

CONSERVATION, LIFE HISTORY AND SYSTEMATICS OF *LEPTOXIS* RAFINESQUE

1819 (GASTROPODA: CERITHIOIDEA: PLEUROCERIDAE)

by

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ABSTRACT

Freshwater gastropods of the family Pleuroceridae are incredibly important to the health of freshwater ecosystems in the southeastern United States. Surprisingly, however, they are one of the most understudied groups of mollusks in North America. Recent data suggest that pleurocerids have an imperilment rate of 79% and many species are under immediate threat of extinction. As such, the time is now to better understand their biology, taxonomy, and phylogeny. Any study that wishes to understand a species group like pleurocerids must start with extensive field work and taxon sampling. As a part of that endeavor I rediscovered *Leptoxis compacta*, a snail that had not been collected live in 76 years. In chapter two, I report on this discovery and I propose a captive propagation plan and potential reintroduction sites for *L. compacta* in an attempt to prevent its extinction. In chapter three, I explore the often reported species level polyphyly on mitochondrial gene trees for gastropods in the family Pleuroceridae and its sister family Semisulcospiridae. Explanations for this paraphyly have ranged from unsatisfying (e.g. that species-level polyphyly is caused by historical introgression) to absurd (e.g. the same species on earth today were here over 65 million years ago). I conclusively demonstrate that the “divergent” haplotypes that have caused this phylogenetic pattern in previous studies are paralogous nuclear copies of mitochondrial genes as that were included in phylogenetic inference as mitochondrial homologs. In chapter four, I present a detailed analysis on egg-laying behaviors in *Leptoxis* species. I also demonstrate that, despite claims to the contrary, shell variation among species is a result of genetic differences, not ecophenotypic plasticity. I also show that three different egg-laying behaviors exist within the genus, single egg-

laying, laying eggs in a line, and clutch formation egg-laying. Finally, I tackle the chaotic *Leptoxis* taxonomy by inferring phylogenetic hypotheses for *Leptoxis* and other pleurocerids outgroups using three genes. *Leptoxis* is resolved as para- and polyphyletic, and I elevate one genus and describe a new genus. This dissertation is an important first step for better understanding pleurocerid biology, and I hope it will stimulate much future study on this fascinating family of freshwater snails.

DEDICATION

This dissertation is dedicated to my Mom, my Dad, and Nick

LIST OF ABBREVIATIONS AND SYMBOLS

AABC	Alabama Aquatic Biodiversity Center
ADCNR	Alabama Department of Conservation and Natural Resources
ADEM	Alabama Department of Environmental Management
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
BCA	Bayesian concordance analysis
BIC	Bayesian information criteria
bp	Base pair
BSA	Bovine serum albumen
COI	Cytochrome <i>c</i> oxidase
<i>df</i>	Degrees of freedom
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
E	East
e.g.	For example
ESA	Endangered Species Act
<i>et. al.</i>	And others
i.e.	That is to say
FLMNH	Florida Museum of Natural History
NMNH	National Museum of Natural History
USNM	National Museum of Natural History
NHM	Natural History Museum of London
PCR	Polymerase chain reaction

MCMC	Markov chain Monte Carlo
PP	Posterior Probability
OTU	Operational taxon unit
<i>s.l.</i>	<i>sensu lato</i>
<i>s.s.</i>	<i>sensu strict</i>
N	North
W	West
°	Degerees
%	Percent
spp.	Species
L	Liter
mm	Millimeter
°C	Degrees Celsius
Fig.	Figure
Figs.	Figures
n	Number
min	Minute
Δ	Difference
µL	Micro liter
s	Seconds
µm	Micro meter
µM	Micro Molar
mM	Mili Molar
µg	Micro gram

MRB	Mobile River Bain
U.S.	United States
~	Approximately
USFWS	United States Fish and Wildlife Service
MCZ	Museum of Comparative Zoology
USA	United States of America
UMMZ	University of Michigan Museum of Zoology
NCMNS	North Carolina Museum of Natural Sciences
KM	Kilometers
GIS	Geographic information system
S	South
SEM	Scanning electron microscope
pos.	Position
pl.	Plate
HKY	Hasegawa, Kishino, and Yano model
+	Plus
Γ	gamma
GTR	General time reversible model
\leq	Less than or equal to
dN/dS	Ratio of non-synonymous to synonymous mutations
rRNA	Ribosomal ribonucleic acid
DNA	Deoxyribonucleic acid

NUMT	Nuclear copy mitochondrial gene transfer
H3	Histone H3 gene
28S	28S rRNA gene
16S	16S rRNA gene
et al.	and others
lnL	log-likelihood
TBA	To be assigned
SYM	Symmetrical model
F81	Felsenstein 81 model
TIM	Transition model
TVM	Transversion model
I	Invariant sites

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CHAPTER 1

INTRODUCTION

The rivers and streams of the southeastern United States are a global hotspot of molluscan diversity (Bogan, Pierson and Hartfield, 1995; Lydeard and Mayden, 1995; Neves, *et al.*, 1997; Williams, Bogan and Garner, 2008). However, anthropogenic pressures such as river modification for hydropower, channelization for navigation, and pollution have caused many species to undergo drastic range declines or extinction (Johnson, *et al.*, 2013; Strayer and Dudgeon, 2010; Williams, *et al.*, 2008). The majority of freshwater fishes and mollusks in the eastern United States suffer from population fragmentation, and some are under immediate and severe risk of extinction (Strayer and Dudgeon, 2010). Freshwater gastropods are of particular extinction risk because of low dispersal abilities, susceptibility to water pollution, and a generally poor understanding of their biology and systematics (Brown, Lang and Perez, 2008; Johnson, *et al.* 2013; Lysne, *et al.*, 2008).

One of the most imperiled and ecologically important families of freshwater gastropods in North America is the Pleuroceridae. Pleurocerids can be found in rivers east of the Rocky Mountains in North America, sometimes at incredible abundance levels (>90% of macroinvertebrate biomass; Newbold, *et al.*, 1983; Richardson, Scheiring and Brown, 1988). Pleuroceridae is the second most diverse family of freshwater gastropods in North American, but they currently suffer a 79% imperilment rate (Johnson, *et al.*, In press). Moreover, at least 33 pleurocerids species are extinct including all species from the genus *Gyrotoma*, which was endemic to the main stem of the Coosa River. Conservation plans for pleurocerids are hindered

by limited available information about their detailed life history and a chaotic taxonomy (Brown, *et al.*, 2008; Perez and Minton, 2008). Despite the ecological importance of pleurocerids (e.g. nutrient cycling, predator-prey dynamics) few studies have explored their life history, and there have been no systematic studies that utilize both multiple loci and adequate taxon sampling. Unfortunately, information as simple as geographic ranges is not well documented for many species.

Perhaps the primary reason that pleurocerids are so understudied is that the taxonomic problems associated with the family make even simple field identifications difficult. Furthermore, the inconsistent use of generic names and the prevalence of junior synonyms in the literature could easily discourage non-experts and interested students from studying one of the most diverse animal groups in North America. For example, Dillon (2000), a prominent book about freshwater gastropod ecology, uses *Goniobasis*—a junior synonym of *Elimia* (Burch, 2001)—and inconsistently applies species names for pleurocerids (Burch, 2001). How such taxonomic problems associated with pleurocerids came to be is obvious when one considers that there are over 600 nominal species (Graf, 2001), but only 162 species are currently considered valid (Johnson, *et al.*, 2013).

Early malacologists in North America such as Issac Lea, John G. Anthony, Timothy A. Conrad, Thomas Say, and Samuel S. Haldeman were instrumental in initially describing pleurocerid diversity in the 19th century, but many species were described multiple times, and generic names were inconsistently used. The first attempt to compile a comprehensive synonymy of pleurocerids was that of Tryon (1873), which served as the basis for much of Goodrich's revisionary work in the first half of the 20th century starting with his monograph on the *Anculosae* (= *Leptoxis*) of the Alabama River basin in 1922. Goodrich based his taxonomic

revisions on gross shell morphology and geographical distribution, and his work still serves as the basis of current pleurocerid taxonomy despite the fact that it reflects outdated perspectives of species diversity. The last comprehensive taxonomic work on pleurocerids was that of Burch and Tottenham (1980) who clarified Goodrich's taxonomy, synonymized some species, and provided a list of species ranges.

There have been a limited number of phylogenetic studies on pleurocerids, but most have suffered from limited taxon sampling or emphasized a single geographic area rather than whole genera and adequate outgroup sampling. For example, Sides (2005) performed a molecular systematic study on *Pleurocera* species of the Mobile River Basin, but ignored the majority of *Pleurocera* diversity, which is located in the Tennessee River basin (Burch and Tottenham, 1980; Johnson, *et al.*, In press). Furthermore, Dillon and Robinson (2009) only collected pleurocerids in streams of the Appalachian Mountains in Virginia, West Virginia, and Tennessee. Studies like these that stress geography rather than an entire genus or family cannot fully test generic boundaries or elucidate species relationships since so many potential ingroup species are not included. Furthermore, these studies almost never sampled from species or genus type localities. Minton and Lydeard (2003) did sample most of the diversity of *Lithasia*, but only one molecular locus was used (cytochrome *c* oxidase) genus level taxonomic revisions were not put forth despite *Lithasia* being resolved as non-monophyletic. The most recent studies exploring pleurocerid evolution and taxonomy are those of Dillon and others (e.g. (Dillon, 2011; Dillon, Jacquemin and Pyron, 2013; Dillon and Robinson, 2011). These studies have all utilized allozymes to justify drastic and overarching taxonomic revisions. However, allozymes are a population genetic tool, and using them above the population level to justify taxonomic changes such as synonymizing the genus *Elimia* into *Pleurocera* is a gross misapplication of an outdated

method. Obviously, tackling the problem of pleurocerid taxonomy is a massive undertaking, but studies with extensive taxon sampling—both at the generic and species level—and nuclear and mitochondrial loci are desperately needed to advance our understanding of pleurocerid diversity.

The genus *Leptoxis* is of special interest because almost half the genus has gone extinct in the last 70 years and four species are listed on the United States Endangered Species Act. Complicating conservation practices is that the taxonomy of *Leptoxis* at the species level is not applied consistently by different state conservation departments (e.g. Virginia listing *L. subglobosa* as a synonym of *L. praerosa* but Tennessee listing *L. umbilicata* as a subspecies of *L. subglobosa*). Furthermore, *Leptoxis* is putatively non-monophyletic (Holznagel and Lydeard, 2000; Minton and Lydeard, 2003). *Leptoxis* is also known to display a variety of egg laying strategies (Dazo, 1965; Winsor, 1933), but the overall life history of many species has never been analyzed. To date, there has been no study whose focus is the systematics or life history of *Leptoxis*. Clarifying *Leptoxis* systematics and understanding *Leptoxis* life history is essential for current management policies to effectively conserve imperiled *Leptoxis* in a cost efficient manner.

In this dissertation I analyze the life history and systematics of *Leptoxis* with the explicit goal of ensuring that future *Leptoxis* conservation is pursued with the best science available. The first step of this project was analyzing museum records of *Leptoxis* and other pleurocerids to ensure the full range of *Leptoxis* is sampled. Second, I performed thorough field work to collect all extant *Leptoxis* species and document the range of morphologically ambiguous species—particularly from the Tennessee and Cumberland River basins. The majority of this project then included captive observations of life history behaviors, anatomical documentation, and molecular

lab work. Finally, I employed cutting edge analytical tools to infer the phylogenetic history of *Leptoxis*.

Extensive field work that includes sampling and observation in nature is integral for understanding the organisms one wishes to study. I follow this paradigm throughout my dissertation and it resulted in the rediscovery of *L. compacta*—the Oblong Rock snail. This species had not been seen since the 1930s and its rediscovery is the focus of the second chapter. *Leptoxis compacta* was positively identified with comparisons to the holotype and radulae of individuals collected in the late 1800s when the species was much more abundant. In this chapter I also explore potential management plans for *L. compacta* that are currently in the process of being implemented by the Alabama Department of Conservation and Natural Resources (P. Johnson *pers. comm.*).

The third chapter of this dissertation explores the prevalence and potential causes of individuals of the same putative species with highly variable mitochondrial genes. The presence of individuals of the same pleurocerid species that have mitochondrial genes that can differ by up to 20% uncorrected *p*-distance was first documented by Lee et al. (2007) in Semisulcospiridae—the sister family to Pleroceridae—and then in pleurocerids by Dillon and Robinson (2009). It has been hypothesized that these divergent haplotypes are either retained ancestral polymorphisms (Dillon and Robinson, 2009), the result of historical hybridization (Miura, *et al.*, 2013), or cryptic species. To explore the hypothesis that different haplotypes are cryptic species I sample four pleurocerid species (*L. ampla*, *L. praerosa*, *P. prasinata*, and *P. pyrenella*) and document the internal anatomy of individuals from each species with highly different haplotypes. The findings of this chapter advance our understanding of a unique genetic pattern that is not found in other animal groups.

As noted above, little is known about the life history of *Leptoxis*. For example, egg laying behaviors of most species are not known, and it is unclear if the size of egg clutches varies among species or populations of the same species. Data on the life history of *Leptoxis* can be used to improve conservation practices and may be useful as synapomorphic and autapomorphic characters. In chapter four, I document for the first time the egg laying behaviors of all *Leptoxis* species and explore how clutch size differs among species, populations, and age classes.

Finally, in chapter five, I perform a molecular phylogenetic analysis of *Leptoxis*. For this, I utilize four loci (two nuclear genes and two mitochondrial genes) and comprehensive sampling of all extant *Leptoxis* and representative taxa from the other four extant pleurocerid genera. Gene and species tree inference methods are used to infer the phylogeny of *Leptoxis* and test its putative non-monophyly. This is the first phylogenetic study of any pleurocerid genus that includes multiple genetic loci and complete in group taxon sampling. The results are used to make needed taxonomic changes that are indicative of phylogeny and better reflect the true extent of pleurocerid biodiversity. Aside from answering long standing evolutionary questions (e.g. what is the most basal pleurocerid lineage?, is *Leptoxis* monophyletic?), the results of this chapter are expected to better facilitate communication between conservation professionals, scientists, and the public.

To fully understand pleurocerid biology and evolution more studies will be necessary, and this study will not fully clarify the taxonomic problems of pleurocerids that have persisted for over a century. However, this dissertation serves as a cornerstone for understanding the life history and phylogeny of a gastropod family that is essential to the health of freshwater resources throughout the eastern United States. The foundation laid here will hopefully jump start

additional research on pleurocerids and create a new found interest in the study and conservation of *Leptoxis* and the family Pleuroceridae.

CHAPTER 2

REDISCOVERY OF *LEPTOXIS COMPACTA* (ANTHONY, 1854) (GASTROPODA: CERITHIOIDEA: PLEUROCERIDAE)

Abstract

The Mobile River Basin is a hotspot of molluscan endemism, but anthropogenic activities have caused at least 47 molluscan extinctions, 37 of which were gastropods, in the last century. Nine of these suspected extinctions were in the freshwater gastropod genus *Leptoxis* (Cerithioidea: Pleuroceridae). *Leptoxis compacta*, a Cahaba River endemic, has not been collected for >70 years and was formally declared extinct in 2000. Such gastropod extinctions underscore the imperilment of freshwater resources and the current biodiversity crisis in the Mobile River Basin. During a May 2011 gastropod survey of the Cahaba River in central Alabama, USA, *L. compacta* was rediscovered. The identification of snails collected was confirmed through conchological comparisons to the *L. compacta* lectotype, museum records, and radulae morphology of historically collected *L. compacta*. Through observations of *L. compacta* in captivity, we document for the first time that the species lays eggs in short, single lines. *Leptoxis compacta* is restricted to a single location in the Cahaba River, and is highly susceptible to a single catastrophic extinction event. As such, the species deserves immediate conservation attention. Artificial propagation and reintroduction of *L. compacta* into its native range may be a viable recovery strategy to prevent extinction from a single perturbation event.

Introduction

The Mobile River Basin (MRB) in Alabama and Georgia contains the highest levels of freshwater molluscan biodiversity in North America (Johnson, *et al.*, 2013; Lydeard and Mayden, 1995; Neves, *et al.*, 1997; Williams, Bogan and Garner, 2008). Anthropogenic activities, however, have caused massive declines in gastropod biodiversity throughout the basin. At least 47 molluscan extinctions (37 gastropods) and many other local extirpations were the immediate result of inundation for hydropower, channelization for navigation and pollution from mine and urban centers throughout the mid 20th century (Johnson, *et al.*, 2013; Neves, *et al.*, 1997; Ó Foighil, *et al.*, 2011). These extinctions comprise a third of known molluscan extinctions globally (Régnier, Fontaine and Bouchet, 2009), making the MRB a major component of the global biodiversity crisis.

