

PHYLOGENETIC POSITION OF THE GENUS *CRYPTAZECA* AND THE FAMILY AZECIDAE WITHIN THE SYSTEM OF THE STYLOMMATOPHORA

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INTRODUCTION

The first data about the genus *Cryptazeca* were provided by Folin & Bérillon (1877a) when they described *Azeca monodonta* based on the study of two empty shells collected near Bayonne in southwestern France. Later they collected some live specimens and established the genus *Cryptazeca* for it, after study of its anatomy (Folin & Bérillon, 1877b, 1891). This genus was included together with *Ferussacia*, *Cecilioides*, *Azeca* (with *Hypnophila*), *Cochlicopa*, and five additional genera in the family Ferussaciidae (Pilsbry, 1908). Watson (1928) noticed that *Ferussacia* and *Cecilioides* had a sigmurethrous excretory system and placed them and the family Ferussaciidae in the infraorder Sigmurethra, whereas Steenberg (1925) and Zilch (1959) placed *Azeca*, *Cochlicopa*, and *Hypnophila* in the family Cochlicopidae (infraorder Orthurethra).

New live specimens of the genus *Cryptazeca* were collected in 1987 and its excretory system described as being of the orthurethrous type by Gómez & Angulo (1987). Consequently, these authors proposed to change the taxonomic position of this genus to be placed with *Azeca*, *Cochlicopa*, and *Hypnophila* in the family Cochlicopidae. Detailed anatomical and histological studies of the reproductive system also corroborated the close relationships among *Cryptazeca*, *Azeca*, and *Hypnophila* (Gómez & Angulo, 1987, 1990; Gómez, 1990b, 1991). Nevertheless, Schileyko (1976) considered that orthurethria was probably a primary condition for Stylommatophora, which could be retained in some taxa outside orthurethral groups. This led again to the placement of *Cryptazeca* within the family Ferussaciidae. As a result, the classification followed by the CLECOM project (Bank et al., 2001; Falkner et al., 2001) placed *Cryptazeca* (Cryptazecinae) with *Ferussacia*, *Cecilioides*, and *Hohenwartiana* (Ferussaciinae) within the Ferussaciidae, in the superfam-

ily Achatinoidea. The families Cochlicopidae (genus *Cochlicopa*) and Azecidae (genera *Azeca* and *Hypnophila*) were placed in the Cochlicopoidea. On the other hand, Bouchet & Rocroi (2005) classified *Cryptazeca* with *Azeca* and *Hypnophila* in the Cochlicopidae, subfamily Azecinae (syn. Cryptazecinae), sister group of the Cochlicopinae.

In the present work, we have used molecular phylogenetics to reexamine the relationships of the genus *Cryptazeca* with respect to Ferussaciidae, Cochlicopidae and Azecidae. This study gives additional information about the validity of conchological and anatomical characters in the resolution of the taxonomy of these genera and families. It also allows us to determine whether Azecidae and Cochlicopidae are sister groups and to establish the position of the Azecidae within the suborder Stylommatophora. To allow comparison with Wade et al. (2001, 2006), who provided the most comprehensive molecular study of stylommatophoran relationships, we have used part of the nuclear ribosomal RNA gene cluster.

MATERIALS AND METHODS

Approximately 1,460 nucleotides of the rRNA gene-cluster were amplified for four taxa – *Azeca* (sample locality U.T.M. 30TXN78), *Cryptazeca* (30TXN78), *Ferussacia* (30SYJ02), and *Hypnophila* (30SVF56) – using two overlapping primer sets (Wade et al., 2006); 42 additional sequences were obtained from the GenBank database and included in the phylogenetic analyses (details and GenBank accession numbers given in Table 1). Due to the numerous insertions and deletions characterizing sequences of rRNA genes, any position that could not be reliably aligned was excluded from subsequent analyses.

