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Introduction

Despite the increasing use of molecular methods to determine evolutionary relationships among taxa, molecular sequence data have never been used to assess the relationships among the polygyrid snails (Gastropoda:Pulmonata:Polygyridae). This is surprising, considering how large, charismatic and common they are. Polygyrids range across North America, going as far north as parts of Canada and south as Mexico and even deeper into Central America (Pilsbry 1940). There is a particular concentration of these snails in the Appalachian Mountains, where they primarily serve as detritivores and prey for various woodland vertebrates in forest habitats. Yet despite the broad geographic distribution and high abundance of polygyrids in many forest habitats, there is still little known about their phylogeny (evolutionary relationships).

Polygyrids are broadly distributed across North America. Mesodontini and Triodopsini are both found in eastern North America (Hubricht 1985). The other tribes are distributed as follows: Ashmunellini in the American Southwest (Pilsbry 1940), Vespericolini along the West Coast, Polygyrini in the Deep South and Central America (Hubricht 1985), Stenotremini in the eastern U.S.(Hubricht 1985), and Allogonini is found in both the eastern and western U.S. (Hubricht 1985) (Pilsbry 1940). The most notable area they seem to be absent from is parts of the western U.S. (specifically Colorado, Wyoming, Nevada and Utah) (Pilsbry 1940) which makes their presence on the West Coast and throughout the eastern U.S. all the more interesting. With the exception of Allogonini, each tribe seems to be centered in one region.

One of the first, significant attempts to classify Polygyridae was in the 1930s and 1940s. Pilsbry classified Polygyridae into ten different genera divided into two subfamilies (Pilsbry 1940). These two subfamilies were Polygyrinae (*Trilobopsis, Giffordius, Praticolella, Polygyra, Stenotrema,* and *Mesodon)* and Triodopsinae (*Ashmunella, Allogona, Vespericola and*

Triodopsis) (Pilsbry 1940). He used a variety of morphological characteristics such as shell shape, aperture and reproductive organ anatomy (Pilsbry 1940). This was the last attempt to organize the taxonomy of Polygyridae until another attempt was made by Kenneth Emberton nearly 40 years later.

The next individual who worked on polygyrid systematics was Kenneth Emberton. Emberton also set out to establish a phylogeny for polygyrid snails. He published a series of papers on polygyrid phylogenetics using morphological (such as shell shape) and behavioral (such as sperm swapping behavior) characteristics (Emberton 1988, 1991, 1994), ultimately resulting in a phylogenetic hypothesis for the entire family (Emberton 1994) (Figure 2.). He determined that there were seven different tribes in Polygyridae (Mesodontini, Polygyrini, Stenotremini, Ashmunellini, Allogonini, Vespericollini and Triodopsini) (Figure 2.). Of the seven tribes, Triodopsini is the sister group to the rest of the group. The next sister group to the rest is Vespericolini. The next sister group is Allogonini. Ashmunellini is the sister group to the last three tribes (Stenotremini, Polygyrini, Mesodontini); relationships among Stenotremini, Polygyrini and Mesodontini are unresolved, and Emberton was not certain that Stenotremini and Polygyrini are monophyletic. Emberton generated an immense data set comprising morphological, anatomical, behavioral and allozymic data, but he did not include DNA sequence data in his analyses. I wanted to test Emberton's phylogenetic hypothesis by comparing his tree to a tree based on mitochondrial DNA sequences.

Methods

Specimen Acquisition and DNA Extraction: Snails were either gathered by myself or were sent by collaborators. DNA was extracted from small pieces of foot tissue using DNAZol (Molecular Research Center). All snails were preserved following standard protocols (Sturm et

al. 2006). Preserved snails were then sent to Dr. Tim Pearce (Carnegie Museum of Natural History) for identification and vouchering.

Polymerase Chain Reactions and Sequencing: After extraction, each DNA sample would be amplified via polymerase chain reactions (PCRs) using HotStar Master Mix (Qiagen; half-reactions) for four different mitochondrial gene primers. Those four genes included two protein-coding gene regions (cytochrome oxidase subunit 1 [COI] (Folmer et al. 1994) and cytochrome b [cytb] (Merrit et al. 1998)) and two ribosomal RNA gene regions (the small subunit [12S] (Simons et al. 1994) and the large subunit [16S] (Gellar et al 1997)). After amplification, PCR products were either purified (using the gel purification method) or diluted 4:1 in water. All purified/diluted PCR products were sequenced using Applied Biosystems BigDye Ready Reaction Mix (~1/8 reactions) and then run out on a 3130XL automated sequencer.

