RESEARCH NOTES

Molecular phylogeny of the helicoid land snails (Pulmonata: Stylommatophora: Helicoidea), with special emphasis on the Camaenidae

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The superfamily Helicoidea is a large and diverse group of land snails belonging to the Pulmonate suborder Stylommatophora. It has an almost worldwide distribution, absent from only sub-Saharan Africa, southern South America, New Zealand and some Pacific islands (Scott, 1996, 1997). The composition of the superfamily has remained somewhat controversial. Morphological studies have suggested the inclusion of between three and nine families, with only the Helicidae and Bradybaenidae common to all classifications (Thiele, 1931; Zilch, 1959–1960; Taylor & Sohl, 1962; Shileyko, 1979; Solem, 1979; Boss, 1982; Nordsieck, 1985, 1986; Tillier, 1989; Vaught, 1989; Scott, 1997; Bouchet & Rocroi, 2005). Our recent molecular study of the Stylommatophora has demonstrated the monophyly of a 'Helicoidea' comprising the Helicidae, Bradybaenidae, Helminthoglyptidae, Hygromiidae, Camaenidae, Polygyridae and Sagdidae (Wade, Mordan & Naggs, 2006). In this study, we apply molecular phylogenetic techniques to investigate specifically the evolutionary relationships within this 'helicoid' group.

Of particular interest are the relationships among members of the helicoid family Camaenidae. The camaenids have an unusual bihemispheric disjunct distribution in Asia and Australasia (from Sri Lanka and India through Myanmar and southeast Asia to Japan, the Philippines, New Guinea to Australia and some Pacific islands) and Central and South America (from Costa Rica, the Greater and Lesser Antilles to northern Argentina) (Scott, 1996; Cuezzo, 2003). The Camaenidae are defined by the absence of a dart sac and related glands on the female genitalia (Pilsbry, 1939), but there is no unique synapomorphy that is characteristic of the group (Scott, 1996) and morphological studies are contradictory with regards to the monophyly or para/polyphyly of the group (Scott, 1996; Cuezzo, 2003). We use molecular data to specifically investigate this issue here.

We have previously sequenced 48 helicoid species for a partial fragment of the rRNA gene cluster (Table 1, Wade, Mordan & Clarke, 2001; Wade et al., 2006). Here we undertake extensive new phylogenetic analyses of the Helicoidea using these sequences, in order to focus specifically on the evolutionary relationships within the helicoid group. By restricting our analyses to the Helicoidea we are able to recruit new sites into our analyses and we present phylogenies based on 915 (for all Helicoidea) and 1,012 (for a subset of the Helicoidea) unambiguously aligned nucleotide sites. Evolutionary trees were constructed using the neighbour-joining (NJ; Saitou & Nei, 1987) and Fitch-Margoliash (FM; Fitch & Margoliash, 1967) distance methods, maximum-likelihood (ML; Felsenstein, 1981), Bayesian Inference (BI; Larget & Simon, 1999) and maximum parsimony (MP; Fitch, 1971). NJ, FM, ML and

MP analyses were performed using PAUP* (version 4.0d65; Swofford, 1998). For the NJ, FM and ML methods, multiple hits were accounted for using the general time-reversible (GTR) model (Lanave et al., 1984) with between-site rate heterogeneity accounted for by incorporating a proportion of invariant sites (I) and gamma-distributed rates (G) into the model (Gu, Fu & Li, 1995). The rate matrix, base frequencies, proportion of invariant sites (pinvar) and shape parameter (α) of the gamma distribution (based on 16 rate categories) were estimated using likelihood by iteration from an initial neighbour-joining tree. For FM, ML and MP methods, tree searching used a heuristic procedure with tree-bisection-reconnection branch swapping. Bootstrap resampling (Felsenstein, 1985) (NJ only with 1,000 bootstrap replicates) was employed to assign support to particular branches within the tree. BI analysis was performed using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). A GTR+G model was used and the tree space was explored using four chains of a Markov Chain Monte Carlo algorithm for 5 million generations, sampling every 100 generations. In order to ensure adequate chain swapping, the heating parameter was set to 0.05. A consensus tree was built using the last 1,000 trees (burnin = 49,001 samples). Alternative phylogenetic hypotheses were evaluated by likelihood using a Kishino–Hasegawa RELL test (Kishino, Miyata & Hasegawa, 1990) as implemented in PAUP*.