Phylogenetic trees, incorporating all 46 species, were constructed using both neighbour-

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Species	5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
<i>Albinaria xantostoma</i>	AY014048
<i>Archachatina marginata</i>	AY014070, AY014071
<i>Arion hortensis</i>	AY014143
<i>Azeca goodalli*</i>	FJ791121
<i>Carychium tridentatum</i>	AY014148
<i>Cerastus schweinfurthii</i>	AY014040
<i>Chondrina avenacea</i>	AY014032
<i>Chondrina clienta</i>	AY014031
<i>Cochlicopa lubrica</i>	AY014019
<i>Cochlicopa lubricella</i>	AY014020
<i>Cryptazeca monodonta*</i>	FJ791122
<i>Draparnaudia singularis</i>	AY841290, AY841291
<i>Elasmias luakahaense</i>	AY841280
<i>Eostrobilops nipponica</i>	AY841287
<i>Eua zebrina</i>	AY014046
<i>Ferussacia folliculus</i>	AY841302
<i>Ferussacia folliculus*</i>	FJ791120
<i>Gastrocopta armifera</i>	AY841286
<i>Hypnophila malagana*</i>	FJ791123
<i>Lauria cylindracea</i>	AY014023
<i>Lauria fasciolata</i>	AY014024
<i>Leptachatina lepida</i>	AY014021, AY014022
<i>Luchuena reticulata</i>	AY841288
<i>Macaronapaeus vulgaris</i>	AY014036, AY014037
<i>Mandarina ponderosa</i>	AY841320
<i>Mastus pupa</i>	AY014038, AY014039
<i>Melampus luteus</i>	AY014146
<i>Napaeus pruninus</i>	AY841289
<i>Orcula austriaca</i>	AY014028
<i>Otala lactea</i>	AY841336
<i>Oxychilus alliarius</i>	AY014114
<i>Oxychilus helveticus</i>	AY014115
<i>Pachnodus silhouettanus</i>	AY014041
<i>Partula suturalis</i>	AY014042
<i>Partulina proxima</i>	AY841281, AY841282
<i>Pene sidonensis</i>	AY014035
<i>Pronesopupa acanthinula</i>	AY841285
<i>Pupoides albilabris</i>	AY841283, AY841284
<i>Pyramidula rupestris</i>	AY014029, AY014030
<i>Rumina decollata</i>	AY014065
<i>Samoana conica</i>	AY014045
<i>Solatopupa similis</i>	AY014033
<i>Succinea striata</i>	AY841295
<i>Vallonia costata</i>	AY014025
<i>Vallonia excentrica</i>	AY014026
<i>Vertigo antivergo</i>	AY014027

TABLE 1. Samples and GenBank accession numbers for all the sequences used in the phylogenetic analyses. Asterisks (*) represent the four taxa amplified in this work.

←

joining (NJ) and Bayesian inference (BI). Neighbour-joining analysis (Saitou & Nei, 1987) was carried out using the PAUP* (version 4.0d65) package (Swofford, 2002). Distances were corrected for multiple hits 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 et al., 1995). The rate matrix, base frequencies, proportion of invariant sites (pinvar), and shape parameter (α) of the gamma distribution were estimated using ModelTest 3.06 program (Posada & Crandall, 1998) according with Akaike Information Criterion (AIC). Support of the recovered NJ tree was evaluated with non-parametric bootstrap proportions (NJ bootstrap – 1,000 pseudoreplicates). BI analysis was performed using the MrBayes version 3.0b4 package (Huelsenbeck & Ronquist, 2001). A GTR + Gamma model was used. This model uses substitutional information only and excludes gaps (Lanave et al., 1984). Rate heterogeneity between sites was accounted for by incorporating invariant sites (I) and gamma-distributed rates (G) into the model (Gu et al., 1995). The tree space was explored using four chains of a Markov Chain Monte-Carlo algorithm for five million generations, sampling every 100 generations. To ensure adequate chain swapping, the heating parameter was set to 0.05. A consensus tree was built using the last 1,000 trees (burn-in = 49,001 samples). Support of the recovered BI trees was evaluated with Bayesian posterior probabilities (BPPs). The non-stylommatophoran pulmonates *Melampus luteus* and *Carychium tridentatum* were used as outgroups.

RESULTS

The consensus alignment of the four new sequences was initially 1,538 bp long and included the 3' end of the 5.8S ribosomal gene (92 bp), the complete ITS-2 region (609 bp), and the partial sequence of the 28S ribosomal gene (approximately 837 bp). Sequence variation at the ITS-2 region was extremely high, and most of its sites could not be aligned when considering all the taxa. Thus, all ITS-2 sites were excluded from the phylogenetic analyses.

