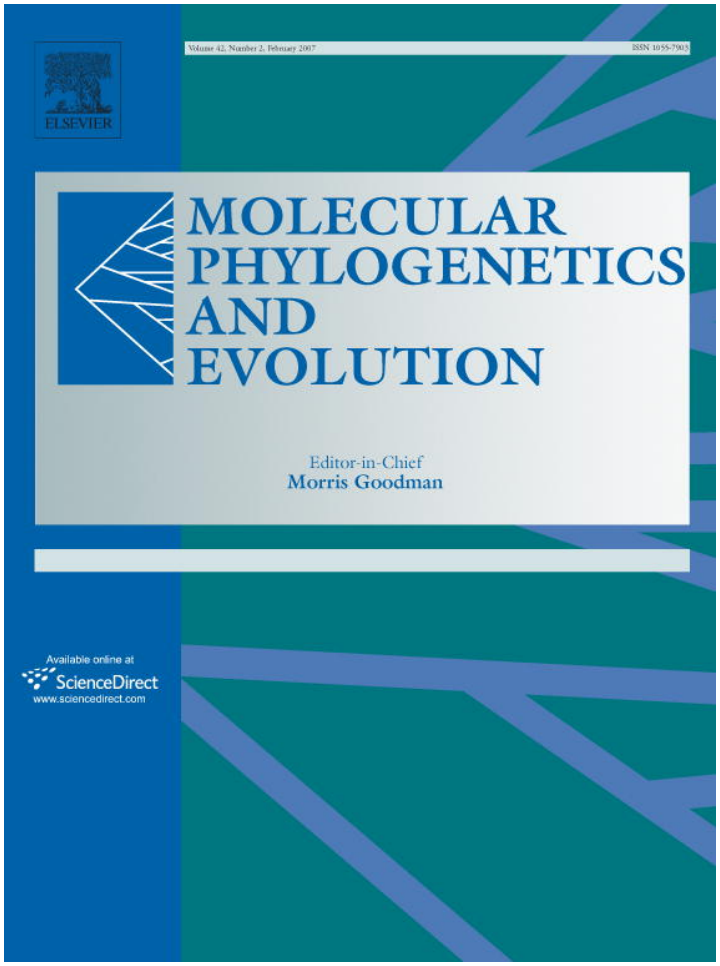

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Lineage divergence of a freshwater snail clade associated with post-Tethys marine basin development

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Abstract

The complex evolutionary history of the Eurasian gastropod lineage *Theodoxus* reflects the evolution of marine basins following the breakup of the Tethys Sea. Today, this clade inhabits the lakes, rivers, streams, and estuaries of Europe, southwestern Asia, and North Africa. Here we present the first phylogenetic hypothesis for this clade. Based upon extensive geographic and taxonomic sampling, portions of the mitochondrial genes for cytochrome c oxidase subunit I and 16S rRNA were sequenced and analysed using maximum likelihood and maximum parsimony methods. Results from bootstrap analyses, Bayesian analysis, and sensitivity analyses lend support to six deep phylogenetic subdivisions within *Theodoxus*. These major clades are geographically associated with the major post-Tethyan marine basins. Estimates of divergence times using a penalized likelihood approach indicate that divergence of these major lineages occurred during the Miocene, simultaneous with the breakup of the Mediterranean and Paratethys Seas. The resulting major subclades later diversified during the Pliocene, primarily within geographic regions associated with the eastern and western Mediterranean Sea, the Pannonian Basin, and the Black Sea, thus producing the extant species assemblages. Finally, these phylogenetic results imply that much of the current taxonomy is flawed, therefore we offer recommendations for revising the classification of *Theodoxus* species based on phylogenetic systematics.

Keywords: *Theodoxus*; Phylogenetic analysis; Historical biogeography; Mediterranean sea; Paratethys; Hypothesis testing; Molecular clock; Phylogenetic systematics

1. Introduction

The closing of the Tethys Sea at the end of the Oligocene (~23 Ma) resulted in changes to distributional patterns in marine and terrestrial taxa as well as major faunal and floral exchanges between Eurasia and Africa (Rögl and Steininger, 1984). The breakup of the Tethys Sea in the Miocene resulted in the formation of the Mediterranean and Paratethys Seas, both of which continued to develop into separate basins that were intermittently connected and disconnected (Steininger and Rögl, 1984). The modern eastern and western Mediterranean Seas, Black Sea, Caspian Sea, and the ancient Lake Pannon are the result of these tectonic processes. The vicariant effects of this breakup on terrestrial taxa have been well-documented (Caccone et al., 1997; Fromhage et al., 2004; Hulva et al., 2004; Lenk et al., 2001; Weisrock et al., 2001). There is also some evidence that vicariance and subsequent dispersal through reconnected waterways has had a major effect on freshwater groups such as mollusks and fish (Glaubrecht, 2000; Hrbek and Meyer, 2003; Ketmaier et al., 2004; Zardoya and Doadrio, 1999). One mollusk that may be particularly informative in the reconstruction of the history of these freshwater faunas is the nerite gastropod genus *Theodoxus*.

Members of the Neritidae are restricted primarily to the southern hemisphere with the exception of *Theodoxus*, which is native to Europe, Southwest Asia, and North Africa. This distribution, surrounding the ancient Tethys Sea, suggests that post-Tethyan marine basin development

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may have influenced the evolution of this group. In addition, *Theodoxus* species are members of Neritopsina—a remarkable, yet under-studied, clade that is well-suited for clarifying many evolutionary and biogeographical questions (Lindberg, 2003). Importantly, taxa within the Neritidae have a demonstrated propensity for habitat shifting, independently evolving a freshwater habit eight times (Holthuis, 1995). The *Theodoxus* invasion of freshwater represents the only non-terrestrial group with an entirely continental distribution. *Theodoxus* species are distributed primarily on the continent of Europe, extending as far west as Ireland, as far east as the Caspian basin, and into North Africa and the Near East (Fig. 1; Al-Dabbagh et al., 1986; Alouf, 1998; Kristensen, 1986; Lucey et al., 1992; Massoud and Hedayeti-Far, 1979). They are the only wholly temperate neritids, occurring at the northernmost reaches of Baltic Sea drainages (Skoog, 1971). No other freshwater neritids are distributed sympatrically with *Theodoxus* (Brown, 1994). Though primarily restricted to well-oxygenated lakes and rivers, populations of *Theodoxus* are also found in brackish water of up to 18‰ salinity in both the Baltic Sea and the Black Sea (Butenko, 2001; Skoog, 1971).

In order to determine the relative contributions of vicariance and dispersal in producing the contemporary distribution of *Theodoxus* species it is necessary to have a hypothesis of phylogenetic relationships. Surprisingly, there has not even been a systematic review of the genus, much less a robust phylogenetic analysis, and the plethora of named species and subspecies renders any patterns derived from current classifications suspect. At least 34 species names have been assigned to *Theodoxus* (Bunje, 2004), the majority of which are from areas surrounding the Mediterranean Sea, in particular the northeast Mediterranean region (Table 1). Circum-Mediterranean lands thus appear to account for most *Theodoxus* species, though regions with numerous named species could be products of bias in taxonomic criteria or the intensity of study of a particular area. For example, many of the named species from Turkey, Syria, and Iraq were studied by Schütt (1987; 1989; Schütt and Sesen, 1992) and subspecies continue to be described from throughout western Europe (Falkner et al., 2002). In contrast to such patterns of high endemicity, Bunje (2005) has argued that only a single species, *T. fluviatilis*, accounts for most distributional records of *Theodoxus*, including for most of western and northern Europe. Therefore, it is uncertain whether the described distributions of *Theodoxus* species are representative of this taxon's true diversity.

