Molecular phylogeny of the Achatinoidea (Mollusca: Gastropoda)

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Abstract

This study presents a multi-gene phylogenetic analysis of the Achatinoidea and provides an initial basis for a taxonomic re-evaluation of family level groups within the superfamily. A total of 5028 nucleotides from the nuclear rRNA, actin and histone 3 genes and the 1^{st} and 2^{nd} codon positions of the mitochondrial cytochrome c oxidase subunit I gene were sequenced from 24 species, representing six currently recognised families. Results from maximum likelihood, neighbour joining, maximum parsimony and Bayesian inference trees revealed that, of currently recognised families, only the Achatinidae are monophyletic. For the Ferussaciidae, *Ferussacia folliculus* fell separately to *Cecilioides gokweanus* and formed a sister taxon to the rest of the Achatinoidea. For the Coeliaxidae, *Coeliaxis blandii* and *Pyrgina umbilicata* did not group together. The Subulinidae was not resolved, with some subulinids clustering with the Coeliaxidae and Thyrophorellidae. Three subfamilies currently included within the Subulinidae based on current taxonomy likewise did not form monophyletic groups.

Keywords: Achatinoidea, rRNA, actin, histone 3, COI

1. Introduction

The Achatinoidea are a large group of terrestrial land snails of the informal group Sigmurethra (Vaught 1989; Bouchet and Rocroi, 2005) in over 100 genera and include the Giant African Snails. Generic placements within the Achatinoidea have varied considerably (Thiele, 1931; Zilch, 1959; Vaught, 1989; Schileyko, 1999, 2001; see also Supplementary Data 1). Taxonomic placements of family level categories have also varied enormously, and there has been little consensus. Solem's (1978) Achatinacea (=Achatinoidea) included the Achatinidae, Ferussaciidae, Megaspiridae, Spiraxidae and Subulinidae. Nordsieck (1986) recognized ten superfamilies within what he classified as the Achatinid Sigmurethra: the Partuloidea, Orthalicodea, Achatinoidea, Aillyoidea, Oleacinoidea, Streptaxoidea, Acavoidea, Rhytidoidea, Plectopylidoidea and the Punctoidea. Within the Achatinoidea, he recognised five families: the Achatinidae, Ferussaciidae, Subulinidae, Coeliaxidae and Thyrophorellidae. Tillier (1989) classified land snails under the superfamily Achatinoidea based on the presence of a closed ureter in the excretory system, symmetrical cerebro-pedal connectives, a short commissure, and contiguous left parietal and visceral ganglia in the nervous system. In addition to the Ferussaciidae, Subulinidae, Achatinidae, and the Streptaxidae, Tillier included the Succineidae within the Achatinoidea. Vaught (1989) followed Nordsieck (1986) in recognising the Ferussaciidae, Subulinidae, Achatinidae, Coeliaxidae and Thyrophorellidae within the Achatinoidea but also provided a full hierarchy of subfamily groups and genera. Schileyko (1999) placed only the Achatinidae in the Achatinoidea. Based on shell characters and, where available, his interpretation of features of the reproductive tract proximal to the genital orifice, Schileyko (1999) introduced substantial changes in his classification of the Subulinidae with nine subfamilies: Subulininae, Petriolinae, Rishetiinae, Rumininae, Opeatinae, Obeliscinae, Perrieriinae, Tristaniinae, and Coeliaxinae (=Coeliaxidae according to Nordsieck, 1986). He also recognised four families within his Subulinoidea: Micractaeonidae, Ferrussaciidae (= Ferussaciidae, see Bouchet and Rocroi, 2005), in which he included the Ferrussaciinae (=Ferussaciinae), and Cryptazecinae.

