

1 **Molecular phylogeny of the Achatinoidea (Mollusca: Gastropoda)**

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13 **Abstract**

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

26

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

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## 29 **1. Introduction**

30           The Achatinoidea are a large group of terrestrial land snails of the informal group  
31 *Sigmurethra* (Vaught 1989; Bouchet and Rocroi, 2005) in over 100 genera and include the Giant  
32 African Snails. Generic placements within the Achatinoidea have varied considerably (Thiele,  
33 1931; Zilch, 1959; Vaught, 1989; Schileyko, 1999, 2001; see also Supplementary Data 1).  
34 Taxonomic placements of family level categories have also varied enormously, and there has  
35 been little consensus. Solem's (1978) Achatinacea (=Achatinoidea) included the Achatinidae,  
36 Ferussaciidae, Megaspiridae, Spiraxidae and Subulinidae. Nordsieck (1986) recognized ten  
37 superfamilies within what he classified as the Achatinid *Sigmurethra*: the Partuloidea,  
38 Orthalicodea, Achatinoidea, Aillyoidea, Oleacinoidea, Streptaxoidea, Acavoidea, Rhytidoidea,  
39 Plectopylidoidea and the Punctoidea. Within the Achatinoidea, he recognised five families: the  
40 Achatinidae, Ferussaciidae, Subulinidae, Coelioxidae and Thyrophorellidae. Tillier (1989)  
41 classified land snails under the superfamily Achatinoidea based on the presence of a closed  
42 ureter in the excretory system, symmetrical cerebro-pedal connectives, a short commissure, and  
43 contiguous left parietal and visceral ganglia in the nervous system. In addition to the  
44 Ferussaciidae, Subulinidae, Achatinidae, and the Streptaxidae, Tillier included the Succineidae  
45 within the Achatinoidea. Vaught (1989) followed Nordsieck (1986) in recognising the  
46 Ferussaciidae, Subulinidae, Achatinidae, Coelioxidae and Thyrophorellidae within the  
47 Achatinoidea but also provided a full hierarchy of subfamily groups and genera. Schileyko  
48 (1999) placed only the Achatinidae in the Achatinoidea. Based on shell characters and, where  
49 available, his interpretation of features of the reproductive tract proximal to the genital orifice,  
50 Schileyko (1999) introduced substantial changes in his classification of the Subulinidae with nine  
51 subfamilies: Subulininae, Petriolinae, Rishetiinae, Rumininae, Opeatinae, Obeliscinae,

52 Perrieriinae, Tristaniinae, and Coeliarinae (=Coeliaridae according to Nordsieck, 1986). He also  
53 recognised four families within his Subulinoidea: Micractaeonidae, Ferrussaciidae (=  
54 Ferrussaciidae, see Bouchet and Rocroi, 2005), in which he included the Ferrussaciinae  
55 (=Ferrussaciinae), and Cryptazecinae.

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

70 To date, molecular phylogenetic studies of the Achatinoidea have been restricted to the  
71 use of a single genetic marker, part of the nuclear rRNA gene cluster (Wade et al., 2001, 2006).  
72 To examine relationships among the Achatinoidea, we utilise a four-fold expanded segment of  
73 the Wade et al. rRNA gene cluster as well as three other markers, the nuclear actin and histone 3  
74 genes and the mitochondrial CO1 gene. We also expand our taxon coverage of the Achatinoidea

75 to include 24 species from all of Vaught's five families. Specifically, this study aimed to: (1)  
76 validate the monophyly of the families within the superfamily Achatinoidea based on available  
77 taxa; and (2) to evaluate five subfamilies within the Subulinidae.