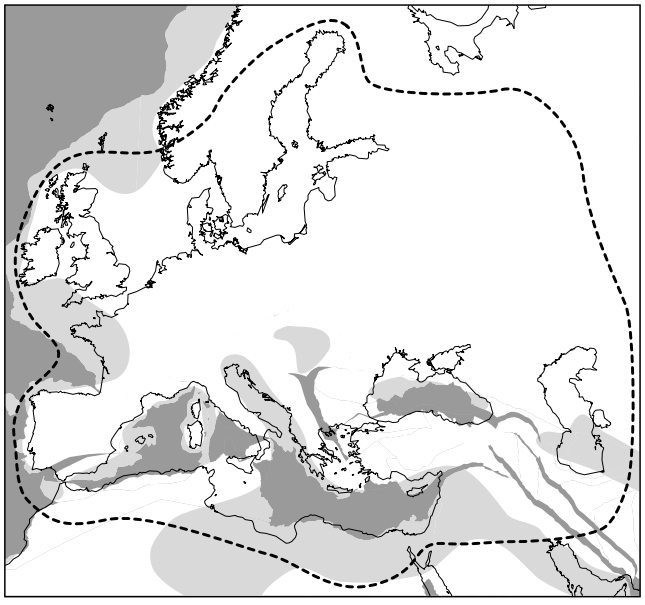


Fig. 1. Reconstructed shorelines of western Eurasia and North Africa during the Miocene. Dark shaded areas represent the position of deep oceanic basins 14 Ma according to the Ocean Drilling Stratigraphic Network (Hay et al., 1999). Sea level fluctuated widely throughout the Neogene, the light gray shading represents the limited extent it may have reached around 23 Ma (Blakely, 2005). This is shortly after the Tethys Sea finally closed, and distinct western Mediterranean, eastern Mediterranean, Pannonian (light grey basin in central Europe), Pontian (Black Sea), and Caspian basins are apparent. Modern shorelines are drawn in solid lines and the approximate range of extant *Theodoxus* is shown by the dashed line.

If the current pattern of species richness is taken at face value, then it appears that most diversification within *Theodoxus* has occurred in regions surrounding the major post-Tethys marine basins. This model would rely largely on vicariant events separating major drainages of post-Tethys Eurasia to produce the modern distribution of *Theodoxus* species. Alternatively, the historical biogeography of *Theodoxus* might be characterized as dispersal driven. Under this model, the relatively high species diversity in Anatolia and the Balkans might represent the center of origin of the clade, with the remainder of the range a result of dispersal, largely westward and northward from this ancestral area. Lastly, it is highly possible that the historical biogeography of *Theodoxus* will represent a mosaic of mechanisms interacting to create its extant distribution and diversity.

Here we test these three non-exclusive hypotheses by constructing a phylogenetic framework outlining evolutionary relationships within the genus *Theodoxus*, testing the probability of relationships among major clades, and dating cladogenic events using a relaxed molecular clock. This framework is then used to evaluate the biogeographic context of the evolution of *Theodoxus* in Paratethyan and early Mediterranean environments and to reconstruct the histories of lineages distributed around post-Tethys marine basins.

2. Materials and methods

2.1. Taxon sampling and specimen collection

We obtained specimens of *Theodoxus* from throughout its current range. A conservative first approach for testing phylogenetic hypotheses involves sampling broadly across the geographic range and collecting specimens from as many named taxa as possible (Omland et al., 1999; Zink et al., 1998). Because many named species (and subspecies) are geographically restricted, our broad sampling regime means that we sometimes sampled individuals that we sus-

Table 1
List of operational taxonomic units (OTUs) used in this study and the species to which they are assigned

OTU ID#	Species assignment	Locus		Collection locality
		16S	COI	
Outgroup				
CS024	*Clithon spinosa*	AY771224	AY820333	Moorea, French Polynesia
Nerita1	*Nerita sp.*	AY771226	AY771272	Tutuila, Samoa
NC050	*Neritina canalis*	AY771225	AY771270	Moorea, French Polynesia
NT100	*N. turrita*	AY771227	AY771273	'Upolu, Samoa
SP005	*Septaria porcellana*	AY771228	AY771274	Moorea, French Polynesia
SS272	*S. sanguisuga*	AY771229	AY771275	Tutuila, Samoa
TA001	*T. anatolicus*	AY771230	AY771276	Vilayet Antalya, Turkey
TA011	*T. anatolicus*	AY771231		Burdur, Turkey
TA012	*T. anatolicus*	AY771232		Burder, Turkey
TA015	*T. anatolicus*	AY771233		Burder, Turkey
TB001	*T. baeticus*	AY771234	AY771277	Quart, Spain
TB007	*T. baeticus*	AY771235	AY771278	Anna, Spain
TB013	*T. baeticus*	AY771234	AY771279	Buñol La Jarra, Spain
TD005	*T. danubialis*	AY771236	AY771280	Garda, Italy
TD093	*T. danubialis*	AY771236	AY771281	Pischelsdorf, Austria
TD121	*T. fluviatilis*	AY771237	AY765336	Esztergom, Hungary
TD137	*T. danubialis*	AY771238	AY771282	Kustány, Hungary
TE001	*T. euxinus*	AY771239	AY765333	Bilhorod-Dnistrovs'kyi, Ukraine
TF009	*T. fluviatilis*	AY771240	AY765306	Erstein, France
TF155	*T. fluviatilis*	AY771240	AY765308	Ascheburg, Germany
TF225	*T. fluviatilis*	AY771241	AY765317	Rheinsberg, Germany
TF251	*T. fluviatilis*	AY771240	AY765319	Garda, Italy
TF295	*T. fluviatilis*	AY771242	AY765325	Goito, Italy
TF328	*T. fluviatilis*	AY771243	AY765328	La Pesta, Italy
TF350	*T. fluviatilis*	AY771240	AY765318	Hammarudda, Finland (also Sweden)
TF377	*T. fluviatilis*	AY771244	AY771283	Chella, Spain
TF389	*T. fluviatilis*	AY771244	AY771284	Ayora, Spain
TF396	*T. fluviatilis*	AY771245	AY765308	Rugia, Germany
TF400	*T. fluviatilis*	AY771256	AY765341	Kherson, Ukraine
TF417	*T. fluviatilis*	AY771240	AY765330	Rockhill, Ireland
TF418	*T. fluviatilis*	AY771246	AY765308	Cheekpoint, Ireland
TF426	*T. fluviatilis*	AY771247	AY765331	Vouvant, France
TF461	*T. fluviatilis*	AY771234	AY771286	Castellón, Spain
TJ001	*T. jordani*	AY771248	AY771287	Banias, Israel
TJ006	*T. jordani*	AY771249	AY771288	Tabha, Israel
TJ012	*T. macrii*	AY771250	AY771289	Nahal Hakibbutzim, Israel
TJ015	*T. macrii*	AY771251	AY771290	Nahal Hakibbutzim, Israel
TM001	*T. meridionalis*	AY771252	AY771291	Cefalù, Sicily, Italy
TM005	*T. meridionalis*	AY771253	AY771292	Sortino, Sicily, Italy
TP001	*T. prevostianus*	AY771254	AY771293	Bad Vöslau, Austria
TP032	*T. prevostianus*	AY771255	AY771294	Kàcs, Hungary
TQ001	*T. velox*	AY771256	AY765340	Kherson, Ukraine
TS001	*T. syriacus*	AY771257		Silifke, Turkey
TS002	*T. syriacus*	AY771240		Silifke, Turkey
TS006	*T. syriacus*	AY771258		Silifke, Turkey
TT001	*T. transversalis*	AY771259	AY771295	Edelény, Hungary
TV001	*T. valentina*	AY771268	AY771296	Massalavés, Spain
TV002	*T. valentina*	AY771234	AY771297	Massalavés, Spain
TX004	*T. peloponnesa*	AY771260		Peloponnesus, Greece
TX011	*T. numidicus*	AY771261		Morocco
TX013	*T. meridionalis*	AY771262		Tunisia
TX018	*T. fluviatilis*	AY771230		Radmanove Mlinice, Croatia
TX019	*T. fluviatilis*	AY771263		Lisimakhia, Greece
TX040	*Theodoxus* sp.	AY771269	AY765339	Xiladiana, Crete, Greece
TX047	*Theodoxus* sp.	AY771264		Panetolio, Greece
TX049	*T. fluviatilis*	AY771240		Karacabey, Turkey
TX053	*Theodoxus* sp.	AY771265	AY765334	Kontra, Greece
TX054	*Theodoxus* sp.	AY771266	AY771298	Agios Floros, Greece
TX059	*Theodoxus* sp.	AY771266	AY771299	Aris, Greece
TX068	*T. euxinus*	AY771267	AY771300	Pervomais'k, Ukraine
TX073	*T. euxinus*	AY771269	AY765337	Odessa, Ukraine
TZ010	*T. velascoi*	AY771261		Massalavés, Spain

GenBank accession numbers are listed for the sequences used in this study. Exact collection localities can be obtained from the authors.

pected of having different nominal names in different regions. Thus, our sampling scheme broadly covered the nomenclatural status of the group as well as its geographic range. In this manner, we also test the monophyly of certain species, such as *T. fluviatilis*, which may have more diversity across the geographic range than is apparent in taxon names (Bunje, 2005). Table 1 summarizes the operational taxonomic units (OTUs) used for this study including the species each specimen is assigned to, the collection locality, and the genes successfully sequenced for each OTU.

