lausen, Basel, Switserland). The *Granaria* bauplan is not diagnostic for the generic placement of these fossils because it also occurs in *Granopupa*. Therefore, we can only say with some confidence that the ancestor of the Chondrinidae had a *Granaria*-like shell. The fragmentary but rather continuous fossil record for the family shows the *Granaria*-bauplan for all the fossils, up to the Pleistocene. Only from pleistocene deposits taxa that can be assigned to other extant genera are known (Gittenberger, 1984 and references therein). The oldest fossil shells of extant species are early Pleistocene, 1.3 Mya, specimens of *Chondrina guiraoensis* Pilsbry, 1918, from Valencia, Spain (Montoya et al., 2001), and an upper Miocene (Pontian) specimen of *Granaria frumentum* from Nagyvazsony (Veszprim, Hungary) from 5.5-9 Mya (Wenz, 1923, p. 943-944). However, since both taxa are absent from the current dataset, these fossils could not be used for calibration.

This leaves us without much information about the nodes to be calibrated on the basis of the fossil record. Two approaches were followed. First we used the oldest known fossils as a calibration point for the root of the tree. A minimum age of 40.4 My was accepted to estimate divergence times in the family, assuming that the radiation of the extant chondrinid taxa is as old as the oldest known fossils. This is disputable, since the oldest fossils might represent an extinct radiation that pre-dates that of the extant species. Therefore, a second scenario was tested, starting from a hypothesis on the historical biogeography of the genus *Rupestrella*. The two main radiations within that genus are found in northern Africa (Algeria and Tunisia) and the Italian island of Sicily. These areas have been connected by a landbridge during the Messinian salinity crisis, when sea levels in the Mediterranean were much lower than today. The Messinian was a period of sea level changes in the Mediterranean, what might have triggered allopatric speciation events in *Rupestrella*. The radiation of the species that occur in Sicily is represented by *R. rupestris* (Philippi, 1836) and *R. occulta gibilfunnensis* (De Gregorio, 1895). We assume a vicariance event between these two taxa and *R. rhodia* from mainland Europe, at the end of the Messinian, i.e. 5.3 Mya ago. This age was used for the ancestral node for these three taxa.

Results and conclusion

The saturation test indicated that both the 28s and the Histone H3 datasets do not suffer from saturation, even when the outgroup is included. However, COI and 16s are heavily saturated when compared across all taxa. They are only slightly or not saturated at the generic level. It was therefore decided not to use these individual datasets for phylogenetic reconstruction, but only in combination with the 28s and Histone H3 data. Hence only a subset of 12 taxa, for which all four markers were available, were used in the subsequent analyses.

The partition homogeneity test revealed no significant difference in phylogenetic signal between the four partitions ($p>0.05$). Therefore they could be combined in a single analysis.

The analysis of the Histone H3-28s dataset including the outgroup, *Ena montana,* supported the deep phylogenetic split that was already observed by Wade et al. (2006). There are three genera in each clade, viz. *Granopupa*, *Granaria* and *Solatopupa* next to *Chondrina*, *Abida* and *Rupestrella* (fig. 2). We will refer to these clades as Granariinae and Chondrininae, respectively, but this text is not issued for purposes of zoological nomenclature (see ICZN Art. 8.2, and Kokshoorn & Gittenberger, [Chapter 8, pp. 115 and 119]).

The basal phylogenetic split is supported by morphological characters. The species of the Granariinae have shells with a single, main, palatal fold in the aperture, whereas in the Chondrininae there are two, about equally prominent, main palatal folds. In both groups however, the palatal folds may be more or less completely reduced (in the taxa with species occurring in the most extreme habitat, on exposed rockfaces). In the Chondrininae the genital flagellum is absent or strongly reduced and the pedunculus is short. The Granariinae always have a (long) flagellum and pedunculus, and the bursa of the bursa copulatrix borders the glandula albumifera.