A maximum-likelihood tree showing the evolutionary relationships among the Helicoidea is presented in Figure 1. The phylogeny is based on the analysis of 915 unambiguously aligned nucleotide sites, of which 221 were variable and 152 were parsimony informative. The tree is rooted on the branch to Haplotrema and Euglandina, shown to fall as sister taxa to the Helicoidea in the Wade et al. (2001, 2006) analyses of stylommatophoran rDNA data. All methods of tree construction employed (ML, BI, NJ, FM and MP) produced trees that were generally consistent, though there were some rearrangements in the relative placement of the basal lineages in the helicoid tree with different methods.

The Helicoidea are consistently resolved as a distinct clade with all tree methods and are supported in 89% of NJ bootstraps ($P = 1$, BI) (Fig. 1). Within the Helicoidea, the molecular phylogenies confirm the monophyly of the Helicidae (currently represented by 12 taxa from nine genera; 82%, NJ bootstraps; $P = 1$, BI), a group well supported in all molecular studies of the Stylommatophora/Helicoidea to date (Wade et al., 2001; Steinke, Albrecht & Pfenninger, 2004; Koene & Schulenburg, 2005; Manganelli, Salomone & Giusti, 2005; Wade et al., 2006). The Hygromiidae, represented by five taxa from four genera (52%, NJ bootstraps; $P = 0.8$, BI), are also monophyletic in our trees, a finding again consistent with other molecular studies (Wade et al., 2001; Steinke et al., 2004; Koene & Schulenburg, 2005; Manganelli et al., 2005; Wade et al., 2006), though

Table 1. Samples, collectors, localities and GenBank accession numbers. The classification broadly follows Vaught, 1989.