Freshwater gastropods in the family Pleuroceridae (Gastropoda: Cerithioidea), suffered the largest number of the aforementioned MRB extinctions (~29)(Johnson, *et al.*, 2013). Of the 14 native MRB *Leptoxis* species, nine are considered extinct, including *L. compacta* (Turgeon, *et al.*, 1998). Four of the remaining five *Leptoxis* are classified under the U.S. Endangered Species Act as either threatened or endangered (USFWS, 1998; USFWS, 2010). Remaining *Leptoxis* species in the MRB are of high conservation concern, and they are the focus of active propagation and reintroduction efforts (Johnson, 2010; Johnson, 2010; Johnson and Evans, 2001).

Leptoxis compacta was formally declared extinct in 2000 (Bogan, 2000), and was not collected in a 1992 survey for the US Fish and Wildlife Service (USFWS) (Bogan and Pierson, 1993), a 2005 ADCNR survey of the Cahaba River (Johnson, *et al.*, 2006)or in a more recent survey by Tolley-Jordan (2008). It is the only pleurocerid endemic to the Cahaba River

considered extinct (Bogan, 2000; Turgeon, *et al.*, 1998), and has not been collected in at least 70 years. Historically, *L. compacta* was most abundant in the central section of the Cahaba River at Lily Shoals in Bibb County, Alabama (Goodrich, 1922; Goodrich, 1941). Exact causes of *L. compacta*'s decline are unknown, but the species was declining in abundance and range by 1935 (Goodrich, 1941). The snail's decline was likely a result of its naturally small range, pollution from mines in the area, and effluent from the Birmingham, Alabama metropolitan area (Shepard, *et al.*, 1994).

In this study, we describe the results of targeted surveys for *L. compacta* in the middle portion of the Cahaba River in Bibb and Shelby Counties, Alabama. Whenever an “extinct” species is putatively re-discovered, special care must be taken to confirm the identity of the species (Roberts, Elphick and Reed, 2009). As such, the conchological and radular morphology of *L. compacta* individuals collected in this study are compared to historically collected material along with other sympatric pleurocerids to confirm the identity and distinctness of *L. compacta*. We also document for the first time, the egg-laying strategy, juvenile morphology, and soft tissue pigmentation of *L. compacta*. Potential threats to the long-term survival of *L. compacta* are also discussed.

Results

Survey

Museum lots of *L. compacta* reviewed are listed in Table 2.1 and historical localities are labeled on Figure 2.1. The historical range of *L. compacta* extended from Centerville, Bibb County, Alabama, USA upriver and into lower Buck Creek in the Valley and Ridge physiogeographic province of the southern Appalachian Mountains. The most recent lots we

analyzed were from 1933 (UMMZ 57871, MCZ 98217), and as far as we are aware this was the last time the species was collected.

Leptoxis compacta was found during the May 2011 survey on an unnamed shoal upstream of the Cahaba River and Shades Creek confluence in Shelby County Alabama (Fig. 2.1; 33.1786°N, 87.0174°W). At this site, we found every pleurocerid species known from the middle Cahaba River including the federally threatened Round Rocksnail, *L. ampla* (Anthony 1855) (Table 2.2) (Burch and Tottenham, 1980; Goodrich, 1941). We also found the endemic limpet *Rhodacmea cahawbensis* Walker, 1917 (Planorbidae) and *Lepyrium showalteri* (Lea, 1861) (Lithoglyphidae), which is federally endangered.

During the first survey we failed to locate *L. compacta* below the Shades Creek confluence. Furthermore, the second survey for *L. compacta* (Fig. 2.1) also failed to locate the species from other historical sites. At every site *L. compacta* was historically found that we sampled, other pleurocerid species were present.

Life history and morphology.

Snails we collected and putatively identified as *L. compacta* possess shells nearly identical to the lectotype (Fig. 2.2) and the original species description (Anthony, 1854). *Leptoxis compacta* shells do not closely resemble those of other sympatric species (Figs. 2.2, 2.3). Juvenile *L. compacta* shells possess one distinct carina on the main body whorl, which is eventually lost as adults (Fig. 2.2). Individuals with shell pigmentation lines are always present in three wide bands. Most wild-caught individuals had purple pigmentation on the columella indentation, but this trait was not observed in juveniles propagated in captivity. The external tissue pigmentation of *L. compacta* is yellow, mottled with black and includes prominent black bands in the middle of the proboscis and on both eyes (Fig. 2.4). This pigmentation banding

pattern is identical to sympatric *L. ampla* (not figured). Pigmentation patterns and the presence of an ocular peduncle are features that distinguish *L. compacta* from sympatric *Elimia* spp. including *E. clara* (Anthony, 1854) (Fig. 2.5), which is conchologically most similar to *L. compacta* (Figs. 2.2, 2.3).

The radular structure of *L. compacta* specimens collected in May 2011 is identical to that of individuals collected in 1881 (Fig. 2.6). The basal margin of the rachidian tooth is widely convex. The central cusp is blunt and flanked by 4-5 denticles, with the outermost being weakly developed in most cases (Fig. 2.6 A, B). The lateral tooth contains one larger rectangular central cusp that is flanked by 4-5 outer denticles and 3-4 inner denticles (Fig. 2.6 B, C). The inner marginal teeth contain 10-12 denticles (Fig. 2.6 D, E). The number of denticles on the outer marginal teeth varies from 12-16, within and among individuals, and the outer denticles are often weakly developed (Fig. 2.6 D, E).

Eggs were laid by female *L. compacta* within three days of being transferred into captive culture. This suggests female snails were laying eggs in the wild when collected in May 2011. Oviposition ceased when the daily maximum water temperature reached 29°C. Eggs were laid either singly or in a linear sequence (Fig. 2.7). Each egg was approximately 0.3 mm in diameter. Average length of the line of eggs was 1.57 eggs (n=51 egg lines) with a maximum observed length of 3 eggs.

Discussion

Only two types of morphological data are available to confirm the putative re-discovery of *L. compacta*: shell and radular. The primary type, shell descriptions in taxonomic works (Anthony, 1854; Goodrich, 1922) and museum records match the *L. compacta* we collected. Furthermore, radulae we extracted from *L. compacta* collected in 1881 are identical to those of

live *L. compacta* collected in this study (Fig. 2.6). Compared to sympatric pleurocerids, *L. compacta* shells most resemble those of Cahaba River *Elimia* spp. (Figs. 2.2, 2.3). Furthermore, the radular morphology is more similar to those of sympatric *Elimia* spp. than *L. ampla* (Minton, 2002; Minton, Garner and Lydeard, 2003). However, body pigmentation of *L. compacta* is most similar to that of *L. ampla* rather than sympatric *Elimia* (Figs. 2.4, 2.5). The aforementioned features of *L. compacta* are similar in some regards to both sympatric *Elimia* spp. and *L. ampla*, but taken in total distinguish *L. compacta* as unique. Molecular systematic analyses are underway to clarify the genetic position of *L. compacta*.

The reduced range of *L. compacta* qualifies the species as critically endangered under International Union for the Conservation of Nature criteria (2011, 2011). It is unclear why *L. compacta* suffered such a drastic range reduction while other sympatric pleurocerid species did not (Goodrich, 1941; Johnson, *et al.*, 2006). However, point-source pollution and urban runoff from the Birmingham, Alabama metropolitan area threaten the long-term survival of *L. compacta*. Furthermore, the lone population of *L. compacta* is found adjacent to a large youth camp currently under construction. The U.S. Fish and Wildlife Service should consider *L. compacta* for protection under the U.S. Endangered Species Act because of its highly restricted range and susceptibility to a single pollution or siltation event. The ADCNR has already undertaken culturing projects for federally endangered *L. plicata* and *L. foremani* to expand their current range (Johnson, 2010; Johnson, 2010; Johnson and Evans, 2001), and we argue that similar efforts should be pursued for *L. compacta*.

There is little literature on the reintroduction of freshwater gastropods (Ahlstedt, 1991), but general conservation rules and guidelines for reintroduction of fish are applicable for freshwater snails (Committee, 2010; George, *et al.*, 2009; Lysne, *et al.*, 2008). There are two

sites within the historical range of *L. compacta* that we consider potentially viable for the reestablishment of a second population. Lily Shoals, which is isolated from development or bridge crossings, is 5.8KM downstream of the remaining *L. compacta* population and supports at least five other species of pleurocerids (Goodrich, 1941). The second potential site for reintroduction is 25.8KM downstream of the site of rediscovery at Pratt's Ferry. Downstream sites are ideal for establishing a second *L. compacta* population because pleurocerid snails have a net upstream movement, thus have potential to naturally colonize upstream localities (Huryn and Denny, 1997; Kappes and Haase, 2012). These localities are also better suited than upstream sites because primary production is generally higher downstream (Vannote, *et al.*, 1980). Furthermore, the lone tributary that historically harbored *L. compacta*, Buck Creek, has three wastewater discharge points and suboptimal habitat (ADEM, 2009; Howard, *et al.*, 2002). Extensive assessments should be performed to identify additional sites for reintroduction that will enhance the survival prospects of propagated *L. compacta* (Johnson, 2010; Seddon, Armstrong and Maloney, 2007).

Through comparisons to the *L. compacta* lectotype, and radulae from fresh and historic collections we present compelling evidence that *L. compacta* has been “re-discovered” in the Cahaba River in Shelby County, Alabama. Why three previous surveys of the Cahaba River—including the site of re-discovery—failed to locate *L. compacta* is unknown (Bogan and Pierson, 1993; Johnson, *et al.*, 2006; Tolley-Jordan, 2008). Because of its restricted range, *L. compacta* should be the focus of immediate conservation attention. Nevertheless, the rediscovery of *L. compacta* is an encouraging moment in the recent history of conservation and biodiversity studies of freshwater mollusks in the MRB.

Materials and Methods

Survey

Alabama Department of Conservation and Natural Resources scientific collecting permits and U.S. Fish and Wildlife Service permits for threatened species were obtained prior to sampling. Since *L. compacta* does not have a formal status under the U.S. Endangered Species Act, federal permit authorization does not apply.

To document the historical range of *L. compacta* and the approximate last collection of the species, museum specimens were analyzed at the National Museum of Natural History (NMNH), North Carolina Museum of Natural Sciences (NCMNS), and the Florida Museum of Natural History (FLMNH). Photographs of *L. compacta* lots housed at the Museum of Comparative Zoology at Harvard University (MCZ) and the University of Michigan Museum of Zoology (UMMZ) were also examined (Table 2.1). Spurious localities represented by only one lot and outside the otherwise documented range of *L. compacta* (e.g. “Alabama River at Selma,” USNM 178542) were excluded from consideration.

In May 2011 gastropods were qualitatively surveyed from a kayak in the Cahaba River (Fig. 2.1) from sites upstream of Shades Creek and Cahaba River confluence to below Piper Bridge. All other sites where *L. compacta* (Fig. 2.1) was historically found were surveyed in August 2011 to confirm range contraction of *L. compacta*. Live snails collected in May 2011 were transported to the Alabama Aquatic Biodiversity Center in Marion, Alabama for species identifications and preservation. Endangered species that we encountered were not collected. Identification of each species was based on comparison with primary types (Fig. 2.2), museum records, and descriptive literature (Anthony, 1854; Burch and Tottenham, 1980; Goodrich, 1922; Graf, 2001). Snails collected in these surveys were preserved following Fukuda et al. (2008) and deposited at NMNH and FLMNH (Table 2.2).

Morphological and life history analyses

A size range of *L. compacta* individuals was collected from the site of re-discovery, and live *L. compacta* were photographed in an aquarium and compared to other sympatric pleurocerids. We extracted radulae from two *L. compacta* specimens collected in the original May 2011 survey and from two samples of dried tissue left in shells from individuals collected in 1881 (USNM 509539). Radulae were extracted following the procedure of Holznagel (1997). *Leptoxis compacta* radulae were visualized on a Hitachi S-2599 Scanning Electron Microscope at the University of Alabama Optical Analysis Facility.

Approximately thirty *L. compacta* individuals were placed in captivity at the Alabama Aquatic Biodiversity Center to observe egg-laying behavior. Because of the difficulties of recording egg-laying strategies in the wild, a culturing environment is ideal for these observations (Whelan, Johnson and Harris, 2012). Snails were kept in a 20L acrylic tank with a 0.83 cm bulkhead fitting that allowed a constant exchange of thermally ambient well water. A 15 L/min powerhead was attached to the lid of the tank to create a constant flow regime. An airstone was placed in the tank to saturate the water with dissolved oxygen. Water temperature was measured hourly with a Hobo Temp Logger (Onset Computer Corporation).

At least every three days, tanks were checked for eggs and the eggs were counted and the adjacent area of the tank marked with a permanent marker to insure individual eggs were not counted twice. Juveniles were allowed to grow in the culturing environment for 5.5 months. Growth series of wild caught and cultured snails were compared to demonstrate conchological changes during ontogeny.

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Table 2.1: Locality and museum catalogue numbers for *L. compacta* museum lots analyzed in this study. *Lot from which radulae were taken.

Locality	Catalogue #
Alabama (river unspecified), Lectotype	MCZ 072063
Buck Creek	FLMNH 81147; USNM 158595
Cahaba River (location unspecified)	USNM 15874, 218694, 158741, 509539*, 158743, 407631; UMMZ 55741
Cahaba River at Abita	USNM 321957
Cahaba River at Centerville	UMMZ 57871
Cahaba River at Lily Shoals	FLMNH 81172, 81188, 81144, 7136; USNM 590380
Cahaba River near Piper	FLMNH 81137, 81178
Cahaba River at Pratt's Ferry	FLMNH 81165, MCZ 98217

Table 2.2: Species collected in the May 2011 survey at the site of *L. compacta* re-discovery (33.178601°N, 87.017481°W). *Lepyrium showalteri* was found, but not collected due its endangered status under the U.S. Endangered Species Act.

Species	Catalogue #
<i>Elimia annettae</i>	USNM 1186562
<i>Elimia ampla</i>	USNM 1186561
<i>Elimia cahawbensis</i>	USNM 1186563
<i>Elimia clara</i>	USNM 1186564
<i>Elimia showalteri</i>	USNM 1186566
<i>Elimia variata</i>	USNM 1186567
<i>Leptoxis ampla</i>	USNM 1186568
<i>Leptoxis compacta</i>	USNM 1186565 FLMNH 449320
<i>Pleurocera prasinatum</i>	USNM 1186569
<i>Rhodacmea cahabensis</i>	USNM 1186570

Figure 2.1: Map of the Cahaba River and select tributaries. Historical collections sites were sampled in August 2011, but *L. compacta* was found only at the site of rediscovery.

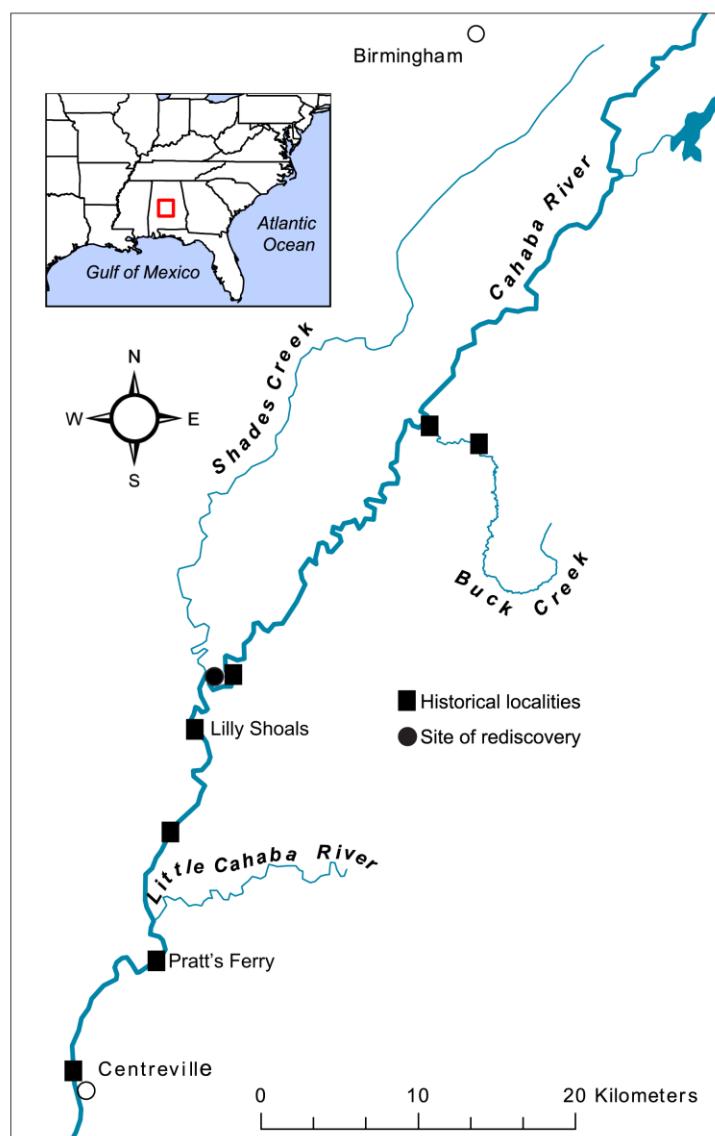


Figure 2.2: Growth series of *L. compacta*. A) *L. compacta* lectotype (MCZ 072063). B-E) Wild caught individuals. F) Juvenile grown in culture, approximately 4 months old. Scale bar=5mm.