Molecular phylogenetic studies (Wade et al., 2001, 2006) based on the nuclear ribosomal (r)RNA gene cluster (parts of the 5.8S and LSU genes) revealed a principal division of the Stylommatophora into two major clades. The 'achatinoid' clade comprises the superfamilies Streptaxoidea, represented by the Streptaxidae, and the Achatinoidea, represented by the Achatinidae, Coeliaxidae, Ferussaciidae, Subulinidae and Thyrophorellidae as adopted by Vaught (1989) and followed by Wade et al. (2006). A study by Sutcharit et al. (2010) now also includes the Diapheridae in the Streptaxoidea. All other stylommatophoran families fall within the 'non-achatinoid' clade. The Spiraxidae (represented by *Euglandina*) and Succineidae (represented by *Succinea*) fell within the 'non-achatinoid' clade, thus contradicting Solem (1978) for his inclusion of the Spiraxidae and Tillier (1989) for his inclusion of the Oleacinidae (Spiraxidae and Testacellidae) and the Succineidae. For the purpose of discussion in the current study, we provisionally adopt an Achatinoidea that largely resembles Nordsieck's family level arrangement as adopted by Vaught, comprising the Achatinidae, Coeliaxidae, Ferussaciidae, Subulinidae and Thyrophorellidae (see Supplementary Data 1).

To date, molecular phylogenetic studies of the Achatinoidea have been restricted to the use of a single genetic marker, part of the nuclear rRNA gene cluster (Wade et al., 2001, 2006). To examine relationships among the Achatinoidea, we utilise a four-fold expanded segment of the Wade et al. rRNA gene cluster as well as three other markers, the nuclear actin and histone 3 genes and the mitochondrial CO1 gene. We also expand our taxon coverage of the Achatinoidea to include 24 species from all of Vaught's five families. Specifically, this study aimed to: (1) validate the monophyly of the families within the superfamily Achatinoidea based on available taxa; and (2) to evaluate five subfamilies within the Subulinidae.

2. Materials and methods

Twenty-four taxa from five achatinoid families (Achatinidae, Coeliaxidae, Ferussaciidae, Subulinidae and Thyrophorellidae) and three streptaxid outgroup taxa were used to evaluate the phylogeny of the Achatinoidea (see Supplementary Material Data 1 and 2). Four subulinid subfamilies recognised from Schileyko's (1999) classification (Petriolinae, Rishetiinae, Rumininae and Subulininae) and the Glessulinae recognized from Vaught (1989) were also represented. Thirteen taxa were entirely new to this study while the remaining 14 taxa were used by Wade et al. (2006).

For all new specimens, tissue slices (approximately eight mm^3) from the foot muscle of the snail were obtained and the DNA was extracted using a CTAB DNA extraction method (Goodacre & Wade, 2001; see also Supplementary Material under section 'DNA extraction').

Amplification of fragments of the ribosomal (r) RNA gene cluster, actin, histone 3, and the cytochrome c oxidase subunit I (COI) genes were carried out following the protocol under 'PCR amplification' in the Supplementary Material and using the primers listed in Supplementary Data 3 and 4. For all fragments, both sense and anti-sense strands were sequenced directly using an Applied Biosystems 3730 DNA sequencer and BigDye version 3.1 termination cycle sequencing chemistry.

Processing of sequences, saturation tests, phylogenetic analyses using maximum likelihood (ML), neighbour joining (NJ), maximum parsimony (MP) and Bayesian inference

(BI), and partition homogeneity tests are described in the Supplementary Material under 'Sequence processing and phylogenetic analyses.'

Nucleotide sequences generated in this study are deposited in GenBank under accession numbers MF415320-MF415391 and MF444863-MF444894.

3. Results

Phylogenetic trees of the Achatinoidea were built using a concatenated dataset comprising 5028 unambiguously aligned nucleotide sites from the rRNA (3435 nucleotides), actin (861 nucleotides), histone 3 (328 nucleotides), and 1^{st} and 2^{nd} codon positions of the cytochrome c oxidase subunit I gene (404 nucleotides) as well as individually for each gene. The 3^{rd} codon position of the COI gene was shown to be oversaturated (Iss=0.7387; value significantly higher than Iss.c=0.6761 for a completely symmetrical tree and Iss.c=0.3992 for an extremely asymmetrical tree; Xia et al., 2003) and excluded from analyses. The following taxa had missing DNA sequence data: *Cecilioides gokweanus* (histone 3 and COI), *Paropeas achatinaceum* (histone 3), *Riebeckia* sp. (histone 3), *Subulina vitrea* (histone 3), *Gonaxis quadrilateralis* (histone 3 and COI) and *Gonospira* sp. (histone 3 and COI). These taxa were still included in the combined dataset but with missing data represented as gaps. A partition homogeneity test of the four regions revealed that these could be concatenated into a single dataset at P=0.001(Cuningham, 1997). For the model-based tree-construction methods (ML, BI and NJ), LRT identified the GTR+Γ as the optimal model. For the non-model based MP method, a total of 533 parsimony-informative sites were used.