| Family	Species	Collection/location	Collector	GenBank Accessions
Helicoidea:				
Helicidae	*Helix pomatia* L., 1758	Pulpit Down, Buckinghamshire, UK	P. Mordan	AY841333
	Helix lucorum L., 1758	Unknown	Unknown	AY841334
	Cantareus aspersus (Müller, 1774) [= *Helix aspersa*]	Kettering, Northants, UK	C. Wade	AY014128
	Cantareus apertus (Born, 1778)	Sicily	A. Davison	AY014129
	Cepaea nemoralis (L., 1758)	Marlborough Downs, Wiltshire, UK	A. Davison	AY014130
	Cepaea hortensis (Müller, 1774)	Marlborough Downs, Wiltshire, UK	A. Davison	AY014131
	Eremina desertorum (Forskål, 1775)	Unknown	Unknown	AY841335
	Marmorana scabriuscula (Deshayes, 1830)	Sicily	A. Davison	AY014132 & AY014133
	Otala lactea (Müller, 1774)	Unknown	Unknown	AY841336
	Theba pisana (Müller, 1774)	Sicily	A. Davison	AY014134 & AY014135
	Arianta arbustorum (L., 1758)	Deepdale, Derbyshire, UK	C. Wade	AY014136
	Helicigona lapicida (L., 1758)	Deepdale, Derbyshire, UK	C. Wade	AY014137
Polygyridae	*Mesodon thyroides* (Say, 1816)	York Co. Pennsylvania, USA	F. Thompson	AY841315
	Triodopsis alleni (Wetherby, 1883)	Williams Creek, Iowa, USA	R. Cameron	AY841316
	Vespericola columbiana (Lea, 1838)	Eugene, Oregon, USA	D. Taylor	AY014120
Hygromiidae	*Trochulus striolatus* (Pfeiffer, 1828) [= *Trichia striolata*]	Deepdale, Derbyshire, UK	C. Wade	AY014124
	Trochulus hispidus (L., 1758) [= *Trichia hispida*]	Deepdale, Derbyshire, UK	C. Wade	AY014125
	Cochlicella acuta (Müller, 1774)	Porthcurnick, Cornwall, UK	E. Bailes	AY014126
	Cernuella virgata (Da Costa, 1778)	Porthcurnick, Cornwall, UK	E. Bailes	AY014127
	Monacha cantiana (Montagu, 1803)	Pulpit Down, Buckinghamshire, UK	P. Mordan	AY841331 & AY841332
Helminthoglyptidae	*Monadenia fidelis* (Gray, 1834)	Oregon	D. Taylor	AY014142
	Cepolis streatori (Pilsbry, 1889)	Grand Cayman	S. Chiba	AY841346
Bradybaenidae	*Bradybaena similaris* (Férussac, 1821)	Sri Lanka	P. Karunaratne	AY014138
	Acusta despecta chinensis (Sowerby, 1839)	Japan	S. Chiba	AY841337
	Ainohelix editha (A. Adams, 1868)	Shimamaki, Hokkaido, Japan	S. Chiba	AY841338
	Ezohelix gainesi (Pilsbry, 1900)	Sapporo, Hokkaido, Japan	S. Chiba	AY841339
	Aegista vulgivaga (Schumacher & Boettger, 1890)	Osaka City, Japan	P. Callomon	AY014139
	Paraegista takahidei Kuroda & Azuma, 1951	Hokkaido, Japan	S. Chiba	AY841340
	Euhadra amaliae (Kobelt, 1875)	Osaka City, Japan	P. Callomon	AY014140
	Euhadra sandai Pilsbry, 1928	Osaka City, Japan	P. Callomon	AY014141
	Nesiohelix bipyramidalis Kuroda & Emura, 1943	Ryukyu, Japan	S. Chiba	AY841341