One of us (PMEB) collected the majority of samples, including those from Ukraine, Hungary, Italy, France, Austria, and Germany (Table 1). These specimens were collected by hand from hard substrates in the margins of lakes and rivers. Tissue samples for DNA extraction were collected either from live animals or those preserved in 96% ethanol. Many additional specimens were collected on our behalf from Sweden, Finland, Ireland, France, Spain, Sicily, Morocco, Israel, Turkey, and Greece. These specimens were preserved in 70% or 95% ethanol until genomic DNA extraction. Finally, 16S rDNA sequence from one sample (TX004, *T. peloponnesa*) was obtained from a dried specimen ~70 years old loaned from the Zoologische Staatssammlung München, Germany. Considering the putative position of *Theodoxus* within the Neritidae (Holthuis, 1995), and the lack of confidence in these relationships, we chose to sample several possible outgroups representing other neritid lineages: *Nerita*, *Neritina*, *Septaria*, and *Clithon*.

2.2. DNA sequencing

Total genomic DNA was extracted from each individual using the EasyDNA Animal Tissue Kit (Qiagen, Hilden, Germany). Between 15 and 35 mg of foot and/or head tissue was dissected from each individual and processed according to the manufacturer's specifications. Partial sequences of two mitochondrial genes were amplified by polymerase chain reaction: cytochrome *c* oxidase subunit 1 (COI) and 16S ribosomal DNA (16S). 600 bp of COI were amplified for 148 individuals plus six outgroup samples using the primers PMB-F4d, 5′-TACTTTRTATATTATGTTTGG T-3′, and PMB-R1d, 5′-TGRTAWARAATDGGRTCW CCHCCVCC-3′, which are just internal of the universal primers of Folmer et al. (1994). Each 50 µl PCR included 1 µl genomic DNA, 10 pmol of each primer, 3 nmol dNTPs, 5 µl of 10× PCR buffer, 125 nmol $MgCl_2$, and 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The cycling parameters for COI were an initial cycle of 95°C for 10 min followed by 36 cycles of denaturation at 95°C for 50 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min. PCR was completed with a 7 min final extension at 72°C. For 16S, 99 individuals plus six outgroup specimens were amplified for a 509–512 bp fragment using the universal primers 16Sar, 5′-CGCCTGT TTATCAAAAACAT-3′, and 16Sbr, 5′-CCGGTCTGA ACTCAGATCACGT-3′ (Hillis et al., 1996). For 16S, each 50 µl PCR reaction included 1 µl genomic DNA, 20 pmol of each primer, 3 nmol dNTPs, 5 µl of 10× PCR Buffer, 125 nmol $MgCl_2$, and 1 unit AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). The cycling parameters for 16S were an initial cycle of 95°C for 10 min followed by 36 cycles of denaturation at 95°C for 50 s, annealing at 52°C for 50 s, and extension at 72°C for 1 min. PCR was completed with a 7 min final extension at 72°C.

PCR products were purified using QiaQuick PCR Purification columns (Qiagen, Valencia, CA, USA) or ExoSAP-IT (USB, Cleveland, OH, USA). Purified PCR products were then cycle sequenced with BigDye v 2.0 (Applied Biosystems, Foster City, CA, USA) using the PCR primers. Sequences were visualized on an ABI 377 automatic sequencer in the Molecular Phylogenetics Laboratory at UC Berkeley. Both complements of each sequence were obtained to verify the accuracy of the final sequence.

Many individuals possessed identical haplotypes for COI and/or 16S. Only unique haplotypes were used in subsequent analyses (Table 1). COI, a coding gene, was aligned by hand. 16S contained one indel in the ingroup and three indels present only between the ingroup and the outgroup. Indels present only between the ingroup and outgroup were ignored in all analyses. Alignment of the rest of the gene was straightforward and completed manually with reference to an alignment performed in ClustalX. Sequences of all COI and 16S data are deposited in GenBank, see Table 1 for accession numbers.

2.3. Phylogenetic analyses

Certain specimens could not be amplified for one of the two genes due to degraded genomic DNA or other practical issues of PCR and cycle sequencing; see Table 1 for a summary. In general, however, both genes were analysed for the same individuals. Mutational saturation was examined for the entire 16S dataset and independently for the three codon positions of COI by plotting observed (uncorrected) pairwise distances versus estimated distances using the maximum likelihood parameters described below (Nichols, 2005). No saturation was observed for any of these groups so all sequence data could be used in phylogenetic analysis (results not shown). A partition homogeneity test implemented in PAUP* v 4.0b10 (Swofford, 2000) did not find significant incongruence between the COI and 16S datasets, therefore the two loci could be analysed simultaneously. For completeness, each locus was also analysed separately. Only unique haplotypes from OTUs with both genes were used for the COI and combined datasets, resulting in 42 ingroup OTUs for these analyses. The 16S gene amplified more easily from poorly preserved specimens, therefore this dataset included unique 16S haplotypes from several other specimens, resulting in 38 ingroup OTUs for this dataset (Table 1). For the analysis of the 16S dataset, all individuals with significant uncertainty (i.e. ambiguous nucleotide assignments) were excluded during one analysis. The results with these sequences excluded did not differ

from the analysis when these sequences were included and are not shown. For each dataset (COI alone, 16S alone, and COI plus 16S combined), separate analyses were performed using the optimality criteria of parsimony and maximum likelihood.

For each maximum likelihood (ML) analysis, different models of sequence evolution were tested using Modeltest v 3.06 (Posada and Crandall, 1998). Following the recommendations of Posada and Buckley (2004) and Sober (2002), the best model for each dataset was chosen using the Akaike Information Criterion. ML analyses were performed in PAUP* and used an heuristic search strategy with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping.

Maximum parsimony (MP) analyses of the three datasets were conducted in PAUP*. For these analyses, characters were set to ACCTRAN optimization, all gaps were coded as a new state, TBR branch swapping, and only the best trees were saved. For the combined data, the genes were analysed using different character weighting schemes. The weights for COI:16S were 1:1, 2:1, 1:2, and 2:3. All weighting schemes resulted in identical consensus trees (with different proportions in the majority-rule consensus). Analyses were performed using a heuristic search strategy, saving unlimited trees in each replicate and run for 10 random-addition sequence replicates.

Bootstrap values were calculated for ML trees using 500 bootstrap replicates, the "fast" heuristic search algorithm, and the same model parameters as used for each ML analysis. Bootstrap values were obtained for MP trees using 1000 replicates for 10 random-addition sequence replicates and saving a maximum of 500 trees per bootstrap replicate. Additionally, a Bayesian analysis was performed for each of the three datasets (COI, 16S, and COI+16S). The Bayesian analysis produced posterior probabilities of branches using MrBayes v 3.1 (Ronquist and Huelsenbeck, 2003). This was performed with the following parameters: the model of evolution chosen by Modeltest, a 4 chain (1 cold, three heated; $T = 0.2$) metropolis-coupled monte carlo analysis run twice in parallel for 2×10^6 generations, trees sampled every 100 generations starting after a burn-in of 50,000 generations.

2.4. Hypothesis testing

The phylogenetic analyses described above resulted in slightly different relationships between major subclades within *Theodoxus*. Therefore, we applied three tests of topological pattern in order to assess the probability of these different major clade relationships. First, we performed the non-parametric Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) by creating constraint trees consistent with the reconstructed relationships of major clades but no resolution within clades, then performed a ML search for the best trees under these constraints, and compared the resulting tree topologies using the Shimodaira–Hasegawa (SH) test in PAUP* using the RELL algorithm and 100,000 bootstrap replicates. As a second test of topology, we estimated Bayesian posterior probabilities by determining the proportion of trees found in the Bayesian analyses after stationary that were consistent with the constraint topologies of different relationships between the major clades (Buckley, 2002). Finally, we utilized the likelihood-mapping method of Strimmer and von Haeseler (1997) to assess the probability of different relationships between the major lineages. The latter method uses quartet puzzling to estimate the probability of each of the three possible unrooted trees for four groups of aligned sequences by sampling one taxon from each of the four predefined quartets and assessing the likelihood of each of the three resolutions for these four clades. We used different permutations of the relationships between the six major clades by grouping these clades together in different clusters that were consistent with the results of the phylogenetic analyses. Using Tree-Puzzle v 5.2 (Schmidt et al., 2002) we performed 100,000 replicate samplings of the four clusters with the model of evolution determined by Modeltest.