Data Analysis: Sequences were then edited in Sequencher (GeneCodes) and aligned in either RNASalsa (12S and 16S) (Stocsits et al 2009) or Muscle (COI and Cytb) (Edgar 2004). After editing, all sequence data (including sequence data from GenBank for various outgroups and polygyrid sequences generated by collaborators) were comcatenated in Mesquite (Madison and Madison 2010). I used sequences of several helicoid snails—*Cepaea* and *Iberus* (Helicidae), Satsuma (Camaenidae), *Euhadra* and *Bradybaena* (Bradybaenidae)— from GenBank as outgroups to root the polygyrid phylogeny. The data matrix was analyzed in RAxML (Stamatakis 2006). 5000 bootstrap pseudoreplicates were analyzed using the rapid bootstrapping option (RAxML options: -f d –m GTRCAT –s <input file name> -# 5000 –b <bootstrap random number seed> n <output file name>), and the maximum likelihood topology was estimated using 20 search replicates (RAxML options: -f d –m GTRMIX –s <input data file> -# 20 –n <output file name>).

Results

The resulting tree included data from over 80 different snails, each with at least one of the desired mitochondrial genes sequenced. There were various PCRs and sequences which did not work. Contributions from collaborators did not include all four genes (some were only COI sequences, others were represented by COI and 16S sequences). These collaborator contributions included all of the Ashmunellini in the data set, both *Trilobopsis* species, all *Millerelix*, the one *Lobosculum* specimen and two of four *Fumonelix* species in the alignment. All of the sequences obtained by myself and the Anderson lab in this study are preceded by the code ND# in Figure 1. The tree presented here (Figure 1) shows that Triodopsini is not sister to the rest of Polygyridae, but is closely related to Mesodontini. Furthermore, Triodopsini is not monophyletic on this phylogeny since it includes Mesodontini. The Triodopsini-Mesodontini clade had moderate (64%) ML bootstrap support. The other five tribes grouped together into a clade that was sister to the Triodopsini-Mesodontini clade. Throughout the tree, several clades have moderate (>50%) to high (>70%) levels of bootstrap support. Mesodontini and Polygyrini were monophyletic while Stenotremini and Allogonini were paraphyletic. Ashmunellini, while monophyletic, only included one genus (*Ashmunella)* and while Vespericolini came out as monophyletic there were only two samples from the same species.

Discussion

My tree and the proposed tree by Emberton have some very significant differences. The most significant and interesting is the position of Triodopsini and the new relationship it has with Mesodontini. On the Emberton tree (Figure 2.) Mesodontini and Triodopsini are at opposite ends of the polygyrid phylogeny. On my tree, however, they form a clade, with Mesodontini nested within a paraphyletic Triodopsini. One of the traits that Emberton used to compose his

tree was shell morphology (Emberton 1994). Despite the inclusion of shell data, Emberton attributed similarities in shell morphology in genera between Mesodontini and Triodopsini to convergent evolution since, on his phylogeny, these traits arose independently of each other from an ancestral shell shape (Emberton 1994). Even though these groups are so much closer on my tree, my tree also suggests that these similarities evolved independently in Mesodontini and Triodopsini. The *Triodopsis* shell shape is also present in *Inflectarius* and *Fumonelix*. These are both mesodontin genera and the other mesodontin genera do not have this shell shape. The shell forms found in *Neohelix*, *Xolotrema* and *Webbhelix* (the other triodopsin genera) are found in the rest of the Mesodontini genera that do not have the *Triodopsis* shell shape.

Another notable part of my tree is the Allogonini-Stenotremini-Vespericolini clade. Though they are grouping together on my tree in something of a jumble (from an Emberton perspective, which had Allogonini and Stenotremini as monophyletic ,but did not recover these two tribes in a clade with Vespericolini): addition of taxa and sequence data (especially for Vespericolini) would allow a stronger test of these proposed relationships.