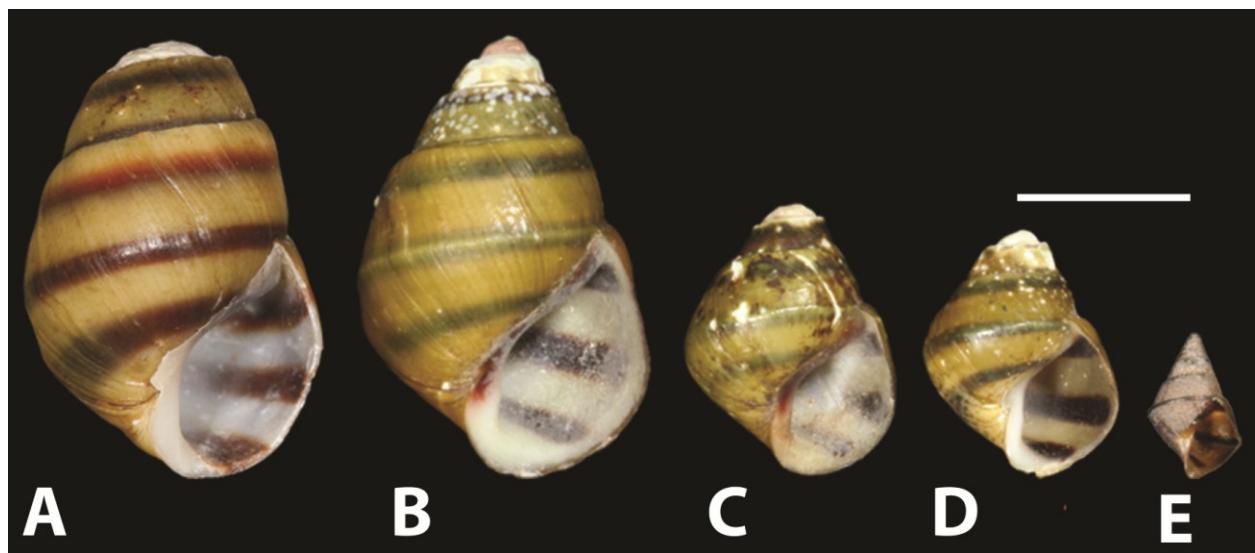


Figure 2.3: Lectotypes of pleurocerids sympatric with *L. compacta*. A) *L. ampla* (MCZ 161803). B) *E. ampla* (MCZ 161735). C) *E. annetae* (UMMZ 128908). D) *E. cahawbensis* (USNM 119055). E) *E. clara* (MCZ 072329) F) *E. showalteri* (ANSP 26881) G) *E. variata* (USNM 118756). F) *P. prasinatum* (USNM 122206).

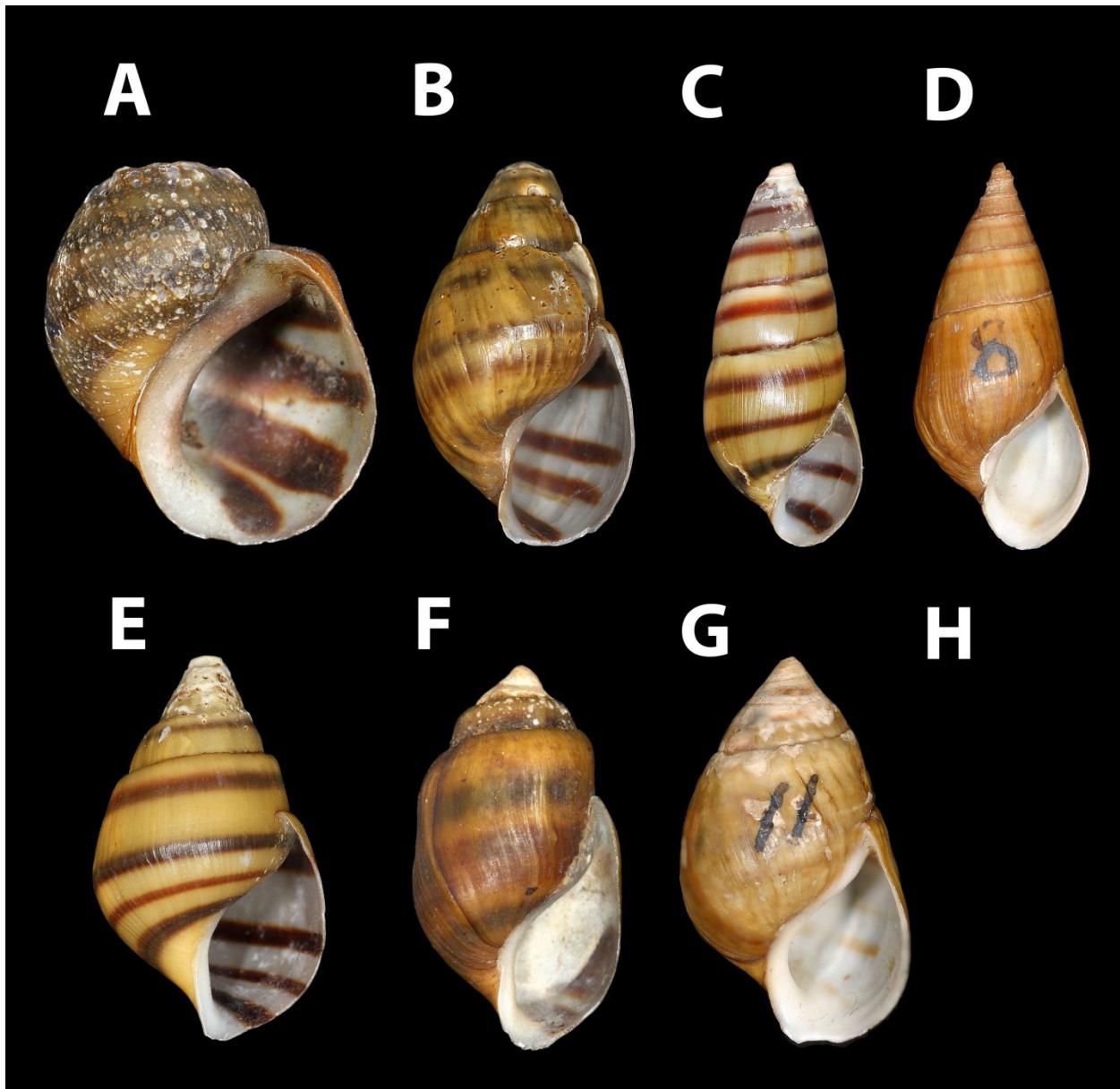


Figure 2.4: Photograph of live *L. compacta* from the Cahaba River, Shelby County, Alabama.



Figure 2.5: Photograph of live *E. clara* from the Cahaba River, Shelby County, Alabama.



Figure 2.6: Scanning electron micrographs of *L. compacta* radulae collected in May 2011 (A-E) and the radula of historically collected individual (F). A) Section of anterior radular ribbon. B) Detailed view of rachidian and lateral teeth. C) View of lateral teeth at 45 degree angle, and slightly rotated counter-clockwise. D-E) Views of marginal teeth showing variation between individuals. F) Rachidian and lateral teeth removed from individual collected in 1881 (USNM 509539).

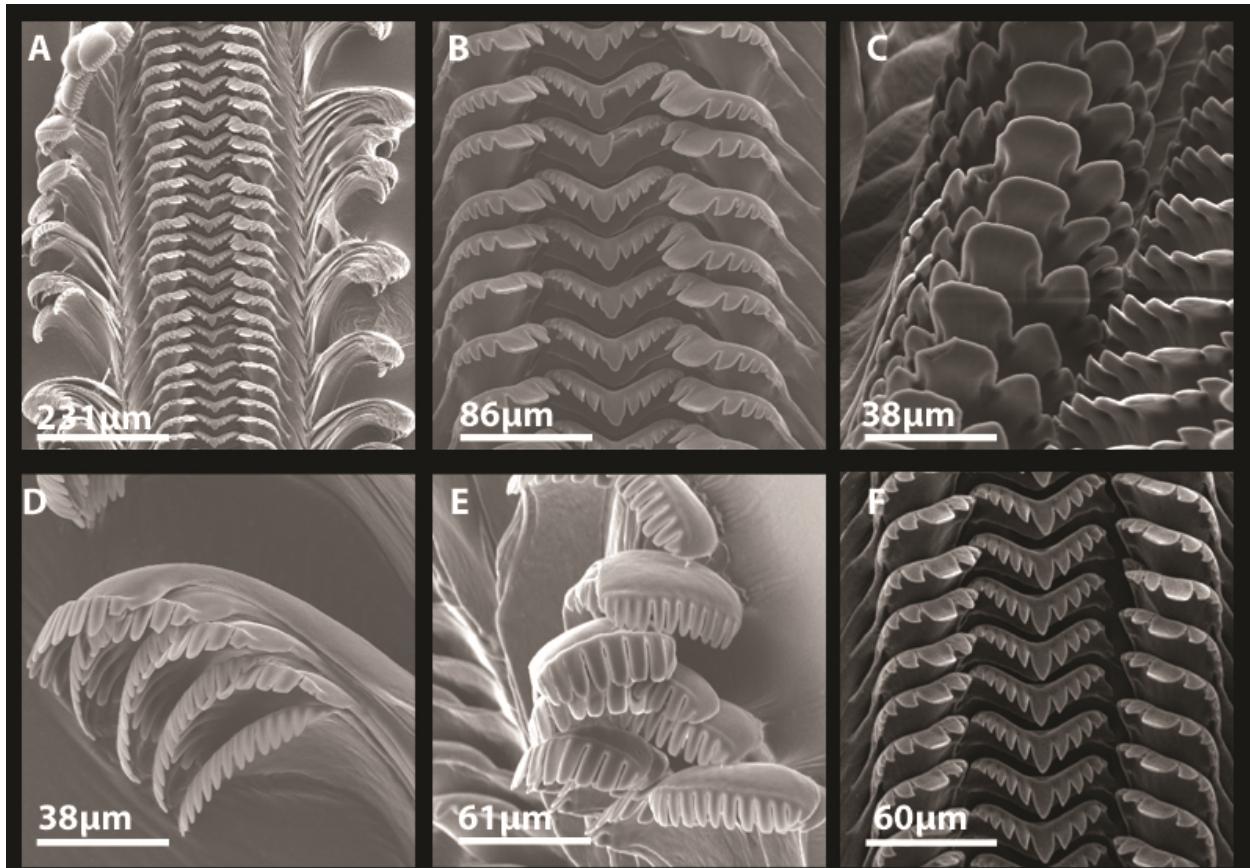
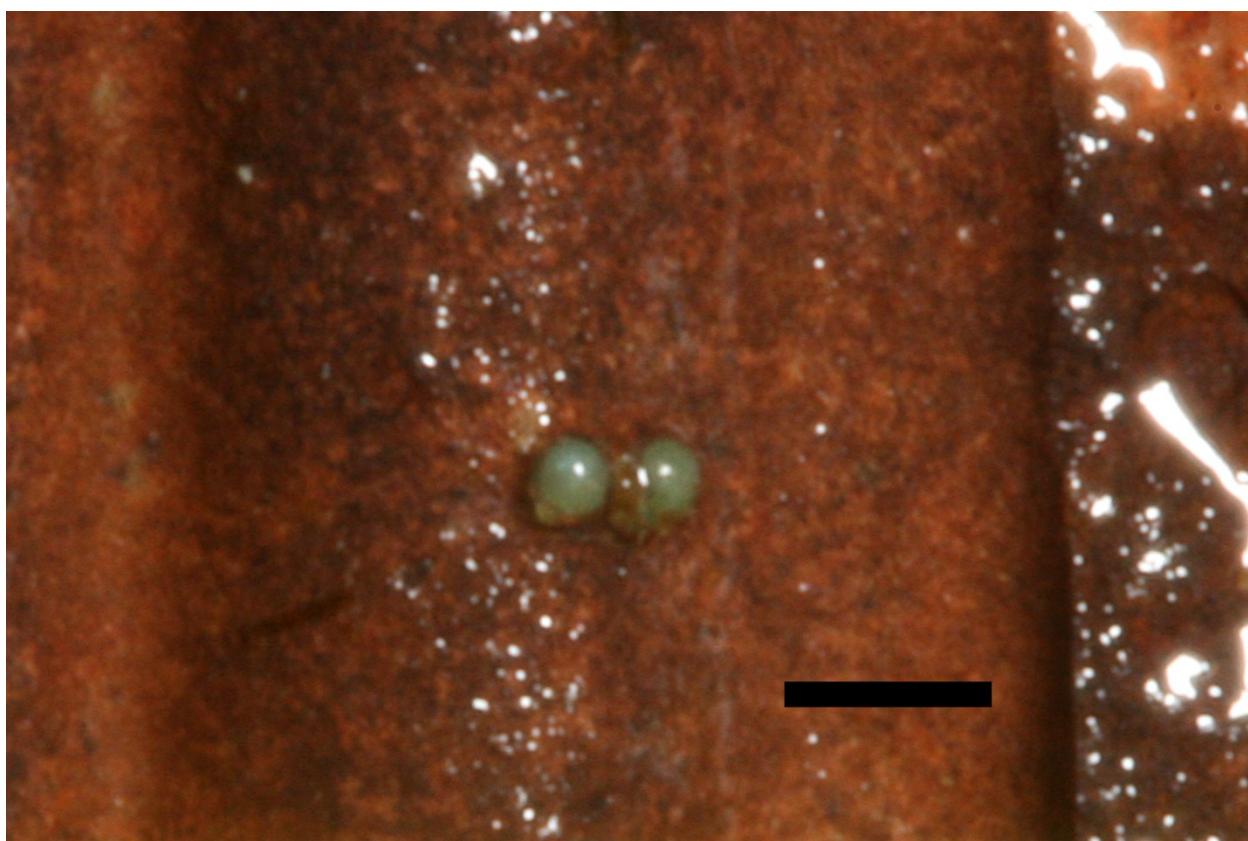


Figure 2.7: Photograph of two eggs that were laid by captive *L. compacta*. Scale bar=1mm.



CHAPTER 3

CHRONIC SPECIES-LEVEL POLYPHYLY IN TWO GASTROPOD FAMILIES IS CAUSED BY CRYPTIC NUMTS

Abstract

Systematic studies of the freshwater gastropod families Pleuroceridae and Semisulcospiridae (Cerithioidea) have long been thwarted by highly divergent mitochondrial haplotypes. These divergent lineages often result in non-monophyletic putative species and/or conflict with nuclear genes on resulting gene trees. In the absence of evidence that these represent nuclear copies of mitochondrial genes (NUMTs) (i.e. heteroplasmy/heterozygosity, indels, stop codons), several hypotheses have been forwarded to explain the origin of these divergent lineages including retention of ancestral polymorphisms, introgression, or that they indicate the presence of cryptic species. However, these explanations have been unsatisfactory given the realities of mitochondrial evolution or the number of cryptic species implied. Consequently, we reanalyzed previous studies and generated a novel dataset of mitochondrial and nuclear gene sequences for four pleurocerid species to explore the possibility that they represent NUMTs. The present results corroborate previous findings that divergent sequences lack the immediate hallmarks of NUMTs. However, they were found to have ratios of non-synonymous to synonymous mutations that were significantly higher than non-divergent lineages. Cloned PCR products revealed that divergent and non-divergent sequences co-amplify in individuals that produce divergent haplotypes with universal primers. These results are consistent with the interpretation that divergent mitochondrial haplotypes are NUMTs. The unusual properties (large size, absence of

indels or frameshift mutations) and prevalence of NUMTs in pleurocerids and semisulcospirids are promising avenues of future investigation and suggest this may be an ideal model system for exploring the evolution and genomic function of NUMTs.