2.5. Divergence time estimation

Likelihood ratio tests (Huelsenbeck and Rannala, 1997) of the two loci both reject the presence of a molecular clock (COI: $-2\ln\Lambda = 66.6234$, $\alpha < 0.05$; 16S: $-2\ln\Lambda = 61.1150$, $\alpha < 0.05$), leading us to utilize a method that allows for lineage-specific rate variation. To estimate the divergence times of major clades, we utilized the penalized likelihood method of Sanderson (2002). Penalized likelihood is a semiparametric approach that allows limited rate variation between branches by applying a smoothing parameter, thus allowing lineage-specific rate variation within a constrained rate space (Sanderson, 2002). We calculated divergence times using this method in the program r8s v 1.70 (Sanderson, 2003). After determining the optimal smoothing value for each dataset using cross-validation (Sanderson, 2002), we estimated divergence times using the TN algorithm. In order to estimate confidence intervals, we created 500 bootstrap replicate datasets of each character matrix in Mesquite v 1.05 (Maddison and Maddison, 2004), estimated branch lengths for each replicate on the optimal tree determined by maximum likelihood, and then used r8s to summarize variation in divergence times for these phylograms. These analyses were performed for two different topologies representing plausible relationships between major clades.

We calibrated the divergence times on two different nodes. Bandel (2001) notes that the earliest species with shell characteristics similar to extant *T. danubialis* appeared early in the history of the freshwater Lake Pannon, around 11.5 Ma. Since *T. danubialis* falls into one major clade ('C') of our analyses, and this clade is the sister group of another clade (clade 'B'), we infer that the split of these two clades occurred during the existence of freshwater Lake Pannon and use minimum and maximum constraints of 5 and 11.5 Ma, respectively, for the split. Bandel (2001) also

summarizes the history of *T. jordani*, a member of another major clade ('A'), in the Near East. The earliest known undoubted fossils of *T. jordani* occur 1.8 Ma. A related lineage (probably *T. macrii*) appeared sometime earlier, but both species are distinct from the related *T. anatolicus* by 3 Ma (Bandel, 2001). Therefore, we constrained the origin of *T. jordani*/*T. macrii* to be 3 Ma. Additionally, r8s allows combined constraints to be used, so we analysed both the COI and 16S datasets under both tree topologies using three constrained nodes: 11.5–5 Ma for the split of clades B and C, a minimum age of 1.8 Ma for the origin of *T. jordani*, and a minimum age of 3 Ma for the origin of *T. anatolicus*.

3. Results

3.1. Phylogenetic analyses

MP and ML analyses all identify the presence of six major clades within *Theodoxus.* All trees have a high degree of congruence with respect to relationships within these clades, but there is some disagreement in the relationships among these clades. The ML tree for the combined dataset is shown in Fig. 2 along with MP bootstrap, ML bootstrap, and Bayesian posterior probability support values. ML trees for 16S and COI analysed separately are shown in Appendix A, Figure S2; all MP analyses were consistent with the ML trees except for some reduced resolution within major clades. Differences in major clade relationships reconstructed using MP for each dataset are noted below. For the combined dataset, sequence divergence ranges from 0% to ~3% between OTUs within the six major clades and ranges from ~6% to ~15% between OTUs in different major clades. The major clades themselves are distinguished by between 5.2% and 13.2% sequence divergence. The results from each analysis are discussed below, followed by a description of the major phylogenetic features.

For the maximum likelihood analysis of the 16S dataset, four trees of score 1993.0160 were found under a GTR+I+Γ model of sequence evolution. Each of these trees differed only in the placement of two specimens of *T. anatolicus* (OTUs TA012 and TA015) and the overall topology of each tree was the same. The tree presented is the most conservative of all four trees, possessing only the relationships present in all trees (Appendix A, Fig. S1a). Parsimony analysis of the 16S dataset found 81,215 trees of length 263 steps (CI = 0.650, RI = 0.828). The primary disagreement between MP and ML trees was among shallow relationships though the strict consensus of MP trees could not fully resolve the relationships of A, B+C, D, and F.

In the analysis of the COI dataset, ML produced one tree of score 3285.9489 under a GTR+I+Γ model (Appendix A, Fig. S1b). In the MP analyses, using equal character weights for the three codon positions produced 234 trees of length 551 steps (CI = 0.537, RI = 0.790). Weighting codon positions differentially did not change the consensus topology or the relationships of major clades (results not shown). A majority of MP trees (56%) reconstruct clades B and C as the sister to clade A, in contrast to the relationships found by ML, which place clade C as the sister group to a combined A+B clade, the only time this relationship was recovered.

In the combined analyses, results were in general accord with the individual COI and 16S analyses. The primary disagreement between MP and ML trees was in the placement of clade F (*T. peloponnesa*, OTU TX004). Just as in the 16S analyses (Appendix A, Fig. S1a), this lineage is reconstructed by likelihood as the sister to all *Theodoxus* except *T. transversalis* (clade E) and by parsimony as the sister to the *T. fluviatilis*/*T. danubialis* clade (clades B and C). Because we could not obtain COI sequence from *T. peloponnesa* (TX004), the tree shown in Fig. 2 does not include this taxon due to a lack of confidence in its placement. However, when we performed analyses that did include this OTU, *T. peloponnesa* was reconstructed by ML as the sister to all *Theodoxus* except clade E. Analysing the combined dataset under a likelihood optimization scheme using a GTR+I+Γ model resulted in one most likely tree of score 5278.3469 (Fig. 2). This tree produced the most common relationships from all analyses. For the parsimony analysis of both genes, 80 trees of length 796 steps were recovered (CI = 0.573, RI = 0.812). Using relative weights of 16S and COI characters resulted in identical consensus trees, indicating that major relationships among *Theodoxus* subclades are not being unduly influenced by differences in the substitution rate of either gene. This is particularly notable in regards to the placement of clade B, which only shifts positions when 16S is ignored.

Our inability to recover the relationship found by ML analysis of COI data (Appendix A, Fig. S1b)—(((A,B)C)D)—places significant doubt on this relationship; even weighting the two genes differently in the combined dataset, such that COI contributed more to MP reconstruction than 16S, failed to recover this relationship. In all other analyses, B and C were found to be sister clades with high statistical support (Fig. 2 and Appendix A, Fig. S1a). This relationship was also highly supported by the likelihood-mapping analysis in which only clades A, B, C, and D were analysed (Appendix A, Fig. S2). Because we could only obtain 16S sequence from *T. peloponnesa* (clade F), we could only perform the SH-test using the other five clades. The results of these analyses are described in Appendix A, Table S1 along with Bayesian posterior probabilities for the three topologies recovered in the phylogenetic analyses. The SH-test, considered a conservative evaluation of possible topologies (Buckley, 2002), is unable to reject any of the three relationships found by ML. Bayesian values, however, reject the relationships reconstructed by ML for COI (Appendix A, Fig. S1b) in all three instances ($p < 0.05$). There is disagreement between the genes in rejecting other hypothesized relationships; only the relationship reconstructed by 16S+COI ML (Fig. 2)—((A(B,C))D)—was not rejected at the $p < 0.05$ level. Clade E (*T. transversalis*) was found at the base of the *Theodoxus* clade in all analyses.