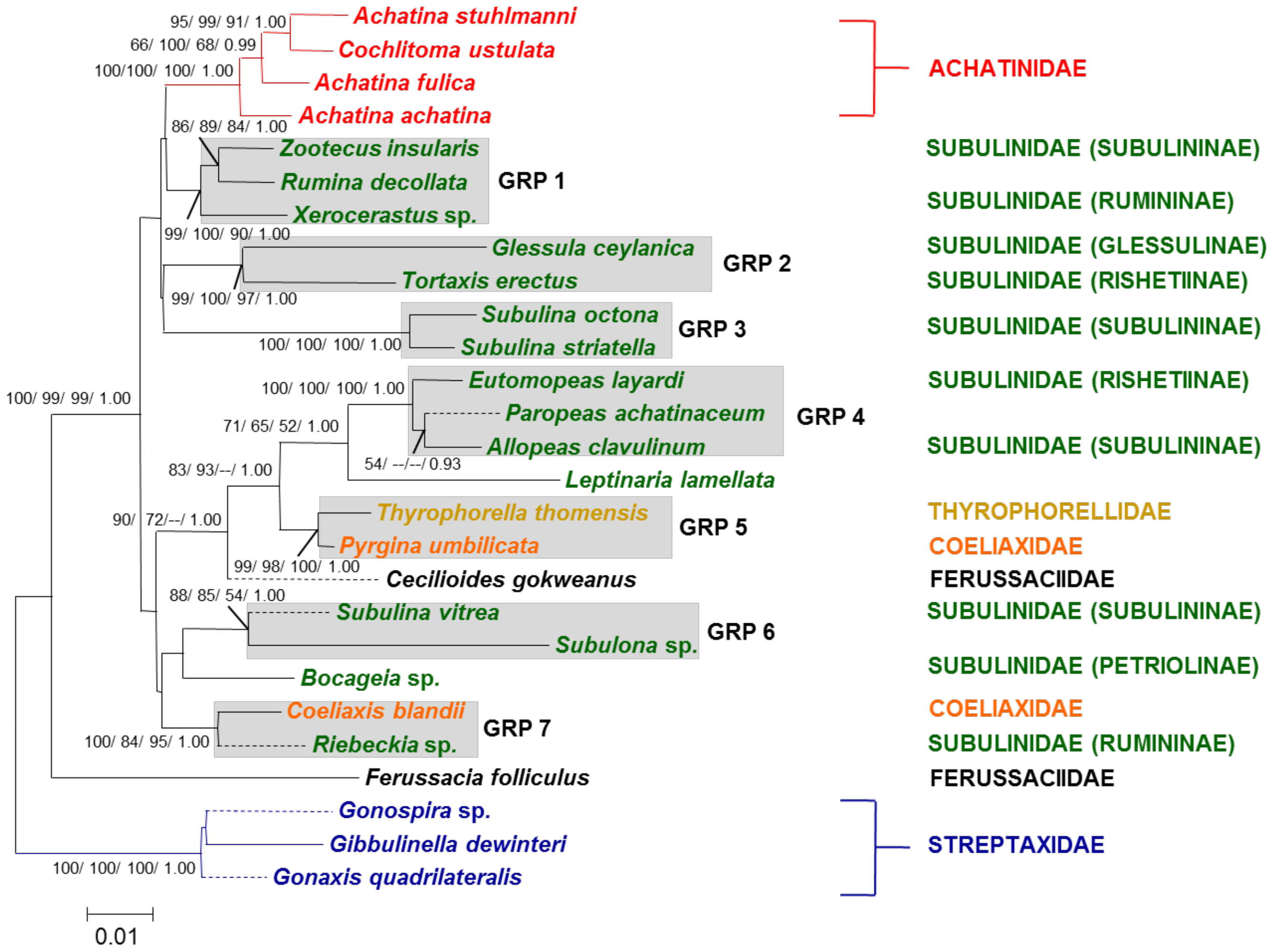

Figure 1. Maximum likelihood phylogenetic tree of the Achatinoidea based on a concatenated sequence of 5028 nucleotides from the combined dataset of the rRNA cluster, actin and H3 genes and the 1st and 2nd codon positions of the COI. For the model-based tree construction methods (ML, NJ and BI), the optimal model used was GTR+Γ. The phylogeny is rooted on the streptaxids *Gibbulinella dewinteri, Gonaxis quadrileteralis* and *Gonospira* sp. Values on the nodes represent bootstrap support (1000 replicates) for ML, NJ and MP, and posterior probabilities (based on the last 1000 trees) for BI, respectively. Bootstrap support less than 50% and posterior probabilities less than 0.7 are not shown. For BI, the optimized number of generations to explore the tree space was 2,000,000 while the optimized heating temperature was 0.125. The scale bar represents 1 substitutional change per 100 nucleotides. Clades in grey (GRPS 1-7) refer to groups discussed in the text. Branches for taxa with missing data are marked with dashes.

The maximum likelihood phylogeny of the Achatinoidea based on the concatenated dataset is shown in Figure 1. Only the Achatinidae was recovered as monophyletic with full support for all four tree methods (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). The other achatinoid families were not recovered in the tree, but seven groupings with strong support were resolved. Group 1 incorporated the subulinids *Xerocerastus* sp., *Rumina decollata* and *Zootecus insularis* (99% ML, 100% NJ, 90% MP bootstraps; PP=1.0 BI). Group 2 contained the subulinid *Tortaxis erectus* and the glessulinid *Glessula ceylanica* (99% ML, 100% NJ, 97% MP bootstraps; PP=1.0 BI). Group 3 consisted of two species of *Subulina*, *S. octona* and *S. striatella* with full support (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). Group 4 included the subulinids *Eutomopeas layardi*, *Paropeas achatinaceum* and *Allopeas clavulinun* with full support (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). Additionally, the subulinid *Leptinaria lamellata* clusters with group 4 with all four tree methods, though support is equivocal (71% ML, 65% NJ, 52% MP bootstraps; PP=1.0 BI). Group 5 clustered the thyrophorellid *Thyrophorella thomensis* and the coeliaxid *Pyrgina umbilicata* (99% ML, 98% NJ, 100% MP bootstraps; PP=1.0 BI). Group 6 incorporated the subulinids *Subulina vitrea* and *Subulona* sp. (88% ML, 85% NJ, 54% MP bootstraps; PP=1.0 BI). Group 7 contained the coeliaxid *Coeliaxis blandii* and the subulinid *Riebeckia* sp. (100% ML, 84% NJ, 95% MP bootstraps; PP=1.0 BI). Finally, the ferussacid *Cecilioides gokweanus* falls as the sister taxon to groups 4 and 5 (90% ML and 72% NJ bootstraps; PP=1.0 BI) and separately to the other ferussacid in the study, *Ferussacia folliculus*, which fell at the base of the Achatinoidea.

A separate maximum likelihood phylogeny of the Achatinoidea but excluding taxa with missing DNA sequence data was constructed. Its topology proved to be very similar to the phylogeny of the Achatinoidea with all taxa included (see Supplementary Data 5). Phylogenies based on each individual gene were also conducted and are shown in Supplementary Data 6.

Seven monophyletic groups proposed in previous classifications (see Supplementary Data 1) that were not recovered in the phylogenetic trees were subjected to hypothesis testing using the Shimodaira-Hasegawa (SH, 1999) test. These groups were the Coeliaxidae, Ferussaciidae, and Subulinidae; within the Subulinidae were the subfamilies Petriolinae, Rishetiinae, Ruminінae and the Subulininae. Except for the Petriolinae, monophyly of all the groups was rejected.