Keywords: Gastropoda; Pleuroceridae; Semisulcospiridae; NUMT; pseudogene; mitochondrial gene tree

Introduction

Molecular systematic studies have revealed the presence of highly divergent mitochondrial lineages within species of the freshwater gastropod families, Pleuroceridae and Semisulcospiridae (Dillon and Robinson, 2009; Lee, *et al.*, 2007; Lee, *et al.*, 2006; Miura, *et al.*, 2013; Sides, 2005). These divergent lineages often fall to the base of gene trees, resulting in non-monophyly of putative species (Dillon and Robinson, 2009; Kim, *et al.*, 2010; Lee, *et al.*, 2007; Lee, *et al.*, 2006; Miura, *et al.*, 2013) and discordance with nuclear gene trees (Lee, *et al.*, 2007; Lee, *et al.*, 2006). Whereas some authors have identified such haplotypes as rare oddities (Dillon and Robinson, 2009; Lee, *et al.*, 2007; Lee, *et al.*, 2006; Miura, *et al.*, 2013), others have failed to recognize them as such and have assumed that they are representative of the genetic diversity of a given species (Holznagel and Lydeard, 2000; Minton, Garner and Lydeard, 2003; Minton, Savarese and Campbell, 2005). Hypotheses about the biological cause of these divergent haplotypes have ranged from retained ancestral polymorphisms and/or historical introgression (Dillon and Robinson, 2009; Lee, *et al.*, 2007; Miura, *et al.*, 2013) to cryptic species (Kim, *et al.*, 2010; Minton, *et al.*, 2005). However, these hypotheses have not been satisfying since it is not immediately obvious how such genetic diversity could be maintained given the realities of mitochondrial molecular evolution and lineage sorting processes (Funk and Omland, 2003) or,

alternatively, because of the sheer number of sympatric cryptic species implied by the sometimes numerous divergent clades.

The presence of these divergent mitochondrial haplotypes in pleurocerids and semisulcospirids has prevented meaningful taxonomic revisions with molecular data. Pleurocerids, in particular, have a complicated taxonomic history with over 800 available species names (Graf, 2001; Garner *et al.* unpubl. data), but only 162 that are currently considered valid (Johnson, *et al.*, 2013). Pleurocerid taxonomy has been mostly unchanged since the first half of the 20th century (Goodrich, 1922; Goodrich, 1940; Johnson, *et al.*, 2013; Tryon, 1873), with current species boundaries reflecting outdated concepts of morphological variation and geographic distribution. Pleurocerids and Semisulcospirids are found throughout North America and eastern Asia, but anthropogenic pressures threaten the survival of many species (Johnson *et al.* 2013). These animals are integral to basic ecosystem processes (e.g. nutrient cycling, predatory/prey dynamics), and the health of freshwater resources in many rivers depends on these snails (Brown *et al.* 2008; Lysne *et al.* 2008; Perez and Minton 2008). Taxonomic confusion and presence of divergent mitochondrial haplotypes complicates conservation efforts and discourages basic research (e.g. toxicology, life history) on an ecologically important and understudied gastropod family in which 79% of species are considered imperiled (Brown, Lang and Perez, 2008; Johnson, *et al.*, 2013; Lysne, *et al.*, 2008; Perez and Minton, 2008).

The most promising, and surprisingly overlooked, hypothesis that may explain divergent mitochondrial haplotypes in these two families is that they are non-functional nuclear copies of mitochondrial genes, or NUMTs, that have been misapprehended as mitochondrial orthologs (Lopez, *et al.*, 1994). If not accurately identified as such, NUMTs can confound estimates of species diversity (Moulton, Song and Whiting, 2010; Song, *et al.*, 2008) and phylogenetic

inference (Bensasson, *et al.*, 2001; Triant and DeWoody, 2009; Williams and Knowlton, 2001). However, NUMTs have been used constructively in a number of studies to explore past gene exchange between lineages (Baldo, *et al.*, 2010), date divergence events (Keller, Bensasson and Nichols, 2006), and explore broad patterns of molecular evolution (Bensasson, *et al.*, 2001; Schmitz, Piskurek and Zischler, 2005). The preponderance of NUMTs in any given genome varies across the eukaryote tree of life and is not necessarily associated with genome size (Richly and Leister, 2004). If divergent haplotypes resolved in pleurocerid and semisulcospirid studies are NUMTs then it would not only help to clarify systematic relationships among species and genera but also provide a useful resource for estimating past gene flow and studying the biogeography of both families (Baldo, *et al.*, 2010; Triant and DeWoody, 2007; Triant and DeWoody, 2009).

NUMTs often are identified by the presence of indels or stop codons (Lopez, *et al.*, 1994). However, in some groups, NUMTs lacking these hallmarks have been sequenced with some frequency (e.g. crayfish; Song, *et al.*, 2008). NUMTs of protein coding genes (e.g. cytochrome *c* oxidase I; COI) can also be identified by a higher ratio of non-synonymous to synonymous mutations (dN/dS) than the functional copy due to the relaxation of selective constraints that act to maintain gene structure and function (Baldo, *et al.*, 2010; Perna and Kocher, 1996). Phylogenetic analyses that include functional and non-functional copies of genes often resolve NUMTs as basal to lineages of functional sequences on inferred gene trees, since their implied relatedness to coding sequences corresponds to the age of NUMT duplication (Bensasson, *et al.*, 2001; Song, *et al.*, 2008). For instance, NUMTs that arose before speciation events will appear basal to the speciation event if included as a mitochondrial homolog in

phylogenetic inference. This property can be used to help identify NUMTs of non-coding genes, such as mitochondrial rRNA genes (e.g. 16S).

Despite that NUMTs are well known to cause some of the molecular signatures seen in published pleurocerid and semisulcospirid mitochondrial gene trees, only Lee et al. (2007) briefly mentioned them as a potential explanation for divergent lineages of Asian *Semisulcospira*, but they dismissed the hypothesis due to the absence of heteroplasmy in sequence chromatograms. Here, we revisit previous studies to explore if there is a signature of NUMTs in previously published data sets, generate a novel population-level dataset using mitochondrial and nuclear markers and use cloning to more thoroughly explore the hypothesis that NUMTs have been confused as mitochondrial orthologs in pleurocerid and semisulcospirid research. There is a pressing need to understand this phenomenon to advance our knowledge about the diversity of these imperiled gastropod families and to better understand the evolution of their genomes.

Materials and Methods

Previous studies

We retrieved COI and 16S sequences for all published molecular phylogenetic studies from GenBank (Dillon and Frankis, 2004; Dillon and Robinson, 2009; Holznagel and Lydeard, 2000; Lee, *et al.*, 2007; Lee, *et al.*, 2006; Lydeard, *et al.*, 1997; Lydeard, *et al.*, 1998; Minton, *et al.*, 2003; Minton and Savarese, 2005; Minton, *et al.*, 2005; Miura, *et al.*, 2013; Sides, 2005); the sequences of Kim et al. (2010) were obtained directly from the authors (Table 3.1). We removed Minton and Savarese (2005) from our analyses since some sequences had unusual anomalies that may have been the result of sequencing error (i.e. concentration of mutations in the last 200 base pairs). Sequences were aligned with MUSCLE (Edgar 2004), and COI was translated into amino

acids to check for stop codons and frameshift mutations. Alignments were collapsed into haplotypes to remove duplicates and COI was subdivided into codon position using Nucleotide Codon Position Parser (Whelan, 2012). Unique mutations (i.e. singletons) were calculated for each codon position using an R (R Development Core Team, 2011) script from Baldo et al. (2010).

Divergent COI haplotypes were identified as possessing at least two second-codon position singleton mutations, and one of the following characteristics: 1) when numerous individuals have been sequenced for a putative species, representing a long branch that is phylogenetically distinct from the common haplotype clade, or, 2) when only one or two individuals from a species have been sequenced, representing an unusually long, isolated branch as compared to other congeneric individuals, typically falling to the base of the ingroup. A few sequences could not be identified as "divergent" branches on the resulting gene trees, but had more than three second-codon position singleton mutations, which was considered sufficient to identify them as "divergent." This approach should yield conservative estimates as an individual with only two unique second-codon position mutations—a rarity in COI—would not be identified as "divergent" unless it was also on a long, isolated branch. This combination of criteria will also minimize false positives (i.e. erroneous identification of divergent lineages) resulting from possible sequencing error. As 16S sequences cannot be analyzed for codon position changes, identification of 16S sequences as "divergent" relied primarily on phylogenetic criteria (i.e. recognition of long, isolated branches on gene trees). Although this criterion is slightly subjective, it was applied as conservatively as possible and likely underestimated the number of divergent lineages.

Codeml in PAML 4.7 (Yang, 2007), was used to compare dN/dS values among COI haplotypes. For simplicity, and because different studies sequenced different COI fragments, each published data set was analyzed separately. A starting tree for each Codeml analysis was inferred in Garli 2.0 (Zwickl, 2006) using the dataset partitioned by codon position and the best fit model for each partition as specified by Partition Finder 1.0 (Lanfear, *et al.*, 2012) using the Bayesian Information Criteria (Table 3.2; Figs. 3.A1-3.A6). We then calculated three dN/dS models for each dataset: 1) all lineages have identical dN/dS rates, 2) non-divergent lineages have one dN/dS rate and divergent lineages have a separate dN/dS rate, 3) non-divergent lineages have one dN/dS rate, branches leading to a clade of divergent lineages have another dN/dS rate, and divergent lineages have a separate dN/dS rate. The rationale behind the third model is as follows: if “divergent” sequences are NUMTs, then for some time along the branch leading to a divergent clade the ancestral haplotype would have been evolving as a functional mitochondrial gene, but once it was transferred to the nuclear genome it would have started evolving as a NUMT. We did not calculate a three-rate model for Minton and Lydeard (2003) since all divergent lineages were represented by only a single sequence. Significant differences among models were tested with a likelihood ratio test in PAML.

New data generation

To survey the prevalence of divergent haplotypes within pleurocerid populations, we generated a population-level dataset for four species that occur in the Cahaba River and Paint Rock River drainages (Fig. 3.1; Table 3.3). Twenty adult individuals were sampled from one population each of *Pleurocera pyrenella* (Conrad, 1834) and *Leptoxis praerosa* (Say, 1821) and twenty individuals from each of four sympatric populations of *P. prasinata* (Conrad, 1934) and *L. ampla* (Anthony, 1855). Snails were removed from their shells following the method of

Fukuda et al. (2008), and a ~ 1 mm² tissue clip was taken from the foot for DNA extractions. Snails were sexed to assess any gender bias among individuals with divergent haplotypes to test if doubly uniparental inheritance (DUI) may be a contributing factor (Breton, *et al.*, 2007; Fisher and Skibinski, 1990). Vouchers have been deposited at the National Museum of Natural History (USNM) in Washington, D.C. (Table 3.3).

Whole genomic DNA was extracted from the foot tissue clip using the Autogenprep965 (Autogen, Holliston, MA) automated phenol:chloroform extraction with a final elution of 50 µL. A 551 base pair (bp) fragment of COI and a ~ 510 bp fragment of 16S were amplified using JGLCO/JGHCO primers (Geller, *et al.*, 2013) and universal 16SAR/BR primers (Palumbi, *et al.*, 1991) respectively. PCR reactions were performed with 1 µL of undiluted DNA template in 20 µL reactions. Reaction conditions for COI were 10µL of Promega Go-Taq Hotstart Master Mix, 0.15 µM each primer, 0.25 µg/µL BSA, 1.25% DMSO and an amplification regime of an initial denaturation at 95°C for 7 min, followed by 45 cycles of denaturation at 95°C for 45 s, annealing at 42°C for 45 s, extension at 72°C for 1 min and then a final extension at 72°C for 5 min. Reaction conditions for 16S were 1X Biolase (Bioline, Taunton, MA) reaction buffer, 500 µM dNTPs, 3 mM MgCl₂, 0.15 µM each primer, 0.25 µg/µL BSA, 1 unit Biolase DNA polymerase and an amplification regime of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 3 min. H3 reactions used similar chemical concentrations as above with an amplification regime of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 15 s and extension at 72°C for 15 s, followed by a final extension at 72°C for 5 min. PCR products were purified using the ExoSAP-IT protocol (GE healthcare, Piscataway, NJ). BigDye (ABI, Foster City, CA) sequencing

reactions and sequencing on an ABI 3730XL DNA analyzer capillary array were done following manufacturer's instructions.

Chromatograms were visually inspected, corrected as necessary, and assembled in Geneious Pro 6.1 (Biomatters); alignments were generated with MUSCLE using default settings (Edgar, 2004). BLAST searches (Altschul, *et al.*, 1997) were used to ensure divergent sequences were not the result of contamination. No conflict in phylogenetic signal was found between the COI and 16S datasets (i.e. all individuals with a divergent COI haplotype had a divergent 16S haplotype), so the two mitochondrial genes were concatenated for phylogenetic inference. The phylogeny was rooted with *Juga silicula* and *Semisulcospira nodiperda*. An independent phylogeny was inferred for the nuclear Histone H3 dataset which was rooted with *J. silicula*.

The best fit partitions and models for phylogenetic analyses were determined with PartitionFinder 1.0 (Table 3.2; Lanfear, *et al.*, 2012). Bayesian phylogenies for the COI+16S and H3 datasets were each inferred using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) on the Alabama Supercomputer Cluster with six independent runs and 8 chains ran for 30,000,000 Markov chain Monte Carlo (MCMC) generations. MCMC convergence and adequate mixing was assessed using Tracer (Rambaut and Drummond, 2007) and AWTY (Nylander, *et al.*, 2008). The first 9,000,000 generations of each run were discarded as burn-in, and a 50% majority rule consensus tree of 126,000,000 trees was constructed.

Divergent COI haplotypes were identified and analyzed as described above. To determine if divergent and non-divergent haplotypes were both amplified in PCR reactions, PCR products were screened by cloning. For cloning, PCR reactions were run as described above, but with a high-fidelity taq polymerase (Kappa Biosystems). A TA TOPO (Invitrogen) vector was used to clone PCR products of two *Leptoxis ampla* individuals with divergent haplotypes and one *L.*

ampla individual with a haplotype from the non-divergent mitochondrial haplotype clade (Fig. 3.2). Twenty clones per individual were sequenced. Coding-sequence specific primers for COI (Forward: TTGGCATGTGATCTGGATTAGTTGG; Reverse: ATAGCCCCAGCTAACACAGGC) were also developed in an attempt to amplify the most non-divergent haplotype in individuals with divergent haplotypes.

Results

Previous studies

Divergent haplotypes (i.e. haplotypes on long isolated branches, or a haplotypes with many second-codon mutations) were identified in every previously published pleurocerid and semisulcospirid study except Lydeard *et al.* (1998; Table 3.1; Table 3.A1). However, some of these represented divergent sequences that had been previously published and recycled from GenBank (e.g. Minton, *et al.*, 2003; Strong and Köhler, 2009). Although chromatograms are not available from GenBank, heteroplasmy was not observed in at least four previous studies (Minton R, O’Foighil D, personal communication). In phylogenetic analyses, almost all divergent sequences were recovered as basal to a larger clade of congeneric individuals, and/or to the entire ingroup. Most previous studies had fewer than 20% divergent haplotypes, with the highest percentages being 50% and 48% in Dillon and Frankis (2004) and Minton *et al.* (2005) respectively; on average 16.7% of sequences in published studies have been divergent.

In all studies, divergent COI haplotypes had more singleton mutations at the first- and second-codon positions than non-divergent sequences except those in Minton and Lydeard (2003), which only had more second-codon mutations (Table 3.4). No COI sequences contained indels or stop codons. Divergent 16S sequences did have indels compared to a consensus sequence, but they were comparable in size and number to those in non-divergent sequences.

In every COI dataset analyzed except Dillon and Frankis (2004), significant support was returned for the two dN/dS rate model over the one dN/dS rate model (Table 3.5). Since the three-rate model was a significantly better fit for only some datasets and could not be calculated for the Minton and Lydeard (2003) dataset, only the two-rate and one-rate models are compared here (Table 3.5). The average dN/dS values of the two-rate model for non-divergent and divergent lineages are 0.004 and 0.043 respectively. Although there is significant support for the two-rate model, no dN/dS value was greater than one.

Newly-generated data

All PCR products sequenced cleanly with chromatograms showing no evidence of double peaks or co-amplification of multiple products (heteroplasmy/heterozygosity). Both males and females produced divergent and non-divergent haplotypes. COI sequences did not contain stop codons or indels, and all BLAST searches returned pleurocerids or semisulcospirids as the most similar sequences. As in the published datasets, there were indels in the aligned 16S sequences compared to a consensus sequence, but they were comparable in size and number to those in non-divergent sequences.

Bayesian analysis resolved seven *L. ampla* clades, one *L. praerosa* clade, five *P. prasinata* clades, and three *P. pyrenella* clades (Fig. 3.2). The number of divergent haplotype clades varied from none (*Leptoxis praerosa*), two to four (*Pleurocera pyrenella*, *P. prasinata*) to as many as six (*L. ampla*). The greatest within species divergence for COI was 19.8% in *L. ampla* and the greatest among species divergence was 24.6% (uncorrected *p*-distance). As noted above, there was no conflict in phylogenetic signal between the COI and 16S datasets. Both *Pleurocera* species are non-monophyletic on the mitochondrial gene tree. *Leptoxis ampla* is resolved as monophyletic (Fig. 3.2), but inclusion of other Alabama River Basin *Leptoxis* would

cause *L. ampla* to be paraphyletic on the gene tree (Whelan NV, unpublished data). Both males and females produced divergent haplotypes. H3 had less phylogenetic signal at the species level than mitochondrial genes as the greatest within species divergence for H3 was 1.5% in *P. prasinata* and the greatest among species divergence was only 2.4% (uncorrected *p*-distance). Individuals with divergent mitochondrial haplotypes were not the same individuals as those outside the largest H3 clade for each species (Fig. 3.3). All H3 mutations were synonymous and nodal support was limited for the H3 gene tree.