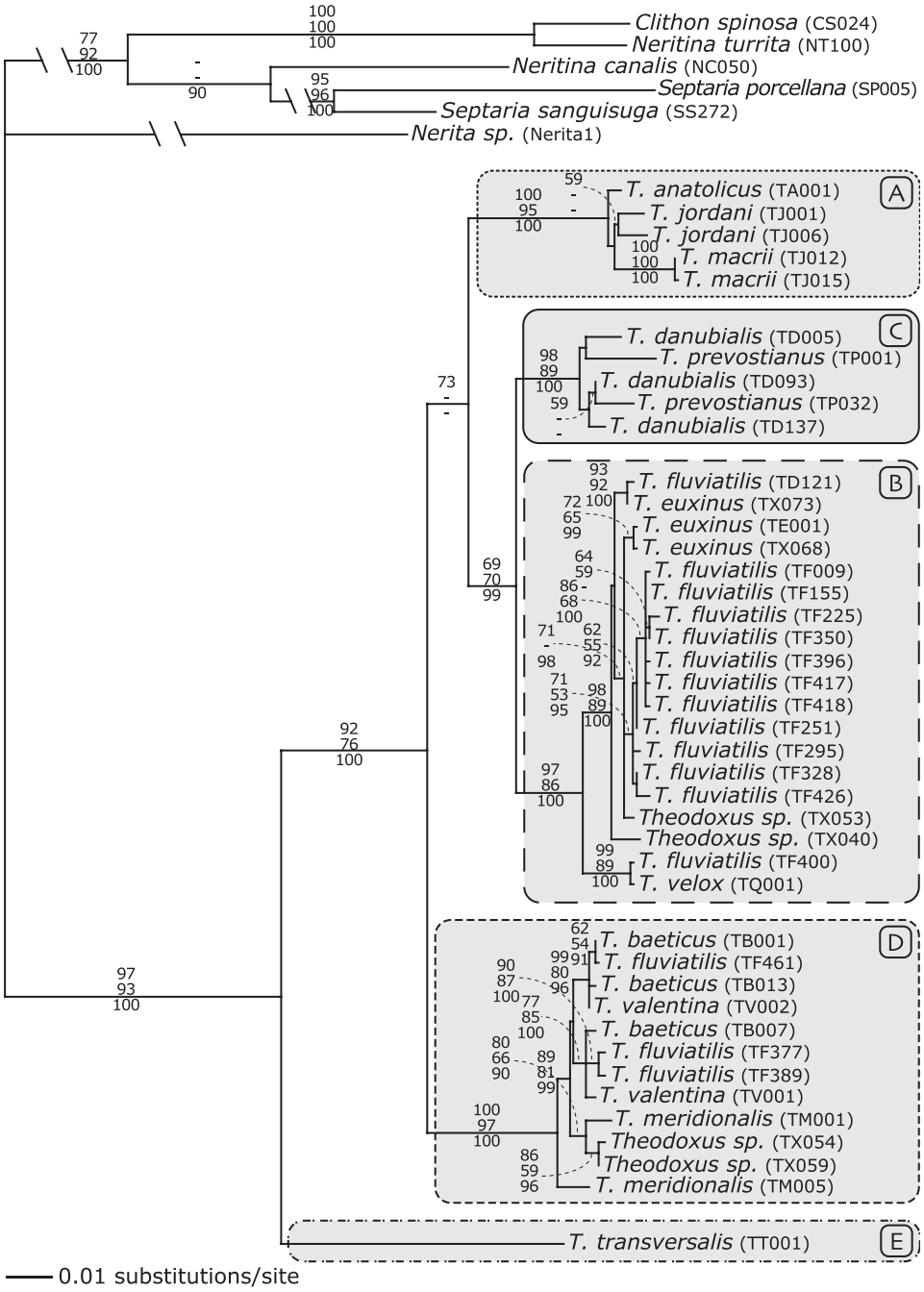
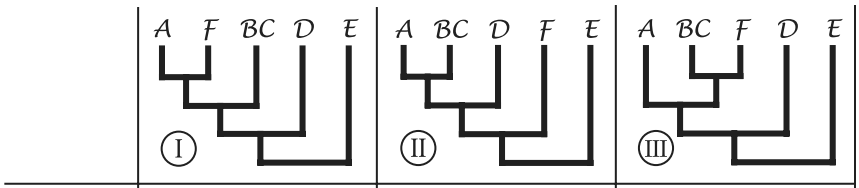


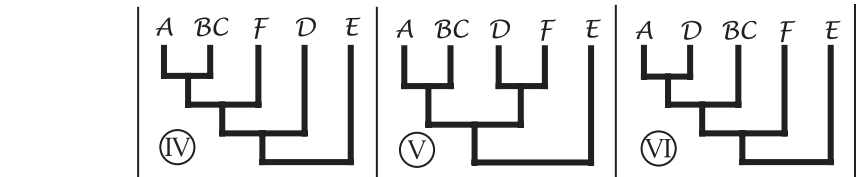
Fig. 2. Results of the maximum likelihood analyses for the combined 16S and COI dataset of *Theodoxus*. Major clades are noted by the circled letters. Terminal labels correspond to the taxon to which the collector assigned each specimen, based primarily on external appearance, and not necessarily to the appropriate taxon (e.g. those labeled "*T. fluviatilis*"). Bootstrap (>50%) and Bayesian (>90%) support values are shown in the following top-to-bottom order: MP bootstrap, ML bootstrap, Bayesian posterior probability.

Therefore, we conclude that B and C are indeed sister groups and that E is the basal lineage of *Theodoxus*.

Disagreement in topology focused on the relationships of A, B+C, D, and F. The low support values for reconstructed relationships (Fig. 2) among these clades was further reason to utilize more intensive analyses in order to assess the probability of different resolutions. Therefore, we performed additional Bayesian and likelihood-mapping analyses on just these clades to clarify their relationships. These tests help to reject some potential resolutions, such as those topologies that do not exclude clade D from the relationship ((A(B,C))F?). Figure S3 of Appendix A describes the likelihood-mapping results for the 16S and 16S+COI datasets in which clades were clustered to test the probability of the possible positions of clade F. Assuming clades B and C are sisters and that clade E forms the root, there are 15 possible rooted trees for the remaining four taxa. Of these, six topologies are consistent with the phylogenetic analyses performed above (Fig. 3). Bayesian posterior probabilities from the 16S and 16S+COI datasets are



Likelihood trees	16S+COI	16S+COI	16S+COI
Parsimony trees	16S COI 16S+COI	16S COI	16S COI 16S+COI
Likelihood-mapping	***A B***	***A*** B D H	A G
Bayesian	***16S 16S+COI***		***16S 16S+COI***



Likelihood trees	16S+COI	16S+COI	16S
Parsimony trees	16S COI 16S+COI	16S COI	16S
Likelihood-mapping	***A*** B ***D H***	***A D H*** I	A F J
Bayesian	***16S 16S+COI***	***16S 16S+COI***	

Fig. 3. Assuming that clades B and C are each others' closest relatives and that clade E is basal to the rest of *Theodoxus*, there are 15 possible rooted trees for the relationships of the remaining four clades (A, B+C, D, and F). Of these, only the six shown are consistent with the results of the maximum parsimony and maximum likelihood analyses. Furthermore, the other nine trees were entirely inconsistent with the results from the likelihood-mapping analysis (letters refer to analyses in Appendix A, Fig. S3). Of these six plausible topologies, we show which are consistent with the phylogenetic reconstructions, which are consistent with the likelihood-mapping analysis and Bayesian analyses (in normal text), and which are inconsistent with these latter two analyses (in bold italic text). Inconsistency is defined as being a Bayesian posterior probability of less than 5% or having a likelihood-mapping probability that is less than 5%, consistency is defined as support greater than 90%.

shown for the six plausible relationships among all six major clades in Appendix A, Table S2.

Fig. 3 summarizes the results of these hypothesis tests in order to clarify which topological hypotheses can be rejected by each analysis. Only topology VI cannot be rejected by any of these topological tests, though topology II was only rejected by a single likelihood-mapping permutation that did not include clade F. Our preferred set of relationships amongst these taxa is ((((A(B,C))D)F?)E) (topology II on Fig. 3). Our reason for preferring this topology is based on its presence in all combined analyses of both genes regardless of the optimality criterion used and its strong support from the topology tests.