	Helicostyla lignaria (Pfeiffer, 1842)	Bohol Island, Phillipines	S. Chiba	AY841342 & AY841343
	Chloraea intorta (Sowerby, 1840)	Bohol Island, Phillipines	S. Chiba	AY841344
	Trishoplita hachijoensis (Pilsbry, 1902)	Niijima Island, Izu Islands, Japan	S. Chiba	AY841345
Camaenidae	*Amphidromus* sp.	Unknown	D. Reid	AY841317 & AY841318
	Nipponchloritis bracteatus (Pilsbry, 1902)	Sendai, Japan	S. Chiba	AY841319
	Mandarina ponderosa (Pilsbry, 1901)	Hahajima, Bonin Islands, Japan	S. Chiba & A. Davison	AY841320
	Theliodomus asper (Férussac, 1821)	Windsor, Jamaica	S. Chiba	AY841321
	Pleurodonte sinuata (Müller, 1773)	Green Grot Cave, Jamaica	S. Chiba	AY841322
	Polydontes undulata (Férussac, 1821)	Dominican Republic	G. Seal	AY014121
	Polydontes lima (Férussac, 1821)	Dorado, Puerto Rico	A. Davison	AY841323
	Satsuma japonica (Pfeiffer, 1847)	Osaka City, Japan	P. Callomon	AY014122
	Coniglobus mercatorius (Pfeiffer, 1854)	Kikai Island, Ryukyu, Japan	S. Chiba	AY841324
	Sphaerospira fraseri (Griffith & Pigeon, 1833)	Brisbane, Queensland, Australia	J. Stanisic	AY841325
	Zachrysia auricoma (Férussac, 1821)	Nr. Dorado, Puerto Rico	A. Davison	AY841326
	Obba rota (Broderip, 1841)	Bohol Island, Phillipines	S. Chiba	AY841327 & AY841328
	Moellendorffia diminuta Pilsbry & Hirase, 1905	Ryukyu, Japan	S. Chiba	AY841329 & AY841330
Sagdidae	*Sagda* sp.	Windsor, Jamaica	S. Chiba	AY841347
Outgroups:				
Spiraxidae	*Euglandina rosea* (Férussac, 1821)	Moorea (Zool. Soc. Lond. colln.)	P. Pearce-Kelly	AY014074
Haplotrematidae	*Haplotrema vancouverense* (Lea, 1839)	Eugene, Oregon, USA	D. Taylor	AY014090
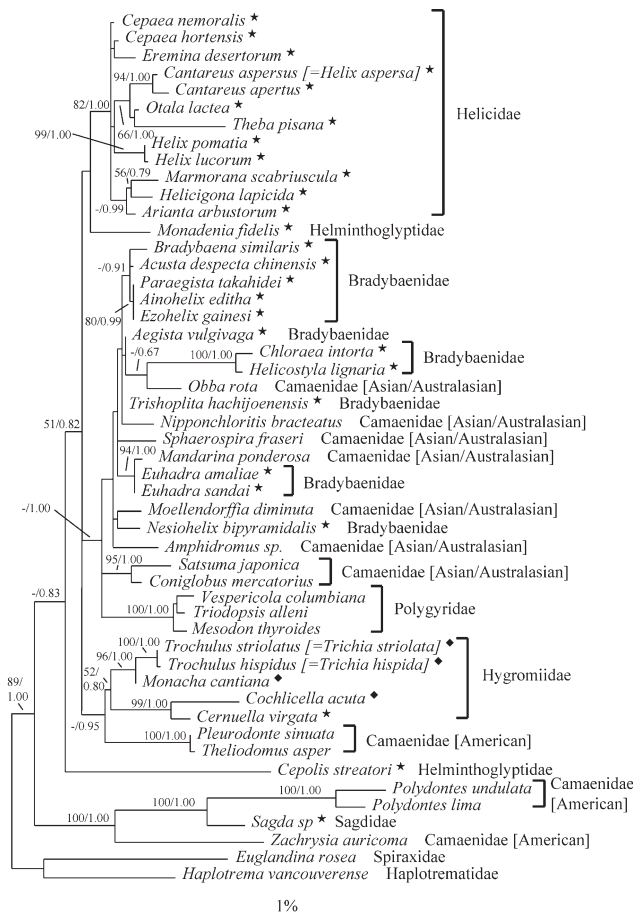
Figure 1. Maximum-likelihood phylogenetic tree showing the evolutionary relationships among the Helicoidea. The phylogeny is based on 915 unambiguously aligned nucleotide sites and the tree is rooted on *Euglandina* and *Haplotrema*, which have previously been shown to form the sister clade to the Helicoidea (Wade *et al.*, 2001, 2006). Correction for multiple hits was employed using a GTR + I+G model (proportion of invariant sites, pinvar = 0.335, and gamma distribution, α = 0.304). The scale bar corresponds to 1 substitutional change per 100 nucleotide positions. Bootstrap values (1,000 NJ bootstraps expressed as a percentage) and Bayesian posterior probabilities indicating support for individual branches are shown on the tree (NJ bootstraps/BI posterior probabilities). Individual taxa marked with a ★ have love darts. ◆ denotes taxa that do not have love darts but do have a dart sac.
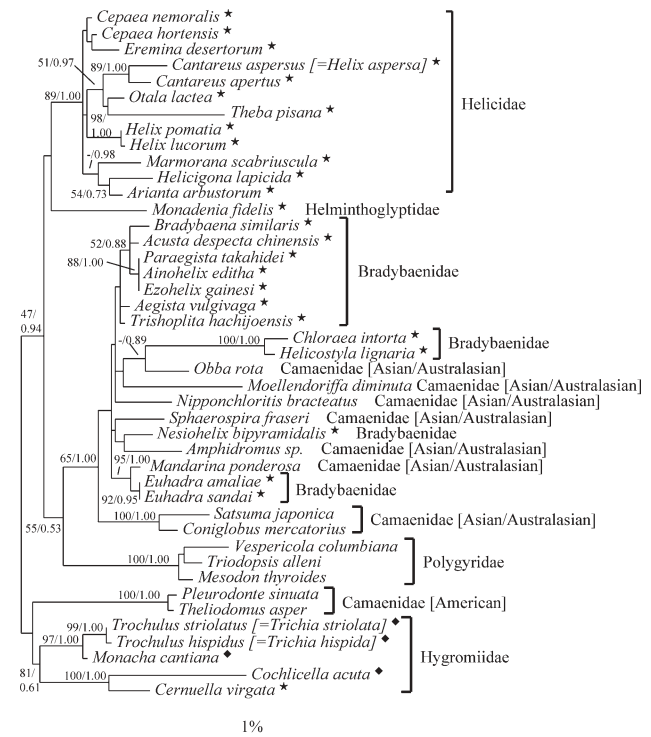