Cloned PCR products of *L. ampla* individuals with divergent haplotypes produced sequences of both the originally sequenced divergent haplotype as well as the non-divergent haplotype. However, specific primers designed to amplify the non-divergent haplotype failed to do so in individuals that produced a divergent haplotype with universal primers. For the individual with a non-divergent haplotype sequenced using standard protocols, cloning did not produce divergent sequences.

Discussion

NUMTs as the causal explanation

Of all hypotheses forwarded to explain divergent mitochondrial haplotypes within pleurocerids and semisulcospirids, the best explanation given the present results is that they are non-functional nuclear copies of mitochondrial genes, or NUMTs. For example, dN/dS values for NUMT lineages are significantly higher than mitochondrial coding sequences. Only the study of Dillon and Frankis (2004) did not produce differences in dN/dS values that were statistically significant; however, that study included only six COI sequences making comparisons difficult. The same sequences were included in Dillon and Robinson's (2009) expanded study, which did return significantly different dN/dS ratios for the six COI sequences included in the earlier study.

The ratio of dN to dS values does appear to be somewhat constrained for the NUMT sequences, but considering the absence of frameshift mutations and that PAML does not model nuclear and mitochondrial lineages differently, support for the interpretation that divergent haplotypes are NUMTs is strong. The bias of unique first- and second-codon mutations in NUMTs compared to coding sequences are consistent with the hypothesis that divergent haplotypes are not subject to the same selective regime as coding mitochondrial genes. Doubtly uniparental inheritance as an explanation is also rejected by our findings since both males and females produced divergent haplotypes and there were more than one divergent haplotype within species. Finally, the cloning results unequivocally support the NUMT hypothesis.

With the benefit of hindsight, it is easy to understand how NUMTs have been chronically overlooked as a causal explanation for what were interpreted as divergent—but coding—mitochondrial sequences. First, many pleurocerid and semisulcospirid species are difficult to identify from shell morphology alone (Ó Foighil, *et al.*, 2009), and the potential for undescribed cryptic species remains a distinct possibility. Second, "divergent" sequences do not possess immediate hallmarks of NUMTs, namely stop codons, indels or frameshift mutations, and chromatograms with heteroplasmy/heterozygosity. Finally, the magnitude of the problem, with the number of NUMT sequences approaching 50% in some studies (e.g., Dillon and Frankis 2004; Minton *et al.* 2005) is unknown within marine cerithioidean families (Strong unpublished data) or indeed, any other gastropod group. It is not clear if this indicates that nuclear copies of mitochondrial genes exist at much higher frequencies in pleurocerids and semisulcospirids compared to other cerithioidean families, or are just preferentially amplified for reasons yet unknown. The increasing use of next generation sequencing technologies in gastropod studies will shed light on this issue.

Previous studies and newly-generated data

The conclusions of past higher-order phylogenetic studies have been mostly unaffected by the inclusion of NUMT sequences. In some cases, this is because of the relatively minor contribution of NUMTs to the dataset and hence the disruptive effect is localized (Holznagel, 1997; Holznagel and Lydeard, 2000), and/or the duplication occurred more recently than the branching events of the focal hierarchical level (Strong and Köhler, 2009). Regardless, many NUMT sequences from early studies have been used as homologs in more recent studies (Lee, *et al.*, 2006; Minton, *et al.*, 2003; Strong and Köhler, 2009), highlighting the problem of using sequences from GenBank without adequate quality control and the domino effect of propagating such data through subsequent analyses.

Studies exploring species-level relationships have been more seriously impacted. For instance, the inclusion of NUMTs as mitochondrial homologs in the study of Dillon and Robinson (2009) resulted in the fantastical claim that pleurocerids were “the snails the dinosaurs saw” (Dillon and Robinson 2009: 1) based on the large molecular divergences within species that NUMT sequences implied. Unsurprisingly, Dillon and Robinson (2009) never offered a plausible mechanism for how such mitochondrial diversity could have been maintained for over 65 my, nor apparently did reviewers of this piece of fiction require one.

Alternatively, non-monophyly and high genetic diversity caused by the inclusion of NUMTs in species-level studies have been interpreted as revealing the presence of cryptic species (Kim, *et al.*, 2010; Minton and Lydeard, 2003; Minton, *et al.*, 2005). Minton *et al.* (2005) formally described *Lithasia spicula* from two sequences with hallmarks of NUMTs (i.e. second-codon position mutations, significantly higher dN/dS value, basal position in the phylogeny). Although, *L. spicula* was also diagnosed as possessing a morphologically distinct radula

compared to sympatric pleurocerids, the genetic basis for its recognition requires re-evaluation.

Lithasia spicula is considered critically imperiled (Johnson, *et al.*, 2013), but its validity must be confirmed before limited management resources are expended on its conservation.

Interpretation of mitochondrial gene trees including NUMTs has also resulted in taxonomic consequences such as the synonymization of species previously considered valid. In their molecular analysis of *Semisulcospira* spp. from Korea, Lee *et al.* (2007) proposed synonymization of six Korean *Semisulcospira* species with *S. libertina* based on the non-monophyly of species caused by inclusion of NUMTs. Miura *et al.* (2013) expanded on the study of Lee *et al.* (2007) with the inclusion of *Semisulcospira* sequences from Japan. They found that divergent haplotypes from Lee *et al.* (2007) nested within a more basal Japanese clade, each with a different, geographically disparate sister taxon. As no divergent haplotypes from Japanese species were found within the Korean clade, Miura *et al.* (2013) proposed that this pattern reflected historical unidirectional dispersal from Japan to Korea. Rather, we suggest this pattern is indicative of the resemblance NUMTs may bear to ancestral sequences as a consequence of slower evolutionary rates following duplication (Song, *et al.*, 2008; Triant and DeWoody, 2007).

The newly-generated dataset corroborated the patterns elaborated in previous studies with regards to pronounced population-level mitochondrial heterogeneity and species non-monophyly. However, the population-level taxon sampling and multi-locus character sampling herein allowed additional characteristics of pleurocerid and semisulcospirid NUMTS to be explored. Following removal of hypothesized NUMT sequences, all putative species were monophyletic indicating that mitochondrial markers are potentially useful for exploring species-level relationships provided that orthologs are correctly identified. The H3 phylogeny was roughly concordant with coding clades in the mitochondrial gene tree, but divergences among

species were small indicating limited utility for elucidating relationships at the species level; incomplete lineage sorting is the likely cause of non-monophyly of *Pleurocera* species. Interestingly, even with NUMTs removed, *Leptoxis* was not monophyletic in either the mitochondrial or nuclear dataset, adding to a growing body of evidence that many pleurocerid genera are not monophyletic.

An attempt to design coding-sequence-specific COI primers failed, and all individuals that produced a COI NUMT also produced a 16S NUMT. Moulton *et al.* (2010) found that, in Orthoptera, specific primers often did not completely prevent NUMT co-amplification. The problem, however, seems to be particularly acute in pleurocerids. Furthermore, the co-sequencing and congruence of COI and 16S NUMTs indicates that mitochondrial fragments incorporated into the nuclear genome must be large and evolving cohesively through time. Furthermore, their copy number or prevalence in the genome could be very large considering the preferential amplification of NUMTs seen in some individuals.

Dense intra-specific sampling revealed considerable variation among populations in the proportion of individuals that preferentially amplified NUMTs and in the number of divergent clades. For example, 19 of 20 individuals from the *Leptoxis ampla* population from the Cahaba River in Helena, Alabama produced NUMTs, while all 20 *L. praerosa* individuals from the Paint Rock River produced only coding sequences. As such, frequency cannot be taken as an indication of functionality, and all mitochondrial sequences in these families should be scrutinized carefully to assess orthology.

Future Studies

The challenge of NUMTs in molecular systematic studies of pleurocerids and semisulcospirids will certainly be diminished in a phylogenomic age. At the moment, however,

the genomic resources available for most gastropod taxa are either lacking entirely or still in their infancy. Nevertheless, there is an urgent need to address systematic questions in many understudied groups, particularly those of immediate conservation concern, faster than genomic resources may be developed. Until such time that genomic tools become routine in gastropod research, all mitochondrial sequences in these families should be scrutinized carefully to assess orthology, and any taxonomic revisions (e.g. Dillon, 2011) and new species descriptions (e.g. Minton, *et al.*, 2005) incorporating analysis of mitochondrial sequences should be approached with appropriate care. The chronic neglect of NUMTs in these families is a cautionary tale for gastropod studies that reveal unexpected levels of sequence divergence within putative species.

Beyond systematic implications, the critical evaluation and removal of NUMTs from pleurocerid and semisulcospirid studies will now allow the application of calibration techniques and, in contrast to Dillon and Robinson (2009), the reasonable estimation of the age of species and clades. Easily-sequenced NUMTs could serve as a tool for exploring evolutionary patterns such as historical migration (as noted above for Miura *et al.* 2013), genome transfer events, and past mitochondrial diversity (Baldo, *et al.*, 2010; Perna and Kocher, 1996; Triant and DeWoody, 2007). In this regard, NUMTs could be particularly useful in a family that has suffered as many recent extinctions as pleurocerids (i.e. 31; Johnson, *et al.*, 2013). Future sequencing of genomes—not just exomes—and population-level analyses with genomic tools will be necessary to fully investigate the properties of NUMTs in pleurocerids and semisulcospirids. Intriguingly, the freshwater sister group to Pleuroceridae + Semisulcospiridae, the Melanopsidae (Strong, *et al.*, 2011), does not exhibit the same preferential sequencing of NUMTs (Smolen and Falniowski, 2009; Strong unpubl. data), indicating that pleurocerids and semisulcospirids pose unique opportunities for studying NUMTs as a molecular phenomenon. For example, future

promising avenues of exploration include assessing the number, size and location of NUMTs to determine if there have been whole mitochondrial genome transfer events or if NUMTs are concentrated in certain regions of the genome. It should be noted that in rare instances, NUMTs with frameshift mutations have been sequenced for pleurocerids (Strong EE, unpublished data), but the complete lack of stop codons or indels in COI NUMTs in previous studies and in our newly generated dataset is an intriguing property meriting further exploration. Considering the apparently cohesive nature of NUMTs in pleurocerids and semisulcospirids (i.e. no frameshift mutations in COI, concordance of COI and 16S NUMTs in individuals), they may have a functional role such as helping to maintain genome integrity or function as in primates (Hazkani-Covo and Covo, 2008). Pleurocerids and semisulcospirids could serve as an invertebrate model for better understanding how frequently duplication events can occur, whether specific fragment sizes and/or regions of the mitochondrial genome are preferentially duplicated, and how NUMTs affect genome size, function, and evolution.

Conclusions

For over 15 years, paralogs of mitochondrial genes have been misinterpreted and included in pleurocerid and semisulcospirid studies as homologs. The confusion this has created has suffocated molecular systematic research on these families and prevented meaningful progress on their systematics and conservation. We know of no similar example in any animal group and the confusion that has been perpetuated is unprecedented.

These findings restore the potential utility of mitochondrial genes and hence greatly simplify the challenge of confronting pleurocerid and semisulcospirid systematics using available molecular tools. Both families are of great conservation concern and in desperate need of targeted studies to clarify their chaotic classification and inform conservation and

management strategies. Our results should stimulate studies on their biology, systematics and molecular evolution. Many questions still exist about the genomic implications of frequently sequenced NUMTs, but we hope this will be an exciting avenue of future research that will no doubt be enhanced with genomic sequencing tools. This system has great potential to serve as a model for better understanding NUMT and genome evolution in invertebrates.

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Table 3.1: Datasets assessed in the present study

Dataset	Family	Gene(s) used	Divergent haplotype interpretation	# of hypothesized NUMTs / total # of sequences
Lydeard <i>et al.</i> (1997)	Pleuroceridae	16S	Not identified as divergent haplotypes, but conclusions about generic non-monophyly need revisiting	2/15
Lydeard <i>et al.</i> (1998)	Pleuroceridae	16S	None included	0/29
Holznagel and Lydeard (2000)	Pleuroceridae	16S	Not identified as divergent haplotypes, but conclusions about generic non-monophyly need revisiting	4/33
Minton and Lydeard (2003)	Pleuroceridae	COI	Not identified as divergent haplotypes, but hypothesized some were cryptic species	5/47
Minton <i>et al.</i> (2003)	Pleuroceridae	16S	Not identified, but did not affect final conclusions	4/34
Dillon and Frankis (2004)	Pleuroceridae	COI, 16S	Retained ancestral polymorphisms	6/12
Minton <i>et al.</i> (2005)	Pleuroceridae	COI	<i>Lithasia spicula</i> described as new species from two identical divergent haplotypes	16/33
Sides (2005)	Pleuroceridae	COI	Identified divergent haplotypes, but offered little explanation	23/114
Lee <i>et al.</i> (2006)	Pleuroceridae & Semisulcospiridae	16S, 28S	Identified nuclear and mitochondrial incongruence with respect to divergent mt haplotypes, but causal explanation not proposed	7/46

Lee et al. (2007)	Semisulcospiridae	16S, 28S	Retained ancestral polymorphisms or introgression	13/107
Dillon and Robinson (2009)	Pleuroceridae	COI	Retained ancestral polymorphisms	8/43
Strong and Köhler (2009)	Pleuroceridae & Semisulcospiridae	16S	Not identified as divergent haplotypes, but did not affect final conclusions	4/51
Kim et al. (2010)	Semisulcospiridae	COI	Hypothesized to be cryptic species, but none were formally described	4/27
Miura et al. (2013)	Semisulcospiridae	16S	Dispersal and possible introgression	12/241
This study	Pleuroceridae	COI, 16S, H3	NUMTs	40/240

Table 3.2: Singleton mutations for coding and NUMT sequences in each data COI set

Dataset		NUMT	2nd		
			1st pos.	pos.	3rd pos.
Minton and Lydeard (2003)	NUMT coding	4	18	108	
		8	6	61	
Dillon and Frankis (2004)	NUMT coding	93	93	101	
		68	79	77	
Minton <i>et al.</i> (2005)	NUMT coding	16	6	100	
		5	2	39	
Sides (2005)	NUMT coding	13	7	69	
		8	3	74	
Dillon and Robinson (2009)	NUMT coding	13	7	99	
		2	2	28	
Kim <i>et al.</i> (2010)	NUMT coding	13	1	86	
		0	1	21	
This study	NUMT coding	12	4	39	
		5	0	46	

Table 3.3: Comparisons of 2-rate versus 1-rate dN/dS models for COI datasets

Dataset	1-rate dN/dS	2-rate dN/dS	1-rate lnL	2-rate lnL	P
Minton and Lydeard (2003)	0.01131	0.00426 ; 0.20235	-4472.958882	-4411.237882	<0.001*
Dillon and Frankis (2004)	0.00435	0.0050 ; 0.0035	-1780.966739	-1780.842602	0.667
Minton <i>et al.</i> (2005)	0.00631	0.00158 ; 0.00948	-4050.163403	-4042.755836	<0.001*
Sides (2005)	0.00609	0.00233 ; 0.00920	-4901.125541	-4893.99673	<0.001*
Dillon and Robinson (2009)	0.00938	0.00497 ; 0.01168	-3354.692646	-3352.708667	0.046*
Kim <i>et al.</i> (2010)	0.00802	0.00338 ; 0.01154	-2329.8795	-2327.7079	0.037*
This study	0.01381	0.00772 ; 0.01640	-2911.915402	-2909.980359	0.049*

Table 3.4: Models of molecular evolution used to create PAML starting trees in GARLI

Dataset	Model
Minton and Lydeard (2003)	TrNef+I ; F81 ; GTR
Minton <i>et al.</i> (2005)	TrNef+G ; TrN+I ; K81uf+G
Sides (2005)	TrNef+G ; F81 ; GTR+G
Dillon and Robinson (2009)	TIM+I ; TIM+G ; TrNef+G
Kim <i>et al.</i> (2010)	TrNef+I ; HKY+I ; TrN+G
This study	TrNef+G ; F81+I ; K81uf+G

Table 3.5: Sampling localities, GenBank accession numbers and USNM catalogue numbers for newly generated data.