The genus *Granaria* appears to be paraphyletic (Fig. 3a), but not in all analyses. Therefore, the consensus tree shows low support values for that scenario. *Solatopupa* shows up as monophyletic in the analyses with the data from Ketmaier et al. (2006) included. The phylogenetic relations within the Chondrininae are well supported. In all analyses the combined *Abida* and *Rupestrella* form the sister clade of *Chondrina*.

The Bayesian analyses using the constraint topologies resulted in two sets of 200 likelihood values for each analysis. The 400 likelihood values from each analysis were pooled. The differences between the likelihood values of the three alternative phylogenetic scenario's for the genus *Solatopupa* (including the results presented by Ketmaier et al. (2006)) were tested. The differences in likelihood values between the three scenario's were not significant ($p=0$). The tree with the highest total posterior probabilities on the internal nodes (from the 'unconstrained' set) is shown in figure 3b.

The test for comparable evolutionary rates in separate branches, showed highly significant ($p=0$) differences. Hence the mutation rate varies substantially between

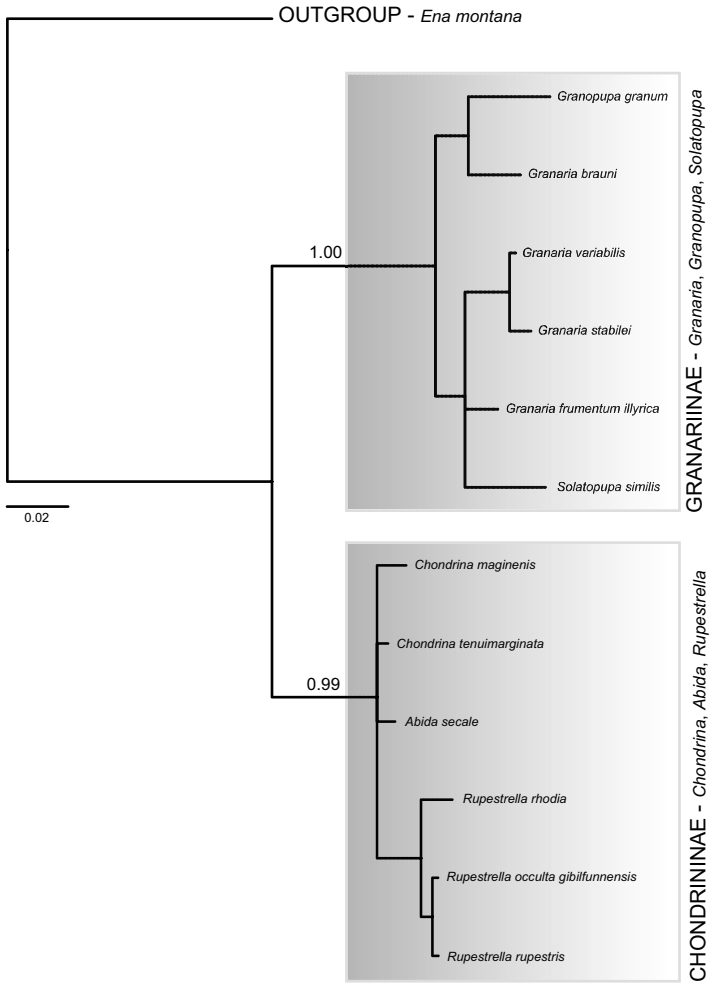
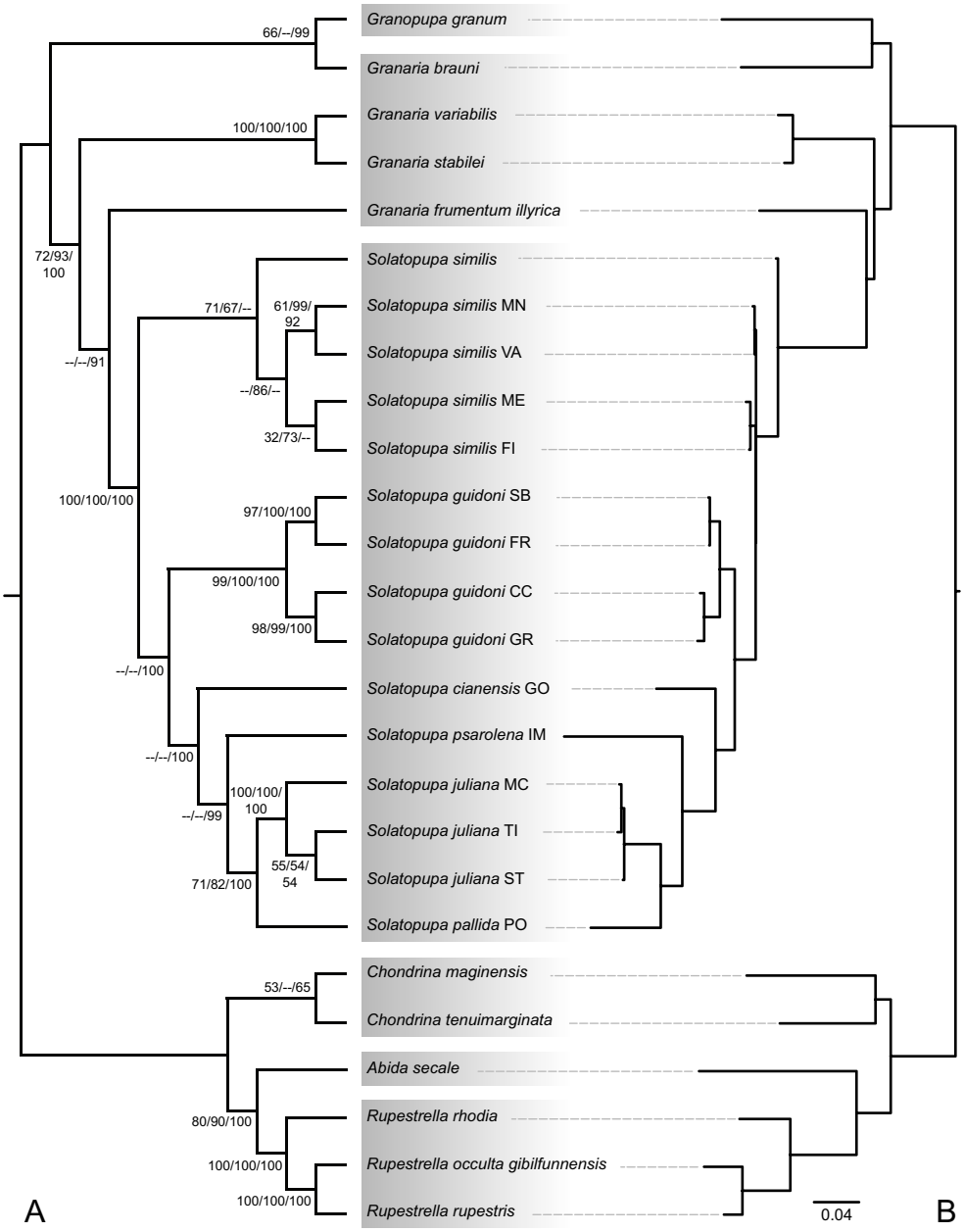


Figure **2**. Bayesian phylogenetic reconstruction of the Chondrinidae based on 28s and Histone H3 data, including the outgroup, *Ena montana*. Scale bar indicates mutations per site.

taxa. Therefore the uncorrelated relaxed lognormal clock model as implemented in BEAST was used (Drummond et al., 2006). This model allows the mutation rate between sistertaxa to vary within certain limits, and does not *a priori* assume a relation between the mutation rate of the taxa and that of their ancestors. The standard deviation and the coefficient of variation (0.302 and 0.269, respectively, in the *Rupestrella*-calibrated run) indicated a usefull 'clocklikeness' in the data based on the selected priors.