The phylogenetic reconstructions were based on a subset of 900 positions (92 positions of the 5.8S and 808 of the 28S). Neighbour-joining and Bayesian phylogenies of the rRNA gene-cluster are given in Figure 1.

The “achatinoid” and “non-achatinoid” clades were recovered as the two basal groups (100% BPP, 100% NJ and 100% BPP, 83% NJ, respectively). Five main phylogroups were obtained within the non-achatinoid clade, including the Elasmognatha, Helicoidea, Clausilioidea, Arionoidea + Limacoidea, and the Orthurethra. As expected, *Ferussacia* belonged to the “achatinoid” clade. Nevertheless, *Cryptazeca* was placed together with *Hypnophila* and *Azeca* in the “non-achatinoid” clade. All these three genera constituted a monophyletic group, the Azecidae, highly supported (100% BPP, 98% NJ). *Cryptazeca* constituted the sister group of *Hypnophila* (94% BPP, 75% NJ). The Azecidae clade was included within the orthurethrous group as an independent lineage. The Orthurethra taxa, including the family Azecidae, formed a monophyletic group only supported by low values in the Bayesian analysis (73% BPP). Three main monophyletic groups highly supported were recovered within the Orthurethra: the Azecidae (100% BPP, 98% NJ), the Chondrinidae (98% BPP, 93% NJ), and a clade joining the rest of the orthurethrous families (100% BPP, 88% NJ). Nevertheless, the phylogenetic relationships of these three basal clades were not fully resolved.

DISCUSSION

Phylogenetic analyses were based on 900 nucleotide positions of the LSU rRNA genes. This number was slightly greater than the 823 nucleotides considered in the work published by Wade et al. (2001, 2006).

The phylogenetic reconstructions clearly demonstrated that *Cryptazeca* is not related with the Ferussaciidae. On the contrary, it was placed far apart from the “achatinoid clade”. This indicated that the elongate, smooth, glass-like, translucent shell, with a pear-shaped aperture, characters that are common to ferussaciids and *Cryptazeca*, are a case of homoplastic combination of characters, and should not be used to join these taxa.