Species	Locality	GenBank 16S	GenBank COI	GenBank H3	USNM catalogue #
<i>Leptoxis ampla</i>	Cahaba River @ US HWY 52 (33.28449°N, 86.88257°W) "Locality B"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Leptoxis ampla</i>	Shades Creek (33.22013°N, 87.03323°W) "Locality C"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Leptoxis ampla</i>	Cahaba River near Bibb and Shelby County line (33.16981°N, 87.01949°W) "Locality D"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Leptoxis ampla</i>	Little Cahaba River (33.05407°N, 86.96919°W) "Locality E"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Leptoxis ampla</i>	Cahaba River @ US HWY 82 (32.95782°N, 87.13971°W) "Locality F"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Leptoxis praerosa</i>	Paint Rock River (34.6873°N 86.3102°W) "Locality A"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera prasinata</i>	Cahaba River at US HWY 52 (33.28449°N, 86.88257°W) "Locality B"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera prasinata</i>	Shades Creek (33.22013°N, 87.03323°W) "Locality C"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera prasinata</i>	Cahaba River near Bibb and Shelby County line (33.16981°N, 87.01949°W) "Locality D"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera prasinata</i>	Little Cahaba River (33.05407°N, 86.96919°W) "Locality E"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera prasinata</i>	Cahaba River @ US HWY 82 (32.95782°N, 87.13971°W) "Locality F"	To be assigned	To be assigned	To be assigned	To be assigned
<i>Pleurocera pyrenella</i>	Paint Rock River (34.6873°N 86.3102°W) "Locality A"	To be	To be	To be	To be assigned

assigned

assigned

assigned

Figure 3.1: Bayesian phylogram constructed using partial COI and 16S sequences with outgroups removed. Letter codes refer to sampling localities (see Fig. 3.3). Clades in bold indicate divergent haplotypes. Asterisks indicate posterior probabilities > 95%.

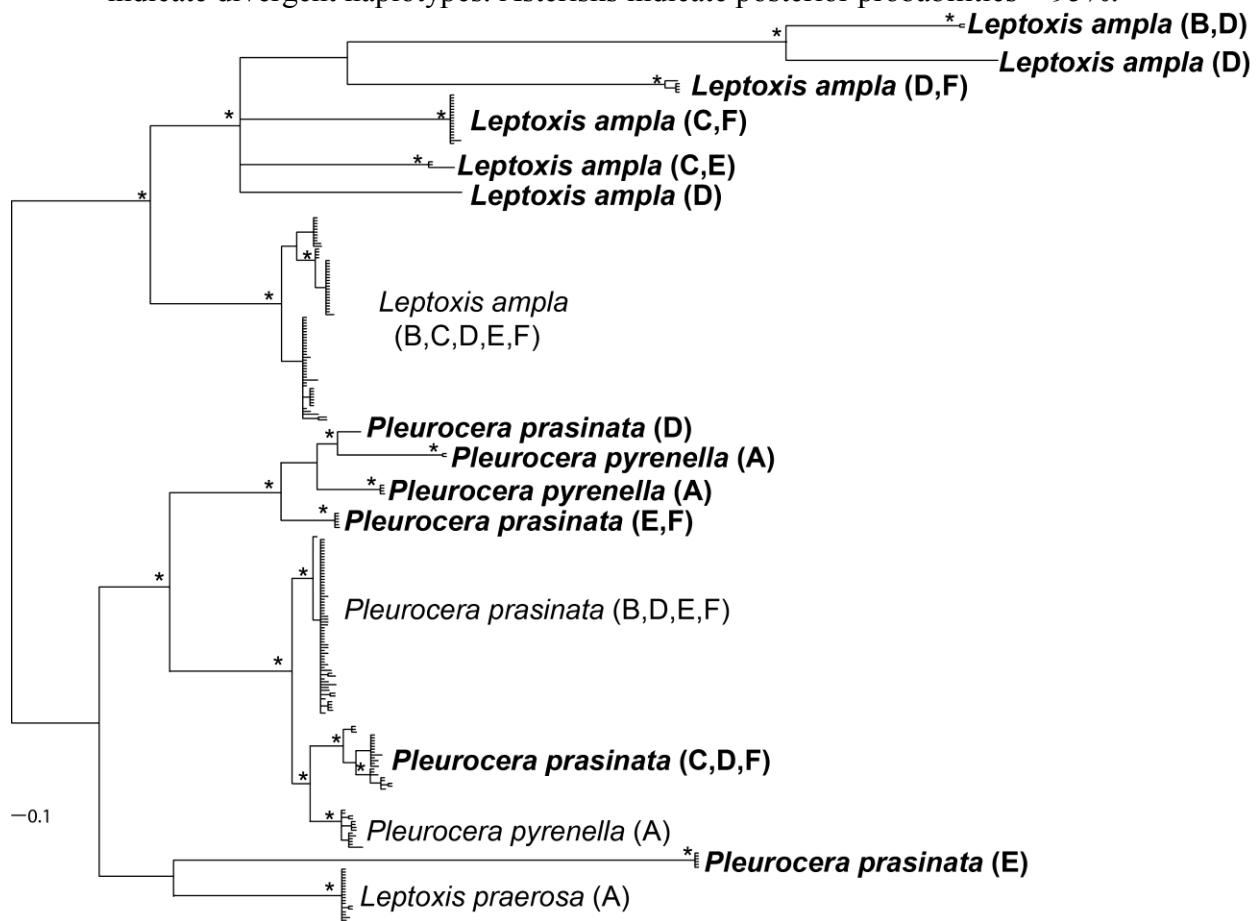


Figure 3.2: Bayesian phylogram constructed using partial H3 sequences with outgroups removed. Letter codes refer to sampling localities (see Fig. 3.3). Asterisks indicate posterior probabilities > 95%.

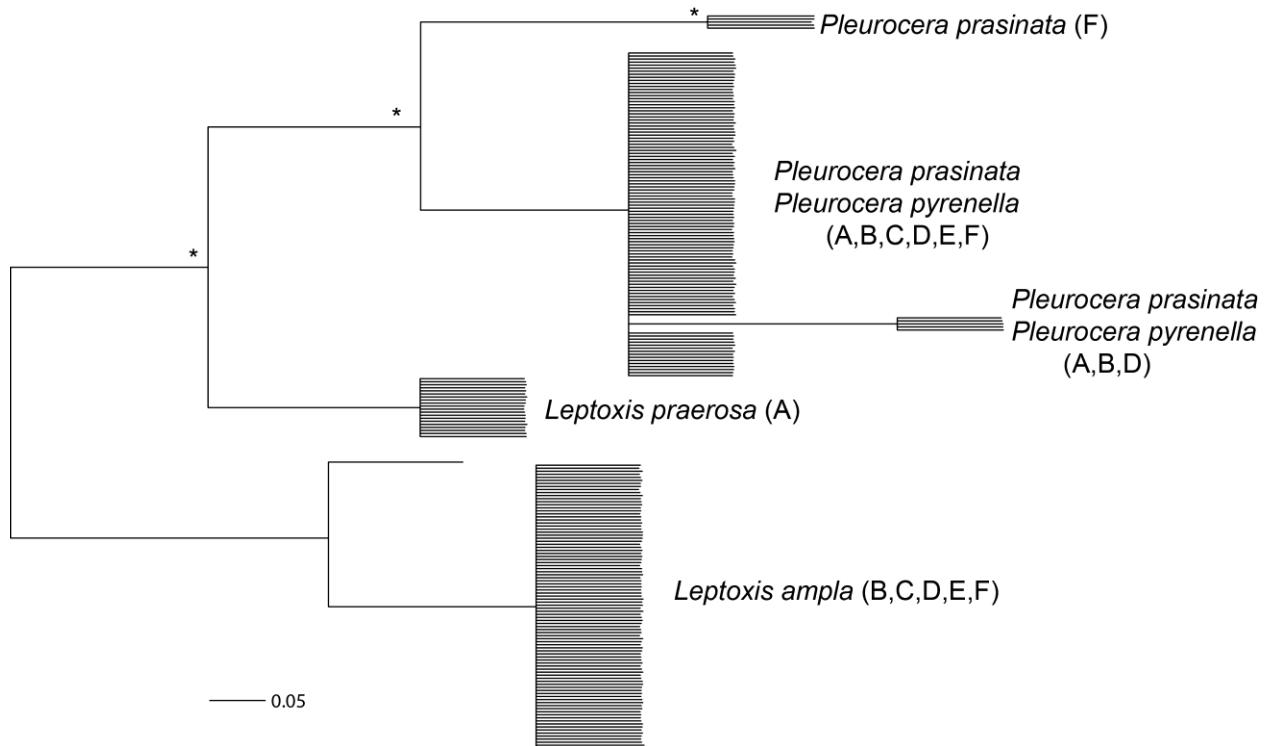
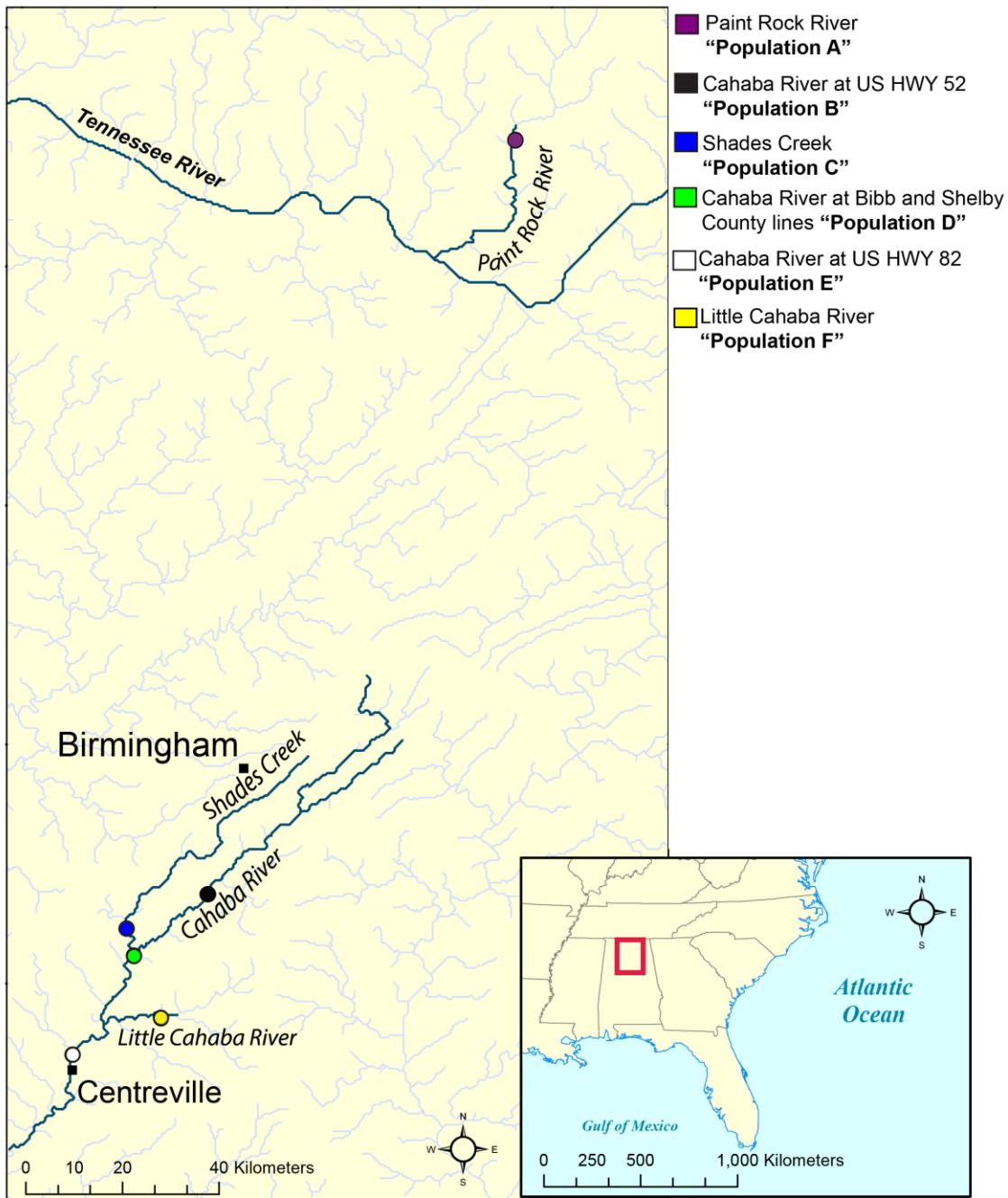


Figure 3.3: Sampling localities for newly generated data. Population letter codes are referenced in Figures 3.1 and 3.2.



Appendix

Table 3.A1: GenBank accession numbers of NUMTs from previous studies

Lydeard *et al.* (1997)

U73770
U73769

Holznagel and Lydeard (2000)

U73770
U73769
AF100998
AF101002

Minton and Lydeard (2003)

AF435747
AF435748
AF435756
AF435761
AF435762

Minton *et al.* (2003); Strong and Kohler (2009)

U73769
U73770
AF100998
AF101002

Dillon and Frankis (2004)

AY063465
AY063467
AY063468
AY063471
AY063472
AY063473

Minton *et al.* (2005)

AF469637
AF469638
AF469639

AF469645
AF469646
AY063464
AY063465
AY063466
AY063467
AY063468
DQ133383
DQ133386
DQ133390
DQ133395
DQ133397
DQ133399

Lee *et al.* (2006)

U73769
U73770
AF100998
AF101002
DQ311122
DQ311123

Lee *et al.* (2007);
(2013)

AY010525
DQ319914
DQ319921
DQ319927
DQ319929
DQ319934
DQ319935
DQ319937
DQ319940

Muirra *et al.*

Dillon and Robinson (2009)

AY063465
AY063466
AY063467
EU414645
EU414667
EU414668
EU414672

Figure 3.A1: ML phylogram of Minton and Lydeard (2003) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.

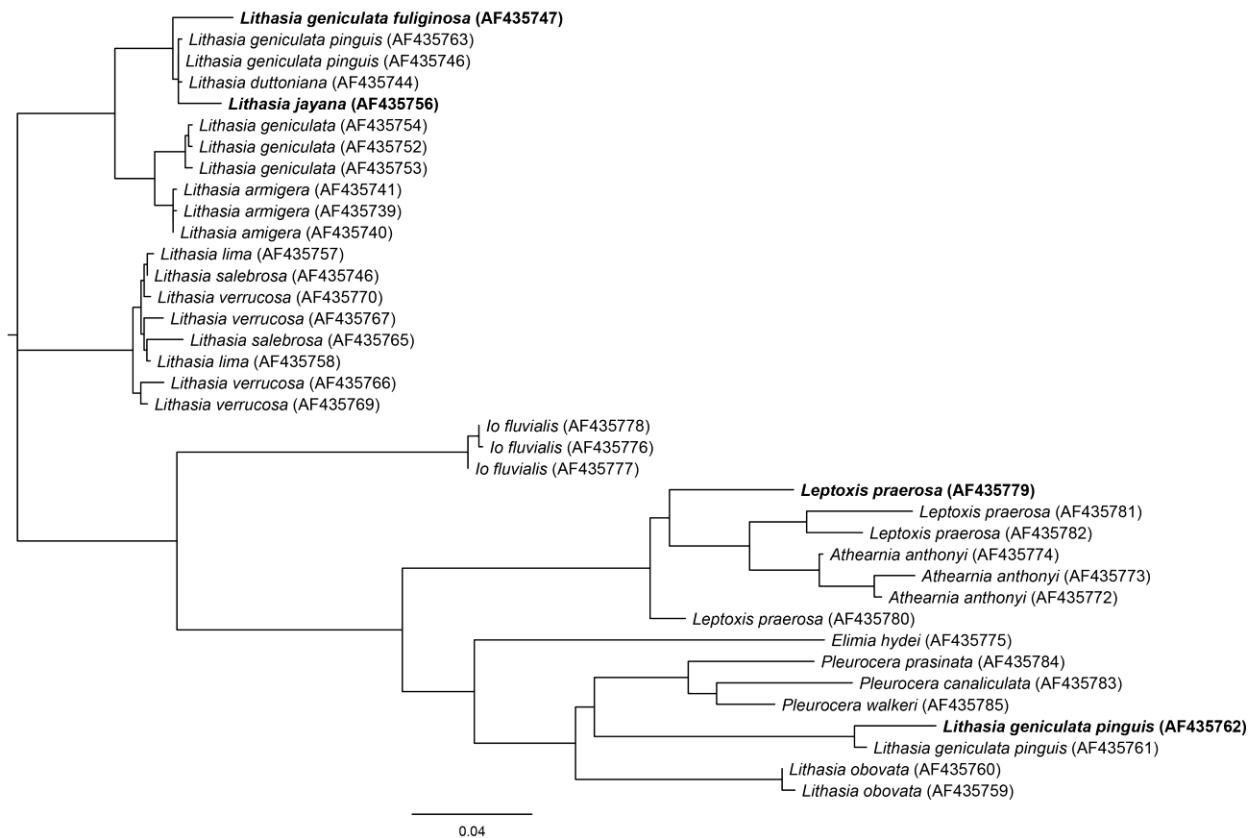


Figure 3.A2: ML phylogram of Dillon and Frankis (2004) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.