More taxa and sequences are always desirable in molecular phylogenetics studies. Of particular interest are the polygyrid genera not represented in my tree (*Giffordius* and *Hochbergallus).* Other goals include collection of additional Vespericolini specimens, more *Fumonelix* sequences (I had particular trouble getting PCRs from these specimens to work), and more members of Polygyrini. Determination of the closest living members of Polygyridae would be helpful for testingthe root position of the polygyrid phylogeny as well. Hopefully, all of these data will give a clearer picture of polygyrid phylogeny.

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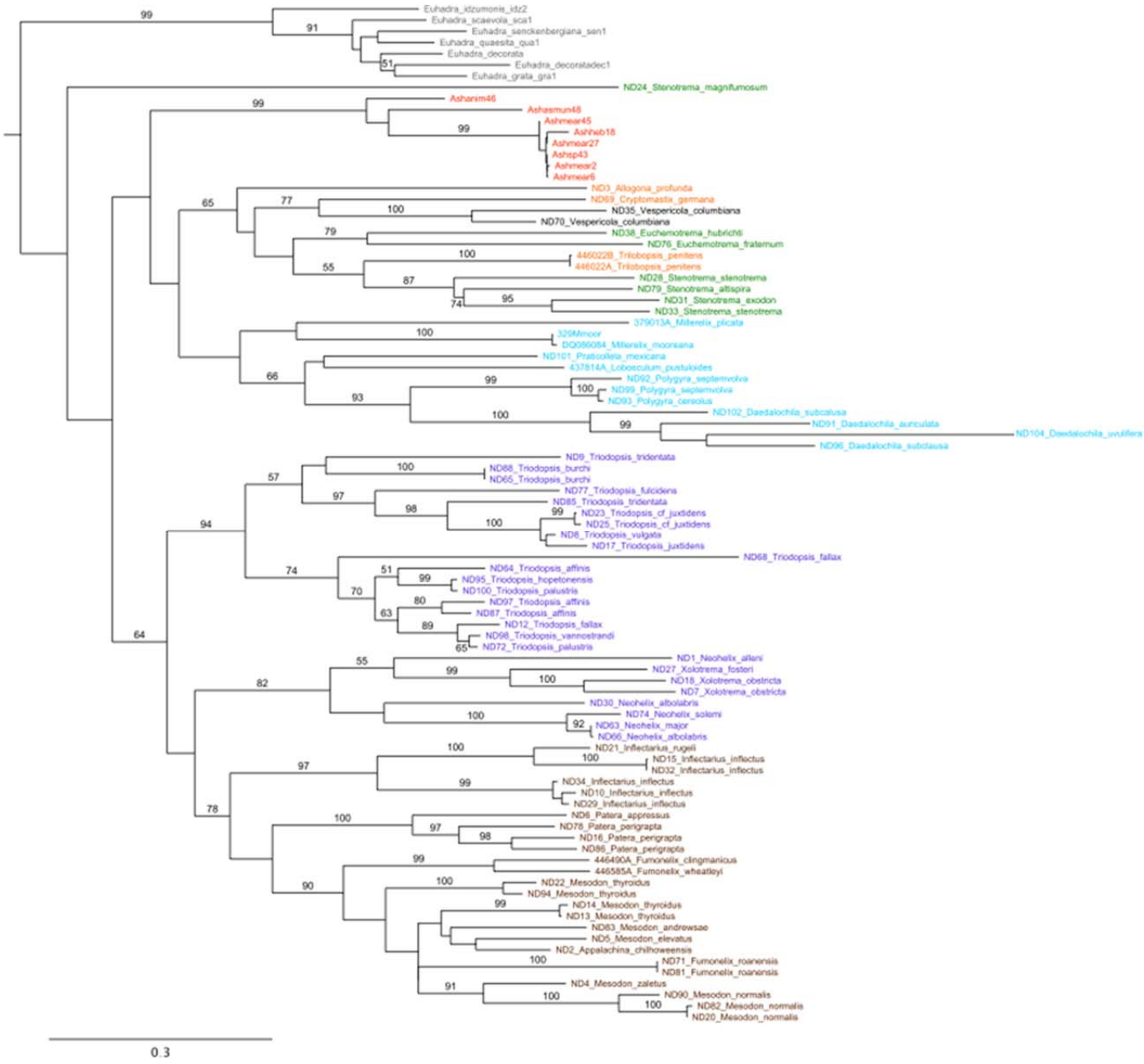

Figure 1. Maximum-likelihood phylogram based on RAxML analysis of the full concatenated data set including four gene regions (COI, cytb, 16S and 12S). Scale bar represents 0.3 estimated substitutions per site. Numbers on the branches represent ML bootstrap support. Taxa denoted "ND" were sequenced in this study; sequences for all other taxa were contributed by collaborators or downloaded from GenBank.