78

## 79 **2. Materials and methods**

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

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

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

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

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

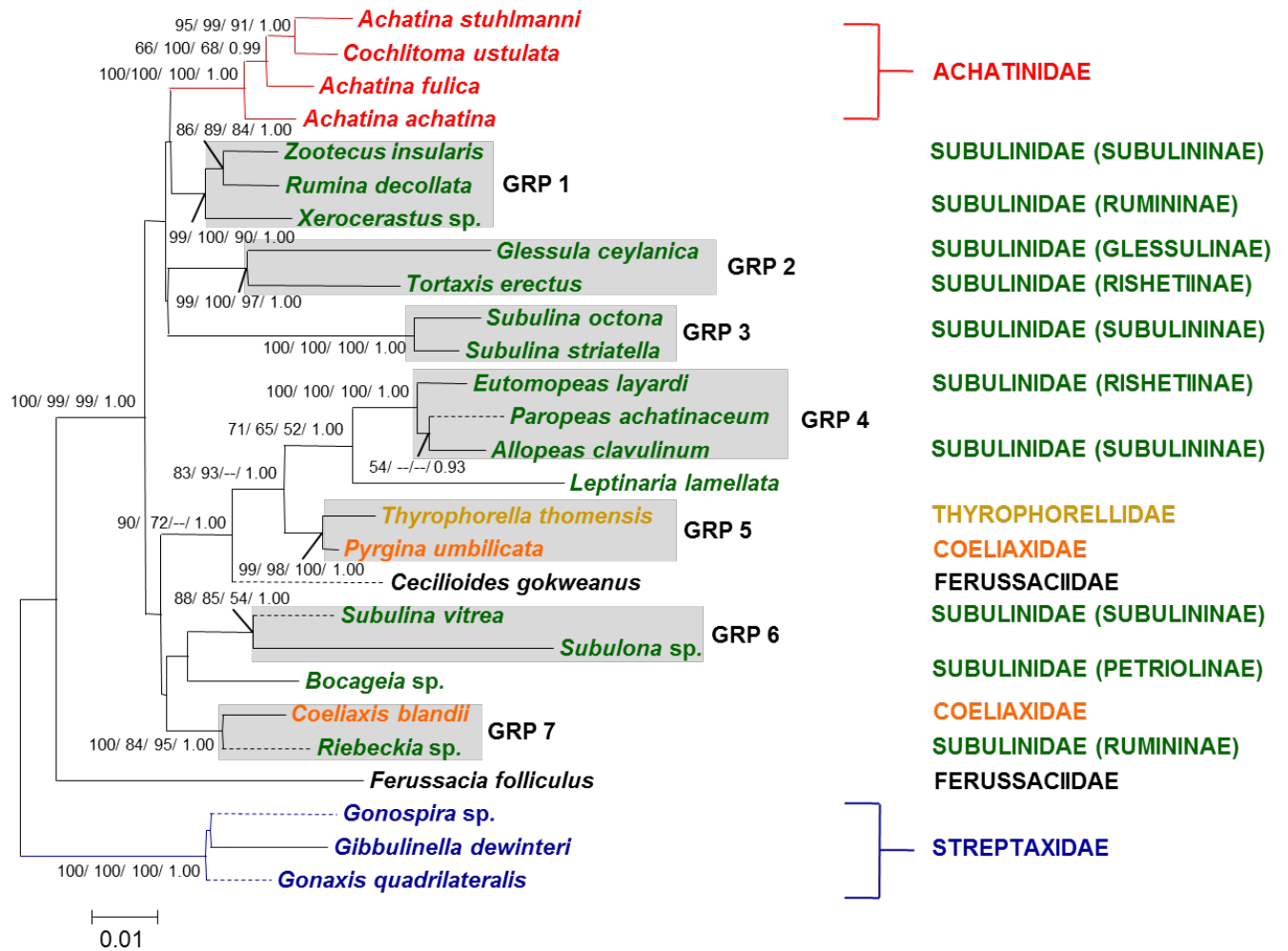
100 Nucleotide sequences generated in this study are deposited in GenBank under accession  
101 numbers MF415320-MF415391 and MF444863-MF444894.

102

### 103 **3. Results**

104 Phylogenetic trees of the Achatinoidea were built using a concatenated dataset  
105 comprising 5028 unambiguously aligned nucleotide sites from the rRNA (3435 nucleotides),  
106 actin (861 nucleotides), histone 3 (328 nucleotides), and 1<sup>st</sup> and 2<sup>nd</sup> codon positions of the  
107 cytochrome c oxidase subunit I gene (404 nucleotides) as well as individually for each gene. The  
108 3<sup>rd</sup> codon position of the COI gene was shown to be oversaturated (Iss=0.7387; value  
109 significantly higher than Iss.c=0.6761 for a completely symmetrical tree and Iss.c=0.3992 for an  
110 extremely asymmetrical tree; Xia et al., 2003) and excluded from analyses. The following taxa  
111 had missing DNA sequence data: *Ceciliooides gokweanus* (histone 3 and COI), *Paropeas*  
112 *achatinaceum* (histone 3), *Riebeckia* sp. (histone 3), *Subulina vitrea* (histone 3), *Gonaxis*  
113 *quadrilateralis* (histone 3 and COI) and *Gonospira* sp. (histone 3 and COI). These taxa were still  
114 included in the combined dataset but with missing data represented as gaps. A partition  
115 homogeneity test of the four regions revealed that these could be concatenated into a single  
116 dataset at  $P=0.001$  (Cunningham, 1997). For the model-based tree-construction methods (ML, BI  
117 and NJ), LRT identified the GTR+ $\Gamma$  as the optimal model. For the non-model based MP method,  
118 a total of 533 parsimony-informative sites were used.