Figure 2. Maximum-likelihood phylogenetic tree showing the evolutionary relationships among a subset of the Helicoidea. The exclusion of the divergent helicoid taxa, *Zachrysia*, *Sagda*, *Polydontes* and *Cepolis* and the outgroups permits an additional 97 sites to be recruited into analyses and the phylogeny is based on 1012 unambiguously aligned nucleotide sites. Correction for multiple hits was employed using a GTR + I+G model (proportion of invariant sites, pinvar = 0.395, and gamma distribution, α = 0.312). Bootstrap values (1,000 NJ bootstraps expressed as a percentage) and Bayesian posterior probabilities indicating support for individual branches are shown on the tree (NJ bootstraps/BI posterior probabilities). The scale bar corresponds to 1 substitutional change per 100 nucleotide positions. Individual taxa marked with a ★ have love darts. ◆ denotes taxa that do not have love darts but do have a dart sac.

within the Hygromiidae the subfamily Helicellinae (*Cernuella*, *Cochlicella* and *Monacha*) appears to be paraphyletic in that it includes the Trochulinae (=Trichiinae, type genus *Trichia*; *Trochulus* is a replacement name for *Trichia* ICZN ruling, Opinion 2079, 2004). The Polygyroidea, represented by three genera, are also clearly monophyletic (100%, NJ bootstraps; *P* = 1, BI) within the molecular trees.

The helminthoglyptids *sensu* Zilch (1959–60), represented in our trees by the genera *Cepolis* and *Monadenia*, are clearly not monophyletic. However, both taxa were excluded by Roth (1996) from his restricted Helminthoglyptidae and allocated to a Bradybaenidae/Xanthonychidae clade. Clearly a wider taxonomic coverage is required in order to resolve this.

The Camaenidae are of particular interest, with the molecular tree providing strong support for the division of the Camaenidae into three groups (Fig. 1). The first includes the American taxa *Polydontes* and *Zachrysia* (and also *Sagda* from the family Sagdidae) and is supported in 100% of NJ bootstraps (*P* = 1, BI)

and with all methods of tree construction. The second includes the American taxa *Pleurodonte* and *Theliodomus* and is again resolved with all tree methods and supported in 100% of NJ bootstraps (*P* = 1, BI). The remaining Asian and Australasian camaenid taxa appear to form a complex with the Bradybaenidae, with camaenid and bradybaenid taxa interspersed in the molecular tree. The phylogenetic placement of the Polygyridae in relation to the bradybaenids and Asian/Australasian camaenids remains unclear.

In order to elucidate more clearly the evolutionary relationships among the Asian/Australasian camaenids and the bradybaenids, supplementary phylogenetic analyses were undertaken in which the outgroups and the divergent (long-branch) helicoid taxa, *Zachrysia*, *Sagda*, *Polydontes* and *Cepolis*, were excluded. This permitted an additional 97 sites to be recruited into the phylogenetic analyses thereby providing greater resolution within the helicoid tree (Fig. 2). In these analyses the Bradybaenidae and the Asian/Australasian Camaenidae consistently cluster together with all methods of phylogeny reconstruction, to form a complex which is supported in 65% of NJ bootstraps (*P* = 1, BI).

The division of the Camaenidae into American and Asian/Australasian groups is in agreement with Scott's (1996) argument that the American and Asian/Australasian camaenids are not sister groups and is consistent with our earlier molecular analyses of the Stylommatophora (Wade *et al.*, 2001, 2006). The molecular

data provide conclusive evidence for the separation of the American Camaenidae from the Asian/Australasian Camaenidae, with the monophyly of the Camaenidae strongly refuted in Kishino–Hasegawa likelihood tests [−ln Likelihood (L) 4124.60869 (best tree shown in Fig. 1) versus −ln L 4221.26103 (Camaenidae monophyletic), $P < 0.01$]. Our results strongly contradict those of Cuezzo (2003), who concluded from a morphological analysis that the camaenids are monophyletic. Moreover, the Asian/Australasian camaenids are interspersed with the Bradybaenidae in our molecular tree, providing additional support for Scott's (1996) suggestions that the Asian/Australasian Camaenidae and the Bradybaenidae are confamilial. In Kishino–Hasegawa tests, the best tree is one that indicates that the Asian/Australasian camaenids are interspersed with the bradybaenids [−ln L 4124.60869 (Fig. 1)], but this is not significantly better than any tree that assumes that the Asian/Australasian camaenids are monophyletic [−ln L 4145.20601, $P = 0.075$]. The separation of the American camaenids into two groups (with *Polydontes* and *Zachrysia* falling in one group, and *Pleurodonte* and *Theliodomus* in the other) is a novel finding that is of considerable interest. A broader survey of the American camaenids is now required to determine the full picture of diversity within the group. Interestingly, the enigmatic taxon *Sagda* falls with the American camaenids *Polydontes* and *Zachrysia*. Within the Bradybaenidae, *Chloroea* and *Helicostyla* cluster strongly (100%, NJ bootstraps; $P = 1$, BI), consistent with their classification within the subfamily Helicostylinae.