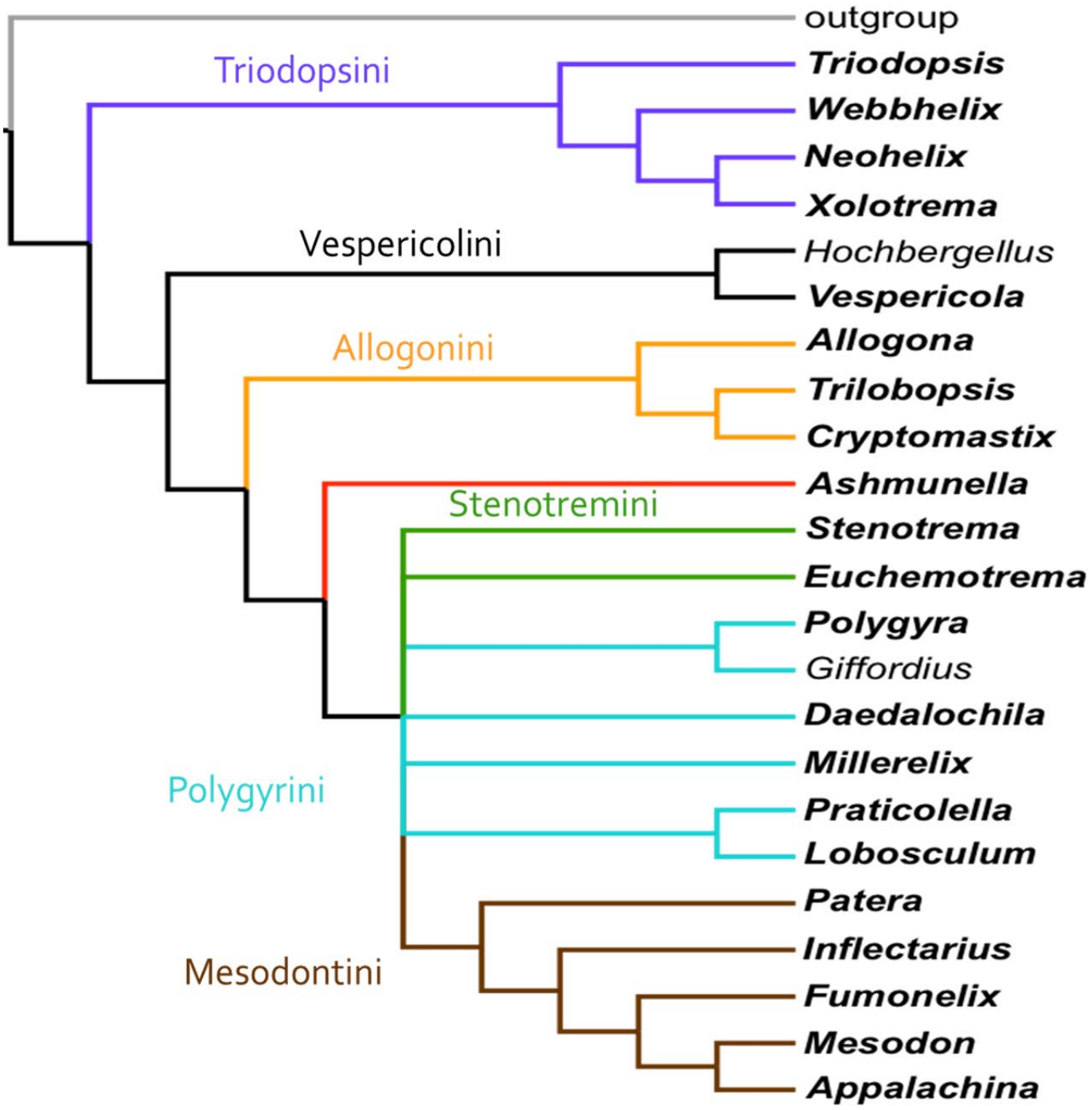

Figure 2. Hypothesis of phylogenetic relationships among the polygyrid genera based on morphological, behavioral and allozyme data (from Emberton, 1994).