119



**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 1<sup>st</sup> and 2<sup>nd</sup> 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 quadrilateralis* 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

137 support for all four tree methods (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). The  
138 other achatinoid families were not recovered in the tree, but seven groupings with strong support  
139 were resolved. Group 1 incorporated the subulinids *Xerocerastus* sp., *Rumina decollata* and  
140 *Zootecus insularis* (99% ML, 100% NJ, 90% MP bootstraps; PP=1.0 BI). Group 2 contained the  
141 subulinid *Tortaxis erectus* and the glessulinid *Glessula ceylanica* (99% ML, 100% NJ, 97% MP  
142 bootstraps; PP=1.0 BI). Group 3 consisted of two species of *Subulina*, *S. octona* and *S. striatella*  
143 with full support (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). Group 4 included the  
144 subulinids *Eutomopeas layardi*, *Paropeas achatinaceum* and *Allopeas clavulinun* with full  
145 support (100% ML, 100% NJ, 100% MP bootstraps; PP=1.0 BI). Additionally, the subulinid  
146 *Leptinaria lamellata* clusters with group 4 with all four tree methods, though support is  
147 equivocal (71% ML, 65% NJ, 52% MP bootstraps; PP=1.0 BI). Group 5 clustered the  
148 thyrophorellid *Thyrophorella thomensis* and the coelioxid *Pyrgina umbilicata* (99% ML, 98%  
149 NJ, 100% MP bootstraps; PP=1.0 BI). Group 6 incorporated the subulinids *Subulina vitrea* and  
150 *Subulona* sp. (88% ML, 85% NJ, 54% MP bootstraps; PP=1.0 BI). Group 7 contained the  
151 coelioxid *Coeliaxis blandii* and the subulinid *Riebeckia* sp. (100% ML, 84% NJ, 95% MP  
152 bootstraps; PP=1.0 BI). Finally, the ferussacid *Cecilioides gokweanus* falls as the sister taxon to  
153 groups 4 and 5 (90% ML and 72% NJ bootstraps; PP=1.0 BI) and separately to the other  
154 ferussacid in the study, *Ferussacia folliculus*, which fell at the base of the Achatinoidea.

155

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



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

166

#### 167 **4. Discussion**

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

174           This study included two representatives from the Ferussaciidae, *Ferussacia folliculus* and  
175 *Cecilioides gokweanus*. The two taxa did not cluster together as expected, and the early  
176 divergence of *F. folliculus* from the rest of the Achatinoidea was strongly supported.  
177 Furthermore, the SH test conclusively rejected the monophyly of the Ferussaciidae. *Cecilioides*  
178 *gokweanus* has only been described conchologically; its internal anatomy has not been studied,  
179 although that of the type species, *Cecilioides acicula*, is available. *Cecilioides acicula*, like  
180 *Ferussacia folliculus*, exhibits characteristics unique to Ferussaciidae such as the short but  
181 transversely elongated kidney (Watson, 1928; Tillier, 1989). However, the kidney of *C. acicula*  
182 is broader and curves gradually forward as it approaches the rectum whereas that of *F. folliculus*

183 bends abruptly where it meets the rectum. Furthermore, *C. acicula* possesses an indistinct penis  
184 papilla (Watson, 1928). There is therefore no compelling evidence from morphological data to  
185 show that the Ferussaciidae is monophyletic, and the molecular data from this study support their  
186 polyphyly.

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

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

196         For instance, *Xerocerastus* sp., *Rumina decollata* and *Zootecus insularis* formed one  
197 group. *Xerocerastus* has a sub-Saharan distribution; *R. decollata* has been widely distributed  
198 through human agency from the Americas to Australia but its natural range is circum-  
199 Mediterrenean; *Z. insularis* is present from the Cape Verde Islands through North Africa, Arabia,  
200 India and Burma (Pilsbry, 1906-1907). This grouping is also in agreement with Zilch's (1959-  
201 1960) premise that *Zootecus* should be placed together with the Rumininae. Most descriptions of  
202 *Xerocerastus* species are limited to the shell (Van Bruggen, 1970; Schileyko, 1999), but  
203 according to Schileyko (1999), both *R. decollata* and *Z. insularis* have a very short to nearly  
204 absent oviduct. *Xerocerastus* should be evaluated anatomically to determine if it shares any  
205 morphological features with *R. decollata* and *Z. insularis*.

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

217           *Pyrgina umbilicata* (Coeliacidae) formed a group with *Thyrophorella thomensis*  
218 (Thyrophorellidae). The position of Thyrophorellidae has been speculatively placed in a wide  
219 range of systematic positions (Thiele, 1931; Zilch, 1959; Solem, 1978; Tillier, 1989; Schileyko,  
220 2001) without consensus. Nordsieck (1986) was the first to suggest provisional placement of the  
221 Thyrophorellidae in the Achatinoidea, although he did not provide a justification. In Wade *et al*'s  
222 (2006) molecular phylogenetic tree of the Stylommatophora, *Thyrophorella* clustered with  
223 *Pyrgina* as achatinoid sister taxa. Here the *Thyrophorella/Pyrgina* group is robustly retained  
224 (Figure 1) and forms a sister group relationship with *Eutomopeas*, *Paropeas*, *Allopeas* and  
225 *Leptinaria*. The sister group relationship between *Thyrophorella* and *Pyrgina* is quite remarkable  
226 because of the extreme morphological divergence. While *Pyrgina* possesses a typical subulinid  
227 high spired dextral shell, *Thyrophorella* has a dorsally domed, almost discoid sinistral shell,  
228 probably unique within the Achatinoidea. Proximal to the aperture, the body whorl of

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

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

238

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

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

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

262

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267

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