Granopupa granum
Granaria brauni
Granaria variabilis
Granaria stabilei
Granaria frumentum illyrica
Solatopupa similis
Solatopupa similis MN
Solatopupa similis VA
Solatopupa similis ME
Solatopupa similis FI
Solatopupa guidoni SB
Solatopupa guidoni FR
Solatopupa guidoni CC
Solatopupa guidoni GR
Solatopupa cianensis GO
Solatopupa psarolena IM
Solatopupa juliana MC
Solatopupa juliana TI
Solatopupa juliana ST
Solatopupa pallida PO
Chondrina maginensis
Chondrina tenuimarginata
Abida secale
Rupestrella rhodia
Rupestrella occulta gibilfunnensis
Rupestrella rupestris
66/--/99
100/100/100
72/93/
100
71/67/--
61/99/
92
--/--/91
--/86/--
32/73/--
100/100/100
97/100/100
99/100/100
98/99/100
--/--/100
--/--/100
100/100/
100
--/--/99
55/54/
54
71/82/100
53/--/65
80/90/100
100/100/100
100/100/100
0.04
A
B

DISCUSSION

The absence in the molecular analyses of the *Granaria* species from the middle and far east, viz. *G. lapidaria*, *G. arabica* and *G. persica*, as well as the lack of additional representatives of *G. frumentum* s.l. (which varies considerably morphologically, and may in fact be a species complex) may have influenced the results of the phylogeny reconstruction. Therefore, pending additional data, we here provisionally accept *Granaria* as maybe a paraphyletic taxon.

According to Ketmaier et al. (2006), *Chondrina avenacea* was used as an outgroup in their analyses of *Solatopupa*. Contrary to the expectation, their *'C. avenacea'* did not cluster with the other representatives of *Chondrina*, but rather with the ingroup, i.e. *Solatopupa*. Detailed analyses of COI, 16s, Histone H3 and 28s revealed that in all cases, the alleged *C. avenacea* clustered with *Solatopupa* instead of *Chondrina*. An incorrect identification is unlikely, since *C. avenacea* differs clearly from all *Solatopupa* species. Contamination cannot be the explanation, since the outgroup sequence differed in all four cases from the ingroup sequences. Whatever the cause may be, the sequence used by Ketmaier et al. (2006) is definitely not *C. avenacea*. Its exclusion changes the structure of the tree markedly, but except for the Bayesian analyses there is still very little support for the deeper nodes (Fig. 3a). This is also evident from the tests that were done for *Solatopupa*, using constraints on the topology. Since the likelihoods associated with the three topologies were not significantly different, none of these is objectively 'better' than the others. Additional data on *Solatopupa*, preferentially of a marker that is more informative at the species level, should be added to obtain a more robust reconstruction of the phylogeny of the genus.

Allthough the data showed sufficient 'clocklikeness', the 95% confidence intervals on the node ages are quite large (Fig. 4). This is most likely due to the use of only a single calibration point.

Since these confidence intervals are very large (fig. 4), they are not significantly different for most nodes. The only nodes differing significantly from the other ones refer to the relatively recent splits between *G. variabilis* and *G. stabilei*, and between *R. rupestris* and *R. o. gibilfunnensis*. This is may be due to the large amount of homoplasy in the trees and the low support for most clades. Since the support for the topology in the Granariinae is very poor, no reliable dating is possible for the divergences between the genera. For the Chondrininae the topology is more stable. Based on a minimum root age of 43.6 Mya (upper boundary of the Lutetian), the split