4. Discussion

Wade et al. (2001) provided the first molecular evidence for the monophyly of the Achatinidae based on the partial fragment of the rRNA cluster. Further molecular evidence for achatinid monophyly is given in this study using an expanded rRNA dataset and the inclusion of three other genes for four taxa. The next logical step is to carry out a comprehensive survey of taxa within the Achatinidae to determine the extent of their monophyly and provide a molecular basis of their inter-relationships.

This study included two representatives from the Ferussaciidae, *Ferussacia folliculus* and *Cecilioides gokweanus*. The two taxa did not cluster together as expected, and the early divergence of *F. folliculus* from the rest of the Achatinoidea was strongly supported. Furthermore, the SH test conclusively rejected the monophyly of the Ferussaciidae. *Cecilioides gokweanus* has only been described conchologically; its internal anatomy has not been studied, although that of the type species, *Cecilioides acicula,* is available. *Cecilioides acicula*, like *Ferussacia folliculus*, exhibits characteristics unique to Ferussaciidae such as the short but transversely elongated kidney (Watson, 1928; Tillier, 1989). However, the kidney of *C. acicula* is broader and curves gradually forward as it approaches the rectum whereas that of *F. folliculus* bends abruptly where it meets the rectum. Furthermore, *C. acicula* possesses an indistinct penis papilla (Watson, 1928). There is therefore no compelling evidence from morphological data to show that the Ferussaciidae is monophyletic, and the molecular data from this study support their polyphyly.

The Subulinidae (represented by 15 taxa) are polyphyletic, with members of the Coeliaxidae and Thyrophorellidae falling among the subulinids and with subulinid monophyly being significantly rejected by the SH test. The phylogenies also revealed that the Coeliaxidae, which are regarded as a subfamily, Coeliaxinae, under the Subulinidae by Schileyko (1999), are likewise not monophyletic, as *Coeliaxis blandii* and *Pyrgina umbilicata* did not cluster together. Coeliaxidae monophyly was also significantly rejected by the SH test.

The polyphyly of the subulinids is not surprising considering several taxa have not been described anatomically and are grouped together based only on their shell morphology and geographic distribution (Schileyko, 1999). However, some fascinating groupings have emerged.

For instance, *Xerocerastus* sp., *Rumina decollata* and *Zootecus insularis* formed one group. *Xerocerastus* has a sub-Saharan distribution; *R. decollata* has been widely distributed through human agency from the Americas to Australia but its natural range is circum-Mediterrenean; *Z. insularis* is present from the Cape Verde Islands through North Africa, Arabia, India and Burma (Pilsbry, 1906-1907). This grouping is also in agreement with Zilch's (1959-1960) premise that *Zootecus* should be placed together with the Rumininae. Most descriptions of *Xerocerastus* species are limited to the shell (Van Bruggen, 1970; Schileyko, 1999), but according to Schileyko (1999), both *R. decollata* and *Z. insularis* have a very short to nearly absent oviduct. *Xerocerastus* should be evaluated anatomically to determine if it shares any morphological features with *R. decollata* and *Z. insularis*.

The subulinids *Allopeas clavulinum* (Subulininae), which has a cosmopolitan synanthropic distribution (Schileyko, 1999), and *Paropeas achatinaceum* (Subulininae), which is distributed in the Indo-Pacific region (Naggs, 1994), clustered with another subulinid, *Eutomopeas layardi* (Rishetiinae), which has a restricted distribution, being found in Sri Lanka, Comoros and Lombok (Schileyko, 1999). Both *Allopeas* and *Eutomopeas* share similar shell characteristics, including shape, size and translucence, which they also share with many subulinids, whereas *Paropeas* tends to be more turrited in shape. The reproductive anatomy of *A. clavulinum* and *P. achatinaceum* have been described, so the logical next step is to examine how the internal structures of *E. layardi* compare with the other two and if these structures are also congruent with molecular data. If so, then the shell characteristics used to distinguish the two taxa into separate subfamilies and even genera need to be revisited.