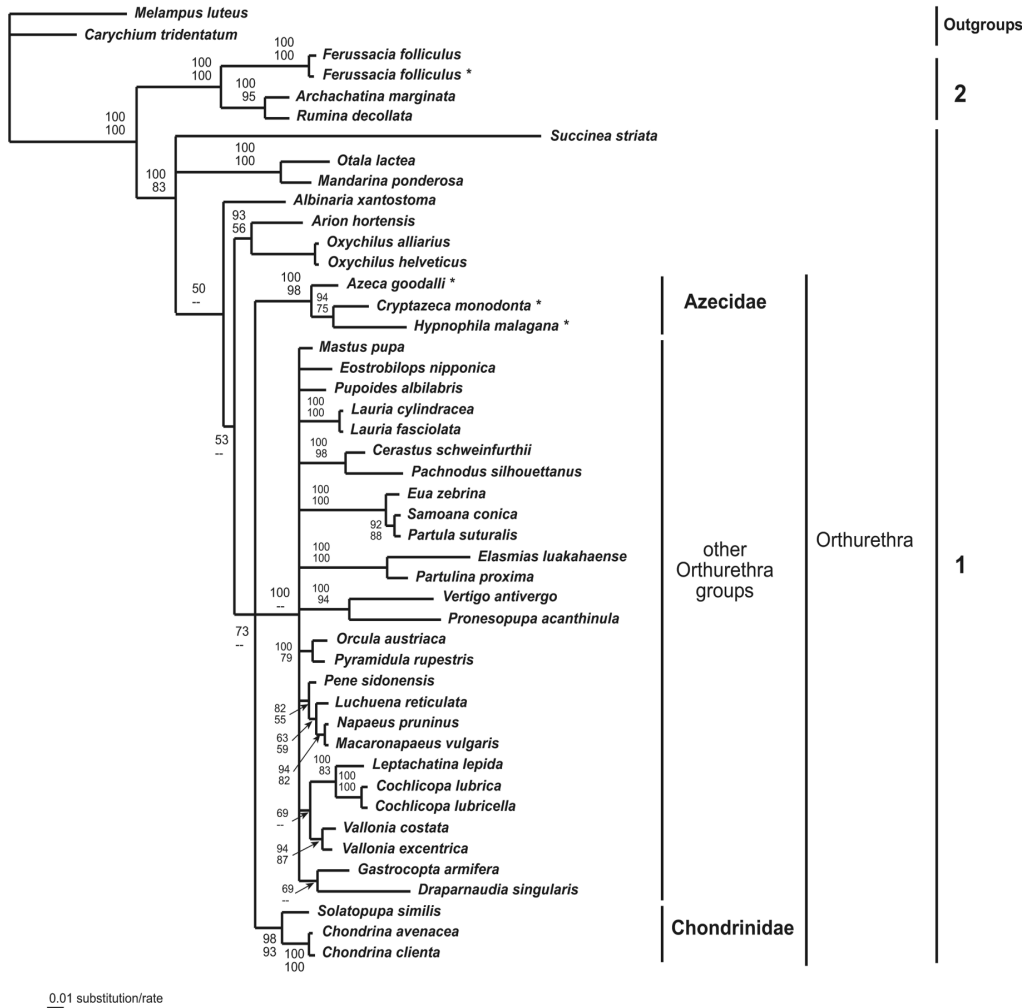


FIG. 1. Phylogenetic relationships (BI phylogram) of *Cryptazeca* and the family Azecidae. Numbers represent support for BI and NJ from top to bottom. Hyphens indicate no support values for NJ. Asterisks (*) represent the four taxa amplified in this work. 1: "non-achatinoid" clade; 2: "achatinoid" clade.

Cryptazeca was recovered as the sister group of *Hypnophila*, as was previously suggested by some authors based on the similarities in shell morphology (Gittenberger, 1983; Gómez & Angulo, 1987), but also by the reproductive system anatomy and shell microsculpture (Gómez & Angulo, 1987; Gómez, 1990a, b). Nevertheless, both genera continued to be classified far apart in different infragroups (Schileyko, 1999; Bank et al., 2001) based on some misinterpretations of *Cryptazeca* morphology.

The phylogenetic analyses showed that *Azeca* is closely related to *Cryptazeca* and *Hypnophila*. These three genera constituted a clade that we propose to be called the Azecidae. The subfamily Azecinae was created by Watson (1920) to highlight the anatomical differences that existed between *Azeca* (absence of diverticulum and penial appendix, among other characters) and *Cochlicopa* (with both reproductive organs). The latter genus constituted the subfamily Cochlicopininae in the classification of Watson (1920). Later, some

classifications have considered both taxa as different families (Kennard & Woodward, 1926; Bank et al., 2001). Current classifications consider Azecidae and Cochlicopidae as sister groups, either as subfamilies of one family (Bouchet & Rocroi, 2005) or as families within the same superfamily (Bank et al., 2001). The new phylogenetic evidence demonstrates that Azecidae and Cochlicopidae are not sister groups. According to Wade et al. (2006), *Cochlicopa* is the sister group of *Leptachatina* (Amastridae), within a large monophyletic group that included all the orthurethrous families, with the exception of Chondrinidae. The Azecidae constituted a third independent main group within the Orthurethra clade, and its monophyly was highly supported by both phylogenetic analyses. Here, again, similarities in shell morphology – glossy, translucent, brown, fusiform-oval shells – led to an inaccurate classification for *Azeca* and *Cochlicopa*.

The monophyly of the Azecidae is supported by shell characters (Gittenberger, 1983), including shell aperture and microsculpture (Gómez, 1990a), reproductive system anatomy (Gómez, 1990b, 1991), and molecular data (present work). Shell characters of this family include: pear shaped aperture, with very narrow parieto-palatal border; slightly curved inward palatal lip, slightly narrowing the aperture; continuous peristome with conspicuous parietal callus extending to columellar bottom; presence of aperture denticles, with at least a columellar tooth (sometimes reduced in *Hypnophila*); and microsculpture of spiral lines on the protoconch and teleoconch. The genital system includes a fertilization chamber surrounded by a voluminous gland formed by goblet gland cells (not studied in *Hypnophila*), a blind-ended allospermidut, and a vagina wall with brown pigmentary cells embedded in it (not a vaginal gland as interpreted by other authors). Male distal organs are very simple, excluding the presence of a sarcobellum in *Cryptazeca*.

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