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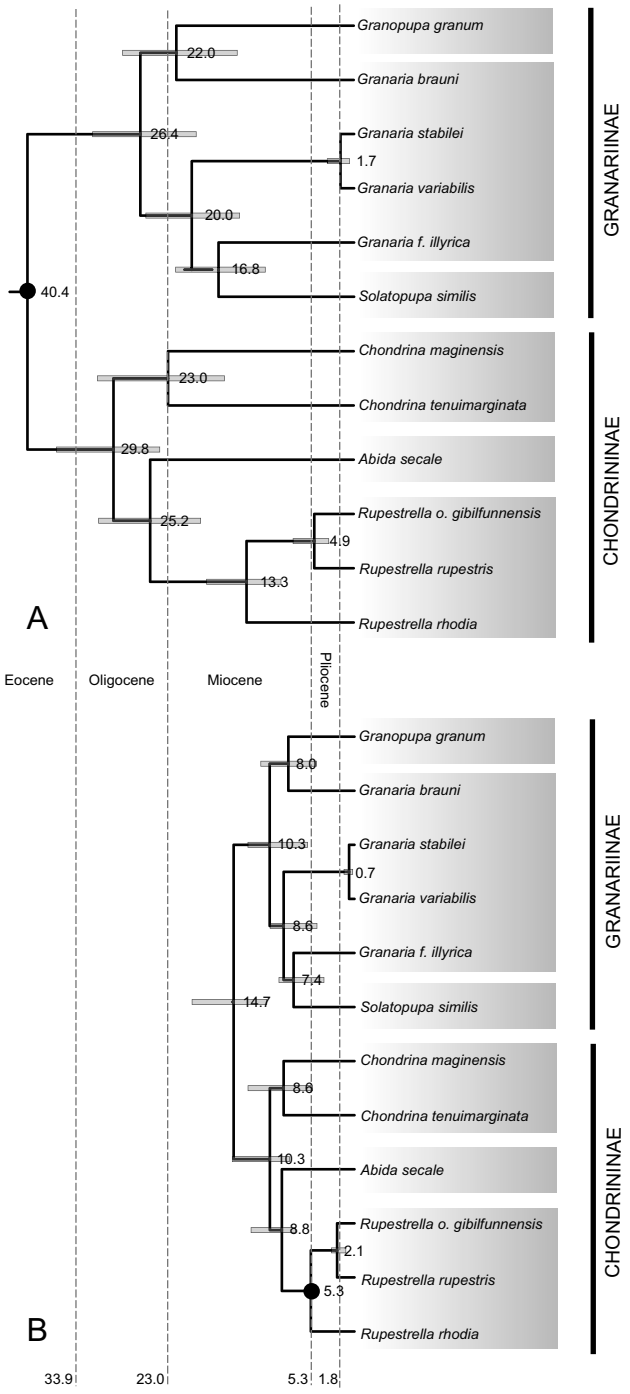
Figure **3**. Phylogeny reconstruction of the Chondrinidae based on all four markers and including data from Ketmaier et al. (2006)(*Solatopupa*). **A**, Consensus tree based on parsimony bootstrap, Neighbor Joining bootstrap and Bayesian analysis (support values resp.). **B**, Bayesian phylogenetic reconstruction (unconstrained) with highest likelihood (lnL -10558.9) from set of 2,000 trees.
Scale indicates mutations per site.


Granopupa granum
22.0
Granaria brauni
26.4
Granaria stabilei
1.7
Granaria variabilis
20.0
Granaria f. illyrica
16.8
40.4
Solatopupa similis
GRANARIINAE
Chondrina maginensis
23.0
Chondrina tenuimarginata
29.8
Abida secale
25.2
Rupestrella o. gibilfunnensis
4.9
Rupestrella rupestris
13.3
Rupestrella rhodia
CHONDRININAE
A
Eocene
Oligocene
Miocene
Pliocene
Granopupa granum
8.0
Granaria brauni
10.3
Granaria stabilei
0.7
Granaria variabilis
8.6
Granaria f. illyrica
7.4
14.7
Solatopupa similis
GRANARIINAE
Chondrina maginensis
8.6
Chondrina tenuimarginata
10.3
Abida secale
8.8
Rupestrella o. gibilfunnensis
2.1
Rupestrella rupestris
5.3
Rupestrella rhodia
CHONDRININAE
B
33.9
23.0
5.3
1.8