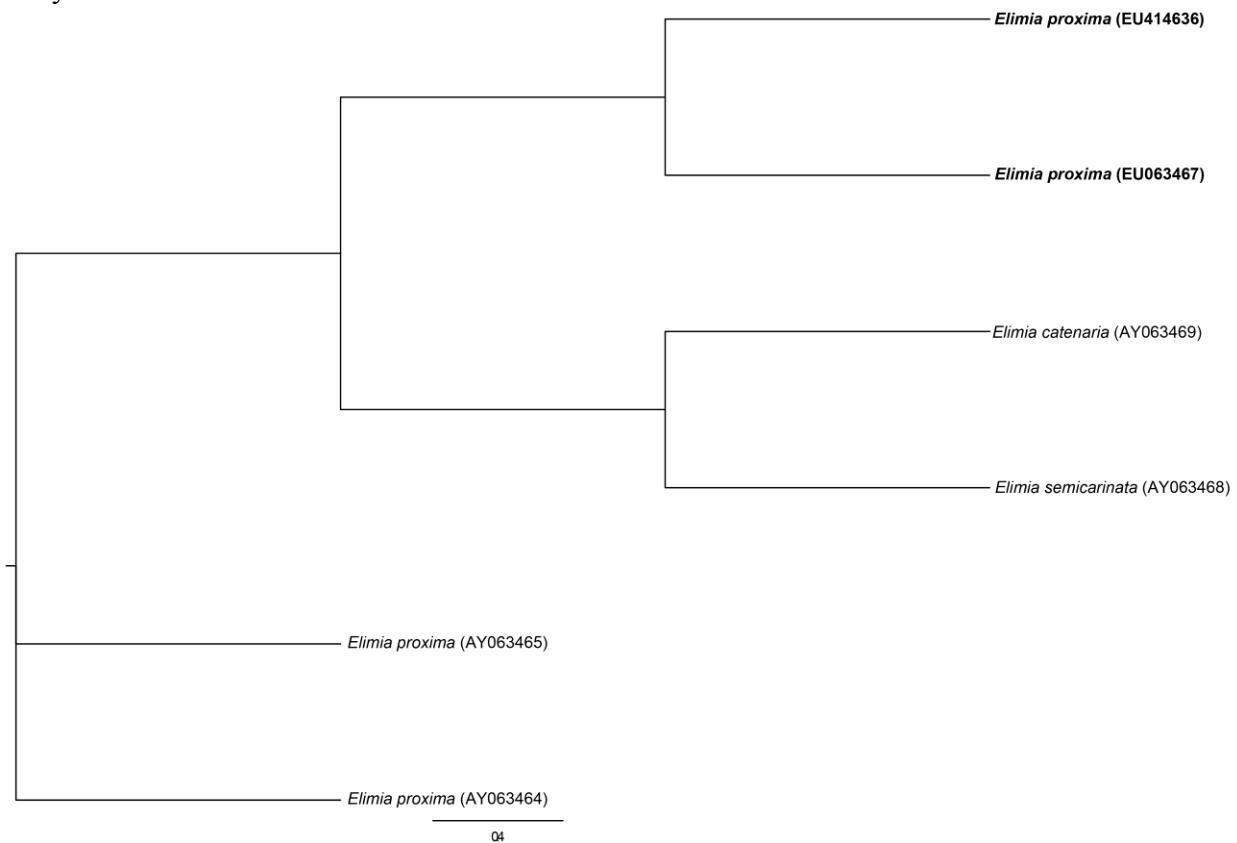


Figure 3.A3: ML phylogram of Sides (2005) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.

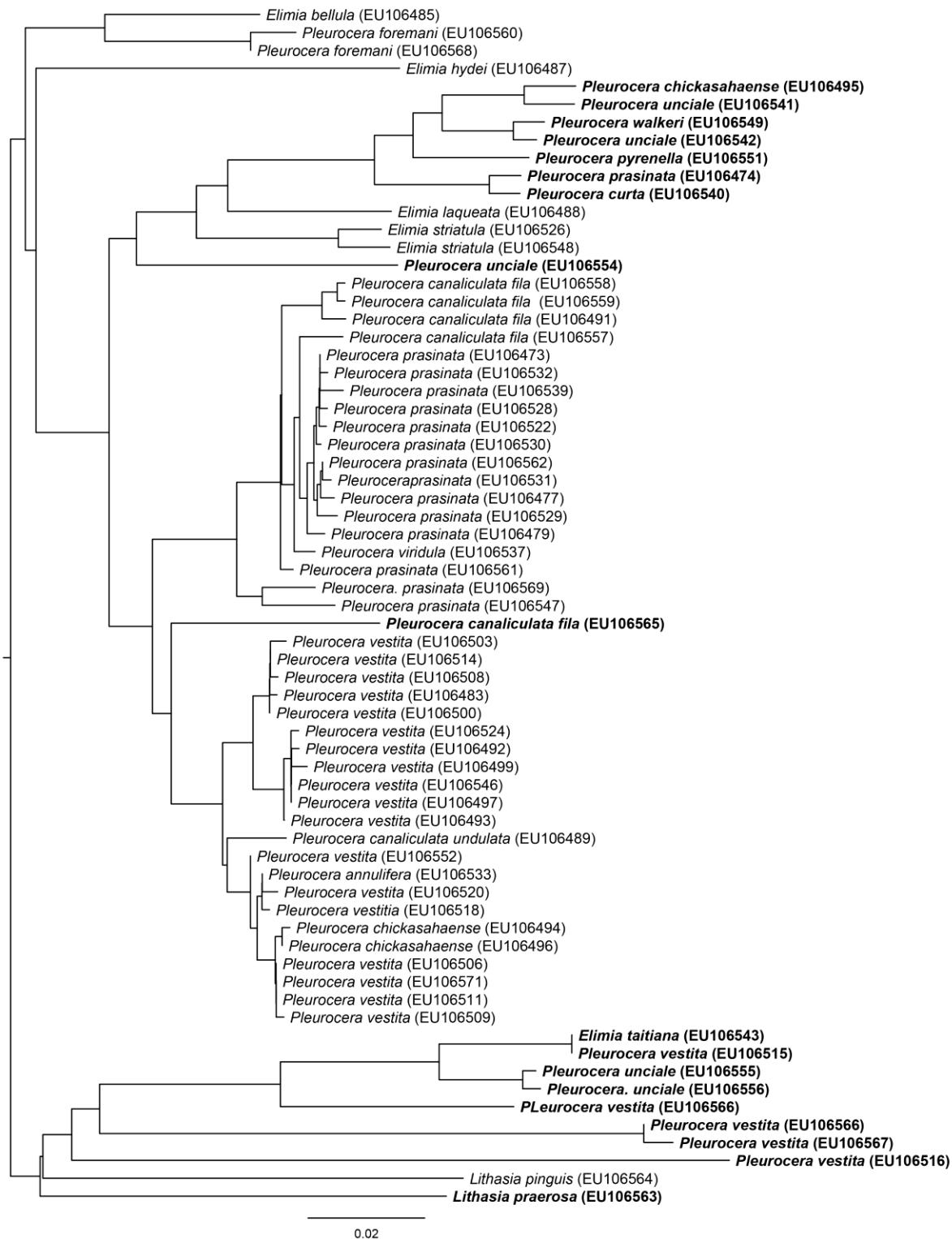


Figure 3.A4: ML phylogram of Minton et al. (2005) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.

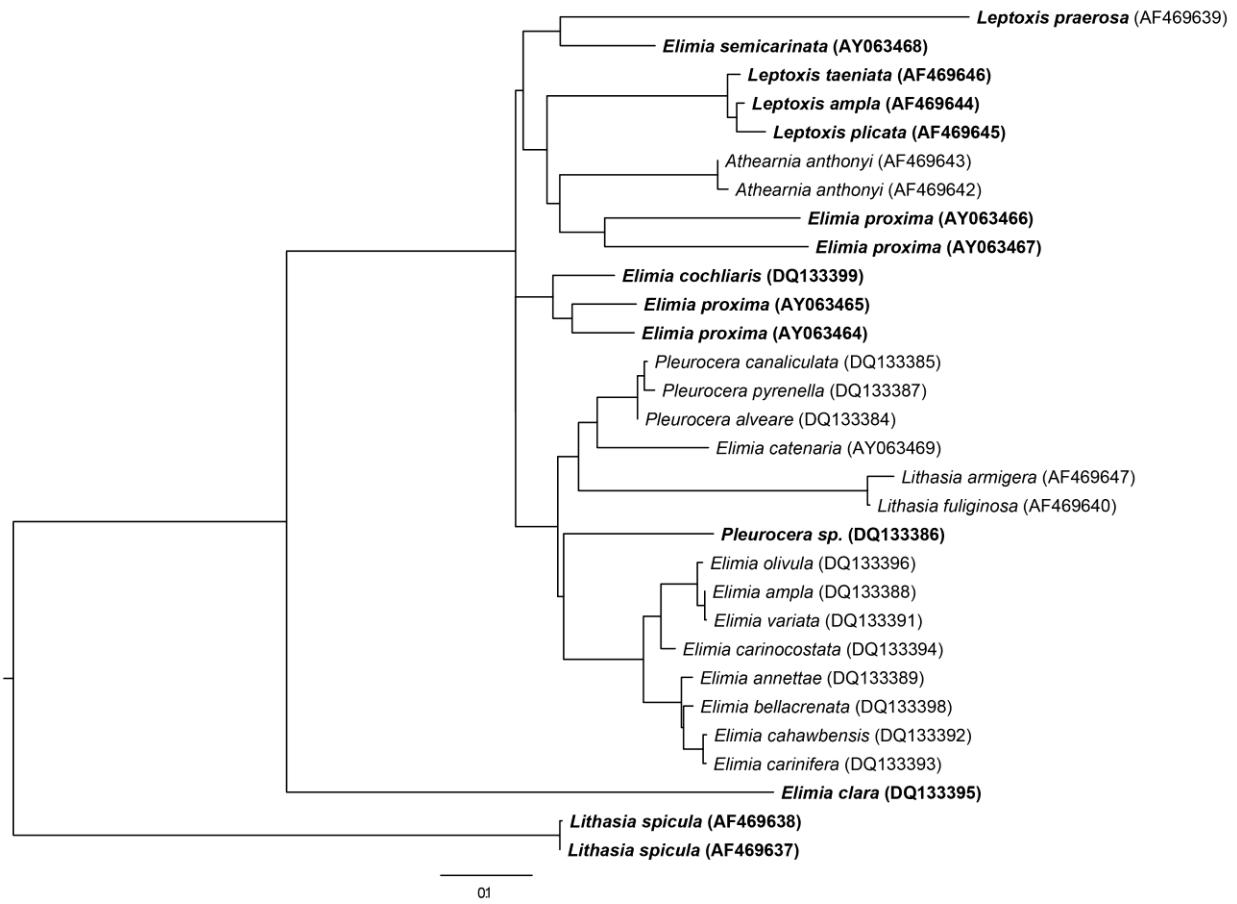


Figure 3.A5: ML phylogram of Dillon and Robinson (2009) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.

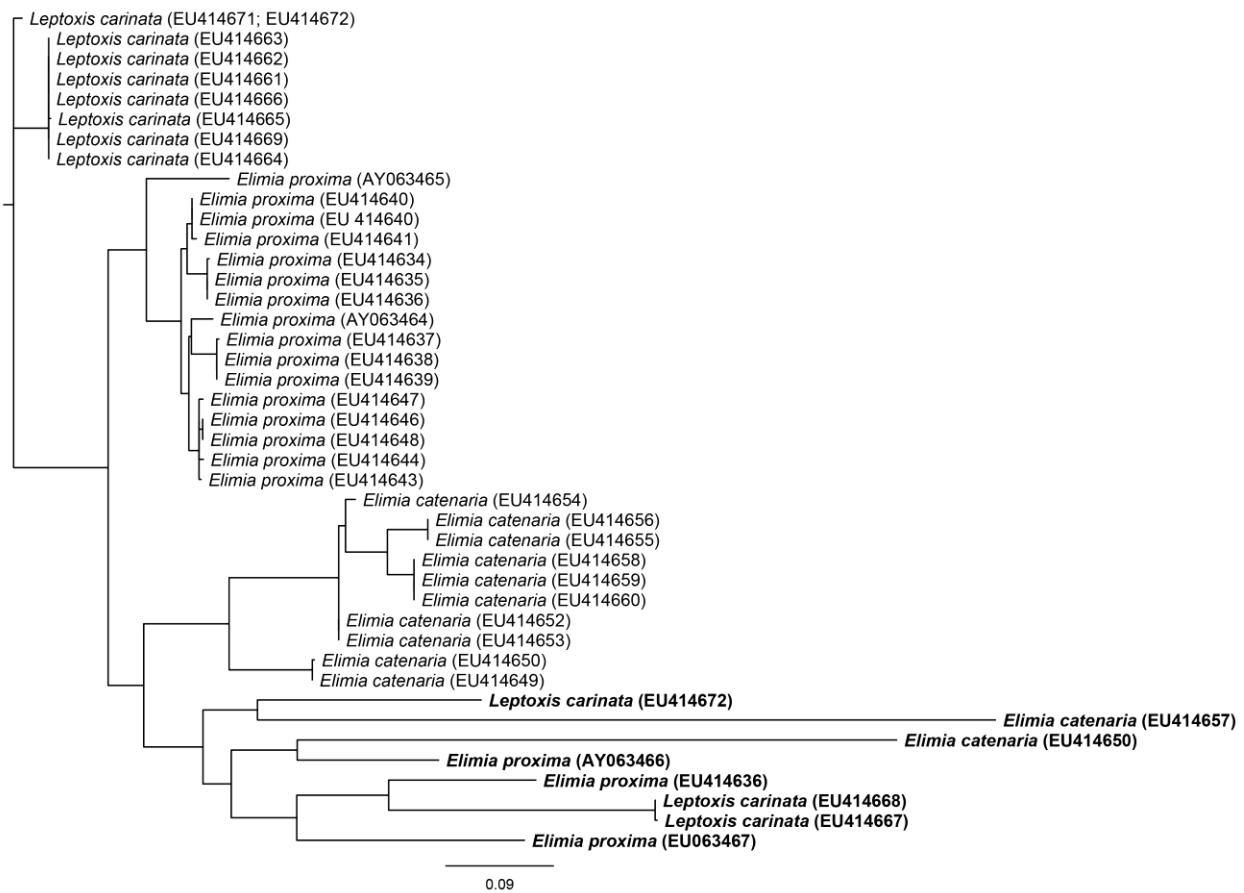
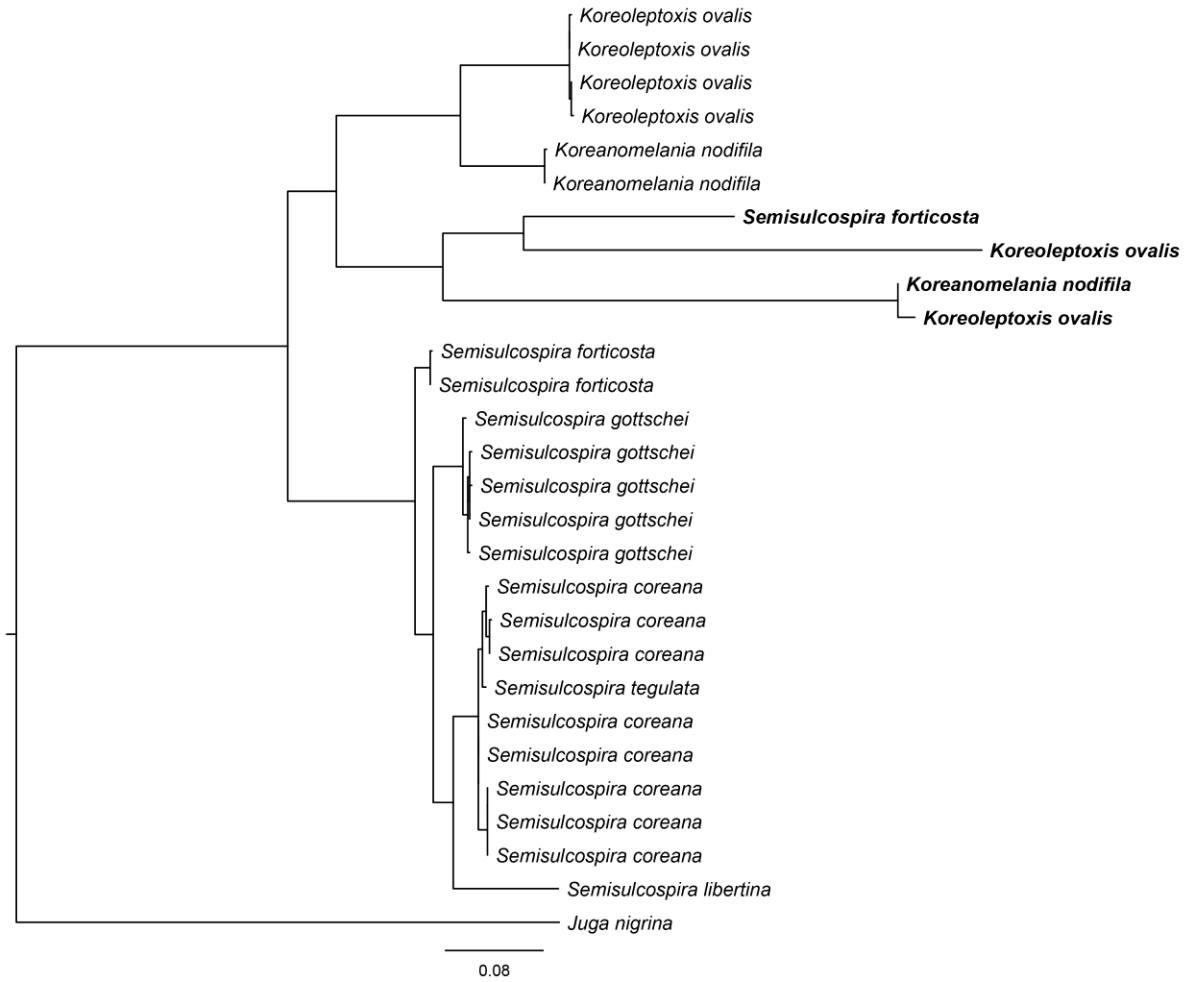


Figure 3.A6: ML phylogram of Kim et al. (2010) used as starting tree in PAML analyses. Bold taxa are inferred NUMTs.