The absence of a comprehensive and consistent set of taxa across all molecular studies of the Helicoidea (Steinke *et al.*, 2004; Koene & Schulenburg, 2005; Manganelli *et al.*, 2005) makes it very difficult to make direct comparisons between our molecular phylogeny and those of other groups. However, our phylogeny appears to disagree with that of Steinke *et al.* (2004), where the helicids and hygromids appeared as sister taxa, with *Bradybaena* falling basal to this clade.

While it was not possible to include helicoid sequences from other molecular studies in our main phylogenetic analyses [due either to the sequencing of nonhomologous gene regions (Steinke *et al.*, 2004; Manganelli *et al.*, 2005) or the sequencing of a substantially shorter fragment of the rRNA gene (Koene & Schulenburg, 2005)], we undertook a supplementary analysis of the Helicoidea in which we incorporated the homologous but substantially shorter rRNA sequences of novel genera sequenced by Koene & Schulenburg (2005). This analysis (based on only 712 sites) permitted the inclusion of two new helicid genera (*Chilostoma* and *Eobania*), nine new hygromiid genera (*Pseudotrichia*, *Leptaxis*, *Xerotrichia*, *Helicella*, *Xeromunda*, *Xerosecta*, *Perforatella*, *Monachoides* and *Hygromia*), one new bradybaenid (*Fruticola*) and four new helminthoglyptid genera (*Helminthoglypta*, *Humboldtiana*, *Xerarionta* and *Polymita*). Resolution and support for clades were considerably reduced in this supplementary analysis (data not shown). However, despite the inclusion of new genera, the Helicoidea, Hygromiidae and Polygyridae remained resolved as monophyletic units. Interestingly, the helminthoglyptid genus *Polymita* clustered with *Cepolis* in this analysis, with the other helminthoglyptids falling with *Monadenia*.

Elsewhere, although the molecular phylogeny provides little resolution of the relationships within the Helicidae, one issue of contention is resolved. The brown garden snail *Helix aspersa* clusters with *Cantareus apertus* in the molecular tree (94%/89%, NJ bootstraps; $P = 1/P = 1$, BI, Figs. 1 and 2) and not with representatives of the genus *Helix*. This supports the study of Giusti, Manganelli & Schembri (1995) in which *Helix aspersa* was placed in the genus *Cantareus*.

Love darts are present in all taxonomic groups in the tree except for the Camaenidae and Polygyridae (Fig. 1; Tompa, 1980; Baur, 1998; Roth, 1996; Davison *et al.*, 2005). Indeed, the Camaenidae are at least in part defined by the absence of a dart-sac and other secondary sexual characters (Pilsbry, 1939). In the Hygromiidae, *Cernuella* possess love darts, *Trochulus* [= *Trichia*] forms only unconsolidated granules in the dart sacs and lacks darts; both *Monacha* and *Cochlicella* lack darts but have a dart sac. The absence of love darts in both the Polygyridae and the polyphyletic Camaenidae (which are scattered across the helicoid tree) provides strong evidence that darts have been lost multiple times in helicoid evolution. Kishino–Hasegawa tests demonstrate that phylogenies in which groups with darts or with darts/dart sacs are constrained to be monophyletic are significantly inferior to the best tree shown in Figure 1 [−ln L 4124.60869 (best tree) versus −ln L 4257.97267 (darts monophyletic), $P < 0.01$, or −ln L 4221.41890 (darts/dart sacs monophyletic), $P < 0.01$]. Darts are also absent from the spiraxid and haplotrematid outgroups, the best candidates for sister taxa of the Helicoidea *sensu lato* (Wade *et al.*, 2001, 2006).

One issue of concern relates to the relatively long branches of the deeper lineages within the Helicoidea. Long-branch attraction is a well-known problem in phylogeny reconstruction (Philippe & Laurent, 1998) and the occurrence of relatively long branches at the base of the helicoid tree could be due to errors in the process of phylogeny construction. Notably, the removal of these sequences (with concomitant increase in sequence length) led to an improvement of bootstrap support values elsewhere in the tree. There is an immediate need to obtain data for new genes (as well as more sequence data for the rRNA) to clarify relationships within the helicoid tree. Nevertheless, the molecular data are beginning to illuminate the evolutionary relationships within the helicoid group.

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