3.2. Biogeography of the major clades

Subclades A–D contain most of the individuals and diversity found within *Theodoxus*. The following descriptions of the distributions of these clades are based primarily on the sampling associated with this study (Table 1), plus additional data from the literature as noted. Clade A contains individuals assigned to the species *T. anatolicus*, *T. jordani*, and *T. macrii*. Based on the published distributions of these species (Al-Dabbagh and Daoud, 1985; Alouf, 1998; Dagan, 1972; Heller, 2001; Massoud and Hedayeti-Far, 1979; Schütt, 1987; Schütt and Sesen, 1989; Schütt and Sesen, 1992; Yildirim, 1999), clade A ranges from Greece through Anatolia and the Near East (Fig. 4). Clade B is the most widely distributed subclade. It includes the wide-ranging *T. fluviatilis*, whose range includes most of western Europe (Bunje, 2005). Clade B also includes lineages from northern Black Sea drainages (Anistratenko et al., 1999), the Balkans (Schütt, 1987), western Anatolia (Yildirim, 1999), the Iberian peninsula (Barsiene et al., 2000; Robles, 1997), and Morocco (Brown, 1994; Kristensen, 1986). Clade B shows substructure in most analyses. Several lineages, representing several apparently paraphyletic taxa, are distributed in eastern Europe (e.g. the northern Black Sea) and there is a single monophyletic species, *T. fluviatilis*, in the west (Fig. 4). Clade C includes *T. danubialis* and *T. prevostianus*. *T. danubialis* is distributed in the Danube watershed (Frank, 1982; Hirschfelder and Hirschfelder, 1998; Leuchs and Tittizer, 1989), northern Italy (Bodon and Giovannelli, 1995), and the northern Balkans (Schütt, 1988). *T. prevostianus* is found in relictual springs surrounding the Pannonian basin (Pintér et al., 1979). Clade D is primarily

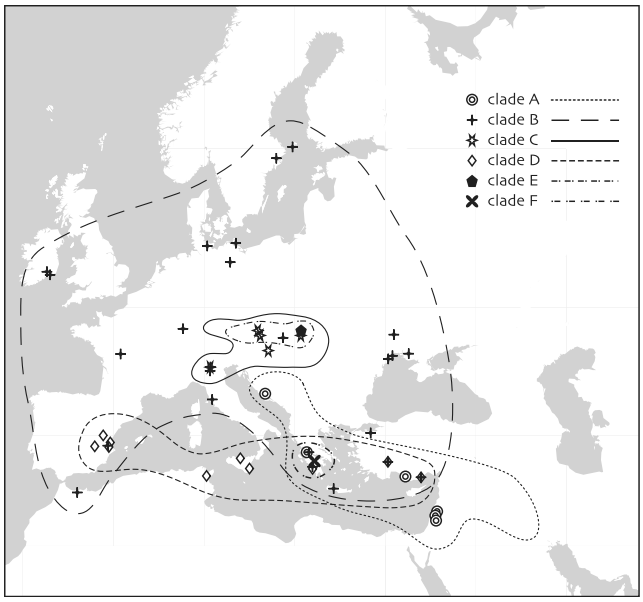


Fig. 4. Modern distribution of the six major clades of *Theodoxus*. Localities of samples used in this study are marked for each clade. Clade ranges are estimated from these localities and published accounts in the literature as described in the text. Note that the distribution of clades C and E cover the area of ancient Lake Pannon (see Fig. 1). Some areas reportedly inhabited by *Theodoxus*, such as northern Caspian Sea drainages, may belong to one of these clades or another, independent lineage, but samples were not available for this study.

distributed in watersheds surrounding the western two–thirds of the Mediterranean Sea, including Tunisia. This interpretation is based primarily on the distribution of sequenced lineages as reported in the literature (Barsiene et al., 2000; Kristensen, 1986; Robles et al., 1996; Yildirim, 1999). Additionally, several specimens referable to this clade were collected from other areas including Sicily, Greece, and western Anatolia (Table 1 and Fig. 4). The monotypic clade E is represented by the species *T. transversalis*, which includes only a few remaining populations in the western Danubian watershed (Peters, 1989; Pintér et al., 1979). The sixth clade, clade F, is only represented by 16S data from a single, old specimen of *T. peloponnesa* and therefore its geographic range cannot be reliably deduced.

3.3. Divergence times of major clades

Estimates of the divergence times of the six major clades are given in Table 2. Modern lineages within the major clades represented by more than one extant species (i.e. clades A–D) appear to have begun diversifying during the Pliocene (5.3–1.8 Ma), with estimated mean ages between 6.9 and 1.3 Ma. Consistent with the branch lengths seen in Fig. 2, the major clades appear to have diverged several million years before modern diversification began. There are some differences between datasets (e.g. 16S tends to reconstruct older divergence dates), but in general these dates are consistent. The divergence events between each of the major lineages appear to have occurred relatively quickly during the Miocene (23.8–5.3 Ma). Mean estimates for splits between clades A–D range from 19.7 to 6.9 Ma, though the estimates for these splits tend to covary (see Table 2). The origin of the extant *Theodoxus* clade was at

Table 2
Divergence time estimates (Ma) for the most recent common ancestor of extant members of major clades and the splits between those clades

	Lake Pannon diversification		Origin of *Theodoxus jordani*		Combined constraints	
	COI	16S	COI	16S	COI	16S
Tree ((A,D)(B,C))						
Clade A	2.5 ± 0.6	4.8 ± 1.6	*3*	*3*	*3.1 ± 0.1*	*4.9 ± 1.5*
Clade B	3.9 ± 1.0	6.4 ± 1.0	4.8 ± 0.6	3.9 ± 1.5	4.4 ± 0.5	6.5 ± 0.7
Clade C	4.0 ± 1.0	2.1 ± 0.7	5.0 ± 0.6	1.3 ± 0.5	4.6 ± 0.3	2.1 ± 0.6
Clade D	1.9 ± 0.5	5.2 ± 1.0	2.3 ± 0.3	3.3 ± 1.2	2.1 ± 0.3	5.3 ± 0.9
(B,C)	*10.2 ± 2.4*	*11.2 ± 1.3*	12.7 ± 1.7	6.9 ± 2.5	*11.4 ± 0.6*	*11.4 ± 0.6*
(A,D)	10.6 ± 2.7	15.5 ± 3.4	13.2 ± 1.9	9.2 ± 2.8	12.1 ± 1.2	15.8 ± 3.0
((A,D)(B,C))	11.2 ± 2.8	17.2 ± 3.9	13.9 ± 1.9	10.1 ± 2.9	12.7 ± 1.2	17.5 ± 3.5
(((A,D)(B,C))F)	—	24.6 ± 4.8	—	14.7 ± 5.2	—	25.1 ± 3.9
MRCA	27.9 ± 7.3	57.5 ± 11.4	34.8 ± 4.2	34.0 ± 13.0	31.9 ± 3.6	58.8 ± 9.1
Tree (((B,C)A)D)						
Clade A	2.9 ± 0.7	4.2 ± 2.0	*3*	*3*	*3.3 ± 0.3*	*5.1 ± 1.5*
Clade B	4.6 ± 1.1	5.6 ± 1.9	4.8 ± 0.6	4.4 ± 1.2	5.0 ± 0.6	6.9 ± 0.7
Clade C	4.5 ± 1.0	1.8 ± 0.8	4.7 ± 0.6	1.4 ± 0.4	4.9 ± 0.4	2.2 ± 0.7
Clade D	2.7 ± 0.9	4.6 ± 1.6	2.8 ± 0.5	3.6 ± 1.0	3.0 ± 0.7	5.7 ± 0.7
(B,C)	*10.4 ± 2.2*	*9.3 ± 2.9*	11.0 ± 1.6	7.2 ± 2.0	*11.4 ± 0.7*	*11.4 ± 0.9*
((B,C)A)	11.6 ± 2.6	14.7 ± 5.9	12.2 ± 1.6	10.8 ± 1.4	12.7 ± 1.2	17.8 ± 3.7
(((B,C)A)D)	14.7 ± 3.7	16.2 ± 6.5	15.5 ± 2.5	11.9 ± 1.5	16.1 ± 2.2	19.7 ± 4.1
((((B,C)A)D)F)	—	22.9 ± 8.3	—	17.2 ± 3.4	—	27.8 ± 4.1
MRCA	30.4 ± 7.1	52.7 ± 19.3	32.1 ± 4.0	39.7 ± 8.7	33.4 ± 3.3	64.0 ± 9.8

MRCA is the most recent common ancestor of all extant *Theodoxus*. Values are described as the mean and standard deviation of divergence times based on 500 bootstrap replicates. Because of uncertainty in the relationships of major clades, estimates based on the two most plausible topologies are presented (II and VI on Fig. 3). COI was not available for clade F and was not included in those analyses. Two divergence estimates were used, one constraining the split of clades B and C to have occurred during the existence of the freshwater Lake Pannon between 11.5 and 5 Ma and another in which *T. jordani* appears at least 1.8 Ma and clade A (the origin of *T. anatolicus*) originated 3 Ma (Bandel, 2001). These constraints were also combined (see Methods). Constrained nodes are italicized.

least 27 Ma, with most estimates for their most recent common ancestor in the Oligocene (33.7–23.8 Ma). These estimates are consistent with the earliest certain *Theodoxus* fossils, which appear about 24 Ma (Bandel, 2001).