CHAPTER 4

EGG-LAYING BEHAVIORS OF *LEPTOXIS* RAFINESQUE, 1819 (GASTROPODA: CERITHIOIDEA: PLEUROCERIDAE)

Abstract

A species overall fitness is directly influenced by life history since it is directly responsible for behaviors essential for survival and recruitment. However, we lack basic data on the life history of most freshwater snails. The imperiled pleurocerid genus *Leptoxis* is of special conservation concern, but almost nothing is known about *Leptoxis* life history. In this study, we document qualitative egg-laying behaviors and conchological growth of all 13 extant *Leptoxis* species and we explore whether intraspecific shell variation is a result of phenotypic plasticity or genetic differences. Furthermore, we test the hypotheses that interspecific differences exist in clutch size among clutch-laying species and that there are not interpopulational differences in egg clutch size within species. *Leptoxis* species were found to lay eggs either singly, in a single egg line, or in circular clutches, and the temperature cues for initiating egg-laying varied from 12°C-26°C depending on species. There are significant differences in clutch size among clutch-laying species and between populations of *L. ampla* and *L. taeniata*. Furthermore, *L. foremani* one and two year old females lay significantly fewer eggs per clutch than females four years or older. Finally, discrete shell morphologies that are characteristic of any given species are genetically controlled and not an ecophenotypic response. Clutch egg-laying likely represents increased parental investment compared to other behaviors and clutches may provide protection from predation or passive dislodgement. Conservationists should use the documented

temperature cues to predict possible shifts in egg-laying period that could result from global climate change.

Introduction

Pleuroceridae is the second most diverse freshwater snail family in North America (~162 species), and these animals are often integral components of stream ecosystems (Brown and Johnson, 2004; Brown, Lang and Perez, 2008; Huryn, Koebel and Benke, 1994; Richardson, Scheiring and Brown, 1988). As a result of widespread human alteration of rivers, over 79% of species in the family are extinct or imperiled (Johnson, *et al.*, 2013). Pleurocerids are dioecious, reach sexual maturity in the wild after one or two years, and lay eggs during species-specific periods in the spring and/or summer (Aldridge, 1982; Brown and Johnson, 2004; Johnson, 2010; Johnson, 2010; Jones and Branson, 1964; Miller-Way and Way, 1989; Stimpson, 1864). Many pleurocerid life history characteristics such as the type of substrate required for egg-laying, period of oviposition, time from egg-laying to hatching, and temperature cues for the commencement of egg-laying are unknown for non-*Elimia* species. Pleurocerids are dioecious and eggs are internally fertilized, but males lack a penis and exact copulatory mechanisms have not been observed. Pleurocerid life histories appear to vary substantially among species, and these differences may have important bearing on life history evolution and conservation. For example, life span varies from two years in *Leptoxis carinata* to over six years in *Elimia* spp. (Aldridge, 1982; Huryn, *et al.*, 1994; Stiven and Walton, 1967); eggs may be laid singly, in clutches, or in lines (Dazo, 1965; Jones Jr. and Branson, 1964; Winsor, 1933), and both semelparity and iteroparity have been documented (Aldridge, 1982; Garner and Haggerty, 2010; Miller-Way and Way, 1989). However, no study has focused on patterns of variation across an entire pleurocerid genus (Burch, 1989).

Differences in life history behaviors are often phenotypic manifestations of genetic variation that can create or enhance reproductive barriers among populations (Lowry and Willis, 2010; Miyatake and Toru, 1999). If egg-laying behaviors like average clutch size and period of oviposition differ among pleurocerid populations and species, then such differences may have facilitated diversification. The required substrate and flow conditions for egg-laying and growth could also influence species and generic distribution patterns and how species respond to anthropogenic habitat modifications (Huryn, Benke and Ward, 1995). For example, certain egg-laying behaviors may be associated with stream size. Furthermore, it is unknown if greater parental investment in some species (e.g., laying clutches rather than single eggs) decreases overall fecundity in pleurocerids. Since many pleurocerid genera—including *Leptoxis*—appear to be paraphyletic (Holznagel and Lydeard, 2000; Minton, Garner and Lydeard, 2003; Strong and Köhler, 2009), differences in egg-laying behaviors could prove to be useful synapomorphies and autapomorphies for future systematic revisions. The current dearth of such information limits our understanding of this diverse and imperiled group.

The genus *Leptoxis* was once widespread in the southeastern United States, but ten of 23 species (we include all species recognized in Johnson *et al.* 2013 plus *L. subglobosa*) have gone extinct in the last 80 years, four are listed under the U.S. Endangered Species Act, and most others are imperiled to some degree (Johnson, *et al.*, 2013). Life history data can aid in predicting how *Leptoxis* will react to future habitat modification (Dudgeon, *et al.*, 2006; Strayer and Dudgeon, 2010). For example, females of several species begin oviposition in response to specific temperature or flow cues (Johnson, 2010; Johnson, 2010), and knowledge of these behaviors is fundamental for understanding potential responses to global climate change, dam

operation, or other factors. However, published life history studies are only available for *L. carinata* and *L. dilatata* (Aldridge, 1982; Miller-Way and Way, 1989; Winsor, 1933).

Juvenile morphology and conchological ontogeny (e.g., shell growth) is unknown for almost every *Leptoxis* species, which complicates efforts to monitor imperiled populations and determine if certain species are recruiting (Mobile River Basin Mollusk Restoration Committee 2010). This is especially true in the Mobile and Tennessee River basins where many pleurocerids sympatrically occur and juvenile morphology can differ greatly from adult morphology (Dillon and Ahlstedt, 1997). Furthermore, there has been recent debate in the literature about whether phenotypic plasticity or genetic variation is the primary cause of conchological variation of discrete characters (e.g., carinae) in pleurocerids (Dillon, 2011; Dillon, Jacquemin and Pyron, 2013; Whelan, Johnson and Harris, 2012). Growing juveniles in common environments is an ideal method for experimentally testing causes of intraspecific shell variation because if phenotypic plasticity causes shell variation then juveniles grown in near identical environments should all look similar rather than resembling their wild type parents.

In an effort to better understand *Leptoxis* and pleurocerid diversity we document egg-laying timing and behaviors of all 13 extant *Leptoxis* species. We present observational data from years of captive propagation as a part of an ongoing pleurocerid management plan (Mobile River Basin Mollusk Restoration Committee 2010), and we detail species life history differences among *Leptoxis* species. Conchological ontogeny is also documented to test if *Leptoxis* morphological variation results from phenotypic plasticity. Since clutch size is a quantifiable metric to test if species and populations differ in egg-laying behaviors we also test three hypotheses about clutch size of clutch-laying *Leptoxis* species: 1) interspecific differences in clutch size exist; 2) there is not an interpopulational effect on clutch size within species; 3) older

individuals lay larger clutches. Testing these hypotheses will allow for inferences about whether life history differences could have attributed to *Leptoxis* diversification and population dynamics.

Methods

We collected approximately 50 individuals from at least one population of each extant *Leptoxis* species and transported them to a captive propagation facility at the Alabama Aquatic Biodiversity Center in Marion, Alabama (AABC; Table 4.1). Sampling sites were as close to the type locality of each species as possible, but in some instances the species has been extirpated from the type locality or the published type locality is prohibitively vague (e.g. *L. ampla*: the state of Alabama; Anthony, 1855). We collected each species in the fall or winter of 2010 or 2011 before oviposition began, except for *L. foremani* and *L. compacta*. The restricted range and highly imperiled status of *L. foremani* required the use of individuals cultured at AABC. *Leptoxis compacta* was considered extinct when this study began, but it was re-discovered after the egg-laying season had begun in May 2011, and only a limited number of individuals were observed over a brief period (Whelan, Johnson and Harris, 2012). For *L. ampla* and *L. taeniata*, we collected individuals from two populations to test for among-population differences in clutch size.

We followed the gastropod culturing protocol developed by Whelan *et al.* (2012a) to observe egg-laying behavior and juvenile growth in captivity. The water supply was untreated well water that had passed through an aeration tower. All tanks were exposed to ambient temperature and natural lighting. Snails grazed on naturally occurring algae and food was not supplemented with an artificial source. All tanks had unglazed terra cotta tiles, which leaned diagonally against the side of the tank for additional egg-laying substrates. We initially held all species in clear, 20 L tanks with a single 18.6 L/min powerhead and 12 randomly selected

individuals of each species distributed among three replicate tanks. However, during the first season of observations (i.e., spring 2010) it became apparent that *Leptoxis* species that lay egg singly (see Results) required a larger tank and higher flow conditions to stimulate oviposition (Table 4.1). For these species, all individuals (~50) were placed in 492 L tanks with four 18.6 L/min powerheads, an airstone, and unglazed terra cotta tiles for additional egg-laying substrates. Four powerheads in one tank created higher flow conditions than in the small tanks, but species kept in smaller tanks will lay eggs in the same qualitative manner if placed in the 492 L tanks with high flow. Fifty individuals were used instead of three replicates of 12 because snails in larger tanks will sometimes not lay eggs if the snail density in each tank is not high enough (Johnson unpubl. data). Water temperatures were measured with Hobo Temp Loggers (Onset Computer Corporation) to document temperature cues for the onset of oviposition. Numerous studies examining snail behavior and growth in captivity have shown few differences from traits observed in the wild (Brown, 1979; Dazo, 1965; Hoverman and Relyea, 2007; Lakowitz, Brönmark and Nyström, 2008; Lam and Calow, 1988; Whelan, *et al.*, 2012), and the relatively homogenous conditions in our facility are analogous to a common garden experiment.

For clutch-laying species, the number of eggs per clutch was counted at least every five days, usually more frequently, throughout the oviposition period. Egg clutches were marked on the outside of each tank with a permanent marker to ensure they were not counted twice. To test the hypothesis that clutch size is related to age, we counted clutch sizes of different age classes of *L. foremani* that had been kept in separate tanks. Age classes observed were captively propagated 1, 2, and 4 year olds, and 5+ year old wild broodstock; the exact age of broodstock was unknown. We use age class instead of body size since older pleurocerids are generally larger (Huryn, *et al.*, 1994), and sexing individuals and tracing which clutch was laid by which female

is virtually impossible with known culturing techniques (e.g., isolated females will not lay eggs).

Age class clutch sizes were counted on a single day in early April, which is near the mid-point of *L. foremani*'s egg-laying period. Counting eggs on a single day removed confounding effects such as time during oviposition and temperature variation throughout the season.

The size of the female that lays an egg clutch may contribute to the overall size of the clutch, but as noted above matching individual females to each clutch is virtually impossible. Furthermore, one would expect inherent size difference among species. Nevertheless, population differences in average clutch size may be most attributable to differences in body size rather than population-level adaptations. Therefore, average body size of each *L. ampla* and *L. taeniata* population was measured by randomly collecting 33 individuals each at the river edge, quarter channel and middle channel at each population and measuring shell-width (SW) and aperture-height (AH) to the nearest hundredth millimeter using digital calipers and mass with a digital scale. Snails were air dried for approximately five minutes before weighing. The ratio of shell-width to aperture-height (SW/AH) was used for comparisons to decrease variability in measurements. After measurements, these snails were returned to the river since they are federally threatened.

To account for the potential effect of phylogeny on differences in clutch size among species, we constructed a phylogeny of clutch-laying *Leptoxis* species (e.g., Felsenstein, 1985; Revell, Harmon and Collar, 2008). Only clutch-laying species were included since non-clutch laying species would have been dropped from phylogenetic signal calculations and inferring a full *Leptoxis* phylogeny with adequate outgroup sampling is outside the scope of this study. Three partial genes (COI, 16S rRNA and Histone H3) were sequenced with published primers and methods (Tabel 4.A1; Colgan, *et al.*, 1998; Folmer, *et al.*, 1994; Palumbi, 1996). The

phylogeny was inferred with *BEAST in BEAST 1.7.1 (Drummond, *et al.*, 2012; Heled and Drummond, 2010) with a lognormal relaxed clock model, and an arbitrary root prior date of 25–35 mya. Blomberg's K (Blomberg, Garland and Ives, 2003) and Pagel's λ (Pagel, 1999) were calculated for log-transformed clutch size with the Phytools package (Revell, 2012) in R (R Development Team, 2011). These test statistics use the inferred phylogeny and a model of Brownian motion to determine if there is statistical non-independence among species traits resulting from their relatedness. Both statistics failed to reject (Pagel's $\lambda = 0.360, p = 1.00$; Blomberg's K = 0.059, $p = 0.813$) phylogenetic independence in the data.

Given the lack of phylogenetic signal in clutch size, differences among species were tested using a nested ANOVA with the two *L. ampla* and *L. taeniata* populations nested within species and log-transformed clutch size. Tukey-Kramer post-hoc comparisons were then computed. A one-way ANOVA was also calculated for log-transformed clutch size of *L. foremani* age classes with Tamhane's T2 post-hoc comparisons. We tested for differences in body size and clutch size between the two populations of *L. ampla* and *L. taeniata* each with a *t*-test.

We evaluated differences in conchological ontogeny among species by culturing juveniles of most species for at least five months under the same conditions as described above. In particular, we took note of discrete conchological characters present in captively propagated juveniles and wild type parents of each species (e.g., carina, costae, plicae, an umbilicus) to determine a possible role of ecophenotypic plasticity in species-specific shell differences. No *L. carinata* or *L. subglobosa* juveniles survived in captivity, possibly because our culture facility is south of their native range and temperatures may have exceeded their upper thermal limit. Shell

series were deposited at the National Museum of Natural History, Washington D.C. (Table 4.A2).

Results

Gross egg-laying behaviors were invariant within species, but varied among species. For example, there were no differences in egg-laying behavior among replicate tanks of the same species, and we never observed a rogue clutch in a tank of single egg-laying species. *Leptoxis* species laid eggs in one of three patterns: a circular clutch, a line of eggs, and single eggs (Fig. 4.1; see subsequent). Eggs of all species were approximately 0.3 mm in diameter and were laid on the walls of tanks or on terra cotta tiles—usually on the underside—and sometimes on shells of other snails. Eggs were rarely, if ever, deposited on the flat bottom of tanks, and all species laid eggs below the water line, except *L. foremani*, which laid eggs at or slightly above or below the water line. Qualitative observations in the wild indicate that eggs are laid on clean, hard substrate (e.g., rocks without siltation or much vegetation) either on the underside or vertical side of cobble-boulder substrates. Eggs hatched about 14 d after oviposition. Regardless of oviposition pattern and/or species, a high percentage of eggs hatched (> about 98%). It is unknown if unhatched eggs were unfertilized or failed to develop for other reasons. Temperature appears to be a strong cue for oviposition, and the temperature at onset of oviposition varied widely among species but was generally concordant with oviposition pattern (Table 4.1). Species that laid eggs in clutches began oviposition at cooler temperatures than most other species; *L. foremani* had the lowest onset temperature and began laying eggs in February when the water temperature reached 12°C, but oviposition ceased temporarily when water temperature dropped below 10°C. With the exception of *L. arkansensis*, all other species