Pyrgina umbilicata (Coeliaxidae) formed a group with *Thyrophorella thomensis* (Thyrophorellidae). The position of Thyrophorellidae has been speculatively placed in a wide range of systematic positions (Thiele, 1931; Zilch, 1959: Solem, 1978; Tillier, 1989; Schileyko, 2001) without consensus. Nordsieck (1986) was the first to suggest provisional placement of the Thyrophorellidae in the Achatinoidea, although he did not provide a justification. In Wade *et al*'s (2006) molecular phylogenetic tree of the Stylommatophora, *Thyrophorella* clustered with *Pyrgina* as achatinoid sister taxa. Here the *Thyrophorella/Pyrgina* group is robustly retained (Figure 1) and forms a sister group relationship with *Eutomopeas, Paropeas, Allopeas* and *Leptinaria.* The sister group relationship between *Thyrophorella* and *Pyrgina* is quite remarkable because of the extreme morphological divergence. While *Pyrgina* possesses a typical subulinid high spired dextral shell, *Thyrophorella* has a dorsally domed, almost discoid sinistral shell, probably unique within the Achatinoidea. Proximal to the aperture, the body whorl of

Thyrophorella forms a plate that flexes forward and establishes a close fitting seal to the aperture, a so-called ‘false operculum’. This is a unique feature within the Gastropoda.

Tortaxis (as represented by the type species *T. erectus*) from Indochina and southern China and *Glessula* (represented by *G. ceylanica*) from South Asia through parts of Myanmar, Thailand, Vietnam, Sumatra, Java and Borneo (Pilsbry, 1906-1907) formed another group. Conchologically, *Tortaxis* differs from *Glessula* in possessing a twisted apertural columella rather than the truncated columella of *Glessula.* There is a need to include a wider range of examples of what are currently understood to be *Glessula* in a molecular phylogeny and to include the likely related genera *Rishetia* and *Bacillum* to establish their status and relationships.

It is clear from this study that while the Achatinoidea sensu Schileyko is a monophyletic group, there is no support for recognising it as a distinct superfamily from the Subulinoidea sensu Schileyko. Schileyko (1999) recognised four families within his Subulinoidea, the Subulinidae, Glessulidae, Microtaenidae and Ferussaciidae. *Micracteon* was not available for this study but from the known anatomy, particularly that of the jaw constructed of discrete plates, its inclusion in the achatinoid clade requires confirmation (Bruggen and de Winter, 1995). It is clear that the Ferussaciidae lies outside of the achatinid/subulinid clade. Glessulidae sensu Schileyko as a monotypic family is not supported. Cryptazecinae Schileyko 1999 is excluded from the Ferussaciidae as its monotypic genus, *Cryptazeca*, has been shown to be a member of the non-achatinoid Cochlicopidae on anatomical criteria (Gomez and Angulo, 1987) and molecular data (Madeira *et al.*, 2010).

Within the Subulinidae, Schileyko (1999) recognised nine subfamilies: the Opeatinae, Obeliscinae, Coeliaxinae, Subulininae, Petriolinae, Rishetinae, Tristaniinae, Rumininae and

Perrieriinae. Within these subfamilies *sensu* Schileyko (1999) and on the basis of examined species, the Ceoliaxinae, Subulininae, Petriolinae, Rishetinae and Ruminnae are not supported as subfamily groups; furthermore, with the exception of the Petriolinae, their monophyly was strongly rejected by the SH Test. On the other hand, Tristaniinae has been shown not to be an achatinoid, *Tristania* having been synonymised with *Balea,* a member of the Clausiliidae in the non-achatinoid clade (Preece and Gittenberger, 2003). There is therefore a need to investigate the morphological features of many anatomically undescribed species to correlate the molecular data with morphological data. Other subfamilies that were not represented in this study (Obeliscinae, Opeatinae, Perrieriinae and Tristaniinae) should likewise be surveyed to determine their validity in light of the rejection of the monophyly of the Rishetiinae, Rumininae and Subulininae

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