4. Discussion

One of the most notable features of this phylogenetic analysis is the presence of several genetically distinct major clades within *Theodoxus* that have well-delineated geographic distributions that correlate with events in the orogeny of the Paratethyan and Mediterranean basins (Fig. 4). This pattern of species richness (sensu de Queiroz, 2005) supports the hypothesis that diversification within the *Theodoxus* clade has occurred in regions surrounding the major post-Tethys marine basins, and that vicariant events separating major drainages of post-Tethys Eurasia have contributed to the modern distribution of *Theodoxus* species. The possibility of additional dispersal events cannot be ruled out and, as discussed below, the physiological plasticity of these snails may have facilitated such events.

Agreement between geological events and the estimated divergence times supports the hypothesis that the evolution of the major clades of *Theodoxus* is coupled with Paratethyan and Mediterranean basin evolution in the Tertiary. These data suggest that the divergence of most major *Theodoxus* subclades occurred during the Miocene (Table 2), as the major post-Tethyan basins broke apart into separate geological and biological provinces (Rögl and Steininger, 1984; Steininger and Rögl, 1984). By the end of the Miocene (5.3 Ma), the major Parathethyan basins as well as the Mediterranean basin were wholly distinct (Steininger et al., 1985). These vicariant events appear to have left their signature on *Theodoxus* in the form of substantial molecular divergence between major lineages.

Paleontological data support the timing and regional setting inferred from molecular data. Fossils that can be confidently assigned to *Theodoxus* first appear in the fossil record around 24 Ma (Bandel, 2001). This timeframe is consistent with our estimates for the origin of the most recent common ancestor of *Theodoxus* (Table 2). In addition, most of the earliest known fossils are found in localities that overlay the basin of the extinct Paratethys (Anistratenko and Goznik, 1995; Bandel, 2001).

The major *Theodoxus* subclades are each broadly associated with one of the major basins of the post-Tethys era (Fig. 4). Clade A is restricted to the eastern Mediterranean basin while clade D is distributed widely across the Mediterranean. The rest of the major clades (except the problematical clade F) are associated with the Ponto-Pannonian region. Within the Paratethyan realm, clade B is distributed throughout Europe, although its basal members are distributed around the Pontian basin (the Black Sea). The group containing *T. danubialis* and *T. prevostianus* (clade C) is a distinctly Pannonian clade, representing another major biogeographic region of the Paratethyan realm.

The basal-most lineage of *Theodoxus* is *T. transversalis* (clade E), which is currently restricted to the western Danube River watershed in the Pannonian Basin. This distinctive lineage probably split from the remainder of the clade during the Early Oligocene (~30 Ma; Table 2), a time when the Tethys Sea was closing and producing vast ecological changes in regional marine and terrestrial environments (Hrbek and Meyer, 2003). As a surviving relict of the earliest diversification of the *Theodoxus* clade, it is important to safeguard the few remaining populations of *T. transversalis* in Hungary, Austria, and Germany.

Diversification of lineages within the major subclades appears to have occurred during the Pliocene (5.3–1.8 Ma; Table 2). By this time the major Paratethyan and Mediterranean basins had formed and were geologically distinct (Steininger et al., 1985). During the Pliocene, seawater transgressions caused by the opening of the Strait of Gibralter created the modern Mediterranean, Pontian, and Caspian basins (Rögl and Steininger, 1984). This event and the major environmental perturbations that such a flood would have propagated likely contributed to the lineage divergence seen within the major clades by further subdividing the ranges of species through sea level fluctuations and long term salinity variability within these basins. This period also coincides with the appearance of numerous lineages of *Theodoxus* in the fossil record (Bandel, 2001), further suggesting that Pliocene basin development was directly tied to the diversification of modern *Theodoxus* species.

The ability of some *Theodoxus* species to live in estuarine environments also informs our understanding of the processes influencing the historical biogeography of *Theodoxus*, as populations may have survived through periods of estuarine inland seas and subsequently dispersed between basins. There are at least two distantly related lineages of modern *Theodoxus* that are able to survive in high salinity environments. Populations that inhabit brackish areas along the margins of the Baltic Sea are referred to as *Theodoxus fluviatilis littoralis* (Carlsson, 2000; Kangas and Skoog, 1978; Skoog, 1971). Another small population of *T. fluviatilis* has been described from an apparently marine environment in the Black Sea near Odessa, Ukraine (Bunje, 2005; Butenko, 2001). Four very small individuals from the population near Odessa were collected for this study (Table 1; OTUs TX067-TX073). The flora and fauna at this site were distinctly marine, but salinity, as measured by a refractometer, was only 8–10‰, well within the range of salinity known for *T. fluviatilis littoralis* in the Baltic Sea (Bondesen, 1941). Specimens referrable to clade B were also collected from estuarine areas in the Dniper River delta, which receives seawater inundation during large tidal fluctuations. Both Dniper lagoon and Black Sea specimens are related to other Ukrainian *T. fluviatilis* of clade B (Fig. 2). In contrast, *T. jordani*, which is often found in brackish environments throughout the Near East (Al-Dabbagh and Daoud, 1985; Dagan, 1972), is not closely related to *T. fluviatilis*. Thus, known brackish populations of *Theodoxus* do not share common ancestry, but are instead nested within clades A

and B. This result suggests that salinity tolerance is a derived character in living *Theodoxus* species, although the immediate ancestor of the freshwater *Theodoxus* was a marine organism (Bandel, 2001; Holthuis, 1995). Consequently, either salinity tolerance has been independently re-evolved twice in *Theodoxus*, or salinity tolerance is a retained plesiomorphy that is not expressed in most lineages.

Extinction has also left a significant signature on the phylogeny of *Theodoxus*. The discovery of the lineage represented by *T. peloponnesa* may represent one of many rare lineages present within this genus. The remaining populations of *T. transversalis* are also indicative of a lineage that may have once been much more species rich. Extinction is a known feature of modern *Theodoxus* population biology (Boger et al., 1979; Bunje, 2005; Frank, 1982; Nesemann, 1985; Robles et al., 1996). Bearing in mind that extinction has shaped the evolutionary history of many European animal clades (Economidis and Banarescu, 1991; Hewitt, 1996; Kotlik and Berrebi, 2002), it is likely that the evolutionary history of *Theodoxus* is also heavily impacted by the repeated loss of lineages.

The phylogenetic reconstruction and divergence time estimates therefore support the hypothesis that the evolution of *Theodoxus* is closely tied to the breakup and development of post-Tethys marine basins. In this scenario, the formation of the Mediterranean and Paratethys coincided with the origin of modern *Theodoxus*. The formation of several major clades was subsequently associated with the division of these basins, and the diversification of modern lineages was tied to habitat changes within each of these basins. Though *Theodoxus* is a freshwater group, it is not illogical to describe its biogeography as associated primarily with marine basin development (Glaubrecht, 2000; Hrbek and Meyer, 2003; Ketmaier et al., 2004). Freshwater systems are linearly related to marine basins because of their flow regimes and mountain chains present extremely strong barriers to gene flow among freshwater animals (Taberlet et al., 1998). *Theodoxus* is represented in the earliest brackish faunas of Paratethyan basins (Harzhauser et al., 2002), supporting our conclusion that the evolution of this clade is linked to the development of distinct post-Tethyan basins.

The ability to distribute across large basins, such as the Paratethys or Mediterranean, underscores the importance of terrestrial barriers to the diversification of freshwater groups (e.g. the Alpine orogenic ridge; Durand et al., 1999). But, the biogeography of the six major clades of *Theodoxus* also indicates substantial within-basin dispersal. It is likely that the pattern of clades distributed across huge distances covering the former Paratethys and Mediterranean (e.g. clades B and D) is real and potentially abetted by the ability of these snails to traverse large distances through "estuary-hopping" or periods of lowered salinity.