between *Chondrina* and *Abida/Rupestrella* originated somewhere in the Oligocene, possibly around the Chattian/Rupelian boundary (30 Mya), i.e. at 31.7 Mya (with a 95% confidence interval of 25.8-39.6 Mya). Between *Abida* and *Rupestrella* the splitting event would be at 26.7 Mya (20.2-33.5 Mya), which is not significantly different from the former branching. Based on these data the initial divergence between *R. occulta gibilfunnensis* and *R. rupestris* is dated at 5.2 Mya (3.4-8.2 Mya). In the absence of reliably dated nodes in the deeper phylogeny of the Granariinae, there is only an inaccurate estimate for the split between *G. stabilei* and *G. variabilis*, dated at 1.8 Mya (0.4-3.4 Mya), i.e. close to the Pliocene-Pleistocene transition.

All estimates based on a minimum root age of 40.4 Mya appear to be very high when compared with the results of an alternative hypothesis, based on the hypothesized biogeographical history of *Rupestrella*. The latter scenario points to an age of the root of 14.7 Mya (10.7-19.7) (fig. 4b), and dates the branching of *G. stabilei* and *G. variabilis* well in the Pleistocene at 0.7 Mya (0.2 – 1.3). This '*Rupestrella* scenario' fits well with the inferences by Ketmaier et al. (2006), who calculated a root age of 11.2-16.3 Mya for *Solatopupa*, based on the geological history of the Corsica-Sardinia microplate. According to this, *G. stabilei* may have evolved in isolated, high-altitude (nunatak) habitats, whereas its sisterspecies *G. variabilis* remained in the lower valleys. An analysis based on an early Pleistocene *Chondrina* sample from Spain, gives a minimum age of the split between *Abida* and *Chondrina* of ca. 3.5 Mya (unpublished data). However, the calibration starts from the root of the clade that contains the fossil (but still extant) species *Chondrina guiraoensis*. Since no sequence data were available for this species, a more exact calibration was not possible. Therefore, most probably this minimum age is is an underestimate of the true minimum age. This means that the calibration on the basis of only the fossil *Chondrina* gives a minimum age of the *Abida-Chondrina* split that is somewhat younger (~3.5 Mya) than that making use of the *Rupestrella* biogeography (~10.3 Mya), but much younger than the age derived from the calibration of the fossil *Granaria* (~32.1 Mya). This second scenario (fig. 4b) implies that the fossil so-called *Granaria* species from the Eocene pre-date the radiation leading to the extant chondrinid genera. Hence *Granaria* sensu Gittenberger (1973) would be paraphyletic. This possibility was already mentioned by Gittenberger (1984). Our molecular phylogeny reconstruction shows that the recent species that are classified in *Granaria* may also form a paraphyletic group, but at the actual state of our knowledge, for the sake of nomenclatorial stability, and without a reasonable alternative, we prefer a continuation of the use of the generic name *Granaria* as it is widely used in the literature.

Figure **4.** Bayesian phylogeny reconstruction with highest likelihood from BEAST, using lognormal uncorrelated relaxed clock model. Bars on the nodes indicate 95% confidence intervals for the age estimates. **A**, fixed age on the root (43.6 Mya). **B**, fixed age on *Rupestrella rhodia - R. rupestris/R. occulta gibilfunnensis* split (5.3Mya).

The absence of fossils for the rock-dwelling taxa (i.e. *Chondrina*, *Rupestrella* and *Solatopupa*) that pre-date the Pleistocene is surprising, considering their suggested origin in the Late Miocene (fig. 4b) according to the second scenario or even much earlier, as indicated by the first scenario. However, the shells of snails occurring in such habitats are known for the paucity in the fossil record (see for instance Evans & Jones, 1973).

An average mutation rate for the genetic markers would not be very useful since the mutation rates vary strongly between the lineages. For the same reason the various mutation rates published for other stylommatophoran taxa (Pinceel et al, 2005 and references therein) have not been used.

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