Other freshwater gastropods inhabiting a similar range have also experienced some of the same evolutionary forces as *Theodoxus*. Glaubrecht (2000) has invoked a complex model to explain the extant, largely circum-Mediterranean distribution of closely related *Melanopsis* species. His hypothesis involves Paratethyan and Mediterranean vicariance followed by subsequent migration between major drainages. Rather than the traditional model of dispersal and accelerated morphological evolution at the margins of the range, Glaubrecht (2000) uses phylogenetic, paleontological, and morphometric evidence to develop a detailed biogeographic hypothesis that involves vicariance and brief periods of accelerated morphological change followed by interchange between temporarily isolated basins.

The breakup of the Tethys Sea into sub-basins has also been invoked as an important biogeographic factor in several other freshwater groups including fish (Choudhury and Dick, 1998; Hrbek and Meyer, 2003; Zardoya and Doadrio, 1999) and crustaceans (Coineau, 1994). Furthermore, there are biogeographic provinces defined for marine gastropods that correlate well with the pattern seen in *Theodoxus* (Harzhauser et al., 2002), offering additional support to a biogeographic model driven by marine basin evolution. Our hypothesis is in close agreement with Zardoya and Doadrio (1999), who predicted that the pattern of division between Mediterranean and Paratethyan Seas, and later among basins within these realms, would be found in many low-dispersal organisms. We find exactly this pattern in *Theodoxus*. Indeed, in this case biogeography and phylogenetic timing offer strong support for the influence of marine geological history on the evolution of a freshwater invertebrate clade.

4.1. Phylogenetic relationships and systematics

The complex vicariance of Mediterranean and Paratethyan basins during the Miocene (Harzhauser et al., 2002; Hrbek and Meyer, 2003; Zardoya and Doadrio, 1999) is reflected in the short branch lengths separating major clades of *Theodoxus* and our difficulty in resolving their relationships. Although there is general agreement between the various phylogenetic hypotheses produced by our different analyses, there is not a single solution to the relationships among the major clades. Our inability to recover the relationship found by ML analysis of COI alone — (((A,B)C)D)—in any other analysis reduces our confidence in this result. Furthermore, even allowing COI to contribute more to parsimony reconstruction than 16S in the combined dataset did not recover this relationship. Only one analysis, ML of 16S, recovered a tree that placed clades A and D as sisters, and A+D as sister to B+C (i.e. topology VI on Fig. 3). Additionally, 43% of most parsimonious trees from the 16S analysis also recovered this less common topology. Since this relationship (topology VI on Fig. 3) could not be rejected by any of the topological tests, we regard it as highly plausible. The other most plausible topology (II on Fig. 3) was only rejected by a single likelihood-mapping permutation in which clade F was ignored. Notably, this topology is very similar to topology V, differing only in their placement of clade E, which we assume to

be the basal member of the *Theodoxus* clade. When clade E is placed at the lineage leading to clade F, topology II is obtained. When clade E is connected to the common ancestor of D and F, topology V is obtained. This indicates that the paucity of characters distinguishing clades A–D makes rooting this group quite difficult.

Recent studies have indicated that choosing the appropriate model of molecular evolution can help to better reconstruct relationships that are obscured by effects such as long-branch attraction (Huelsenbeck, 1998; Swofford et al., 2001). Thus, one approach for choosing between competing trees is to utilize maximum likelihood and Bayesian methods that employ an accurate model of sequence evolution (Steel, 2005). Therefore, the relationships represented by 16S ML (topology VI) would be the best current hypothesis for these relationships. However, discriminating between competing hypotheses when they each attempt to use an appropriate model of sequence evolution (e.g. ML of 16S and of 16S+COI) is difficult when none of the alternative topologies are easily rejected using various best-fit models (i.e. topologies II, III, and VI). Rather, it appears that the vicariance associated with post-Tethys marine basin development produced branching events between these clades that were simply too close in time for these mitochondrial markers to resolve.

Clade F, represented by a single specimen of *T. peloponnesa*, is problematic because of its high 16S sequence divergence and a lack of data for COI. This specimen was collected from the Peloponnesus peninsula in Greece during the middle of the 20th century and preserved as a dried specimen. Because of the poor state of preservation, only 16S could be amplified and it contained some areas of poor sequence that had to be coded ambiguously. The phylogenetic position of this species is therefore unclear because of the uncertainty contributed by sequence divergence and/or missing data. However, its status as a distinct lineage is supported because none of the resolutions of the ambiguous bases nor an analysis excluding these positions placed this species within any of the other major clades.

Molecular studies of ubiquitous molluscan groups typically support some traditional or regional alpha taxonomies, while rendering others nonsensical (e.g. De Weerd et al., 2004; Donald et al., 2005; Duda and Kohn, 2005; Meyer, 2003). *Theodoxus* appears no different and although a full nomenclatural review of the genus is not appropriate here, we do address several issues for future research. Upon first inspection of the geographic distribution of extant species of *Theodoxus*, it is surprising to find that all but one inhabit relatively restricted geographic ranges. The exception, *T. fluviatilis*, has been reported from most of the range of the genus and is absent only in the Caspian drainage of far eastern Europe and North Africa (Bunje, 2005). Having a single outlier like this would appear to indicate either an exceptional species within the genus or that *T. fluviatilis*, the type species of *Theodoxus*, is a nomenclatural waste bin. Our results support both interpretations. Clade B is a monophyletic taxon that corresponds to historical conceptions and use of the name *T. fluviatilis* (Fretter and Graham, 1962). But, specimens assigned by collectors to *T. fluviatilis* are also found in several other major clades. Thus, difficulty in identifying taxonomic affinities based on gross shell characteristics alone and the lack of a thorough systematic review of the genus have resulted in difficulty assigning specimens to species. Since clade B corresponds well to historical and general conceptions, we regard it as the species unit *T. fluviatilis*.

Another nomenclatural problem of particular interest is the nominal species *T. velascoi*. This species was thought to be extinct until recently, when it was rediscovered in orange grove canals in eastern Spain (Robles, 1997). All populations of *T. velascoi* co-occur with *T. valentina*, which is co-distributed in eastern Spain with two other species: *T. fluviatilis* and *T. baeticus* (Robles, 1997). All four species are readily distinguished from one another by eye (A. Martinez-Orti, personal communication) and are thought to represent historically distinct lineages (Barsiene et al., 2000). Our phylogenetic analysis concludes that there are three distinct clades represented by these Spanish specimens, two belonging to clade D and one which nests within clade B. Unfortunately, these taxa do not coincide with species assignments based on shell morphology. One of these monophyletic groups almost certainly represents *T. baeticus*, which has been described from Spain, Greece, and Turkey. Additionally, specimens assigned to *T. baeticus* fall into two distinct subclades within clade D (Fig. 2). These incongruences between nomenclature based on morphology and molecular phylogenetic data highlight the difficulty in correctly classifying molluscan species for which extensive biogeographic, morphologic, and phylogenetic data is unavailable. Indeed, these problems become even more of a concern when conservation efforts are based on taxonomy that lacks a phylogenetic framework, as in the case of *T. velascoi*.

Lastly, the determination that *T. prevostianus* is a diphyletic taxon nested within the greater *T. danubialis* clade C is unanticipated and further demonstrates how gross shell morphology can mask genealogical history. Given the other paraphyletic and polyphyletic taxa that were discovered by these analyses (e.g. *T. meridionalis*, *T. syriacus*, *T. prevostianus*, *T. baeticus*, and *T. fluviatilis*; Fig. 2), this problem may be widespread in *Theodoxus* and morphology may be an especially poor predictor of taxonomy.

This phylogenetic analysis underscores the need for a systematic revision of *Theodoxus*. While it may be argued that it is inadvisable to draw conclusions of taxonomic affinity from a mtDNA phylogeny, particularly at the species level (Davis, 1994), the phylogenetic hypothesis presented here can and must be used to inform future systematic revisions. For example, it is clear from our analyses that using gross morphology and/or geographic area as the diagnostic characters is unwarranted. When diverse and complex cladogenic processes drive lineage divergence, such as in *Theodoxus*, molecular phylogenetic data are critical for defining a consistent and meaningful taxonomy by unraveling the history of evolutionary diversification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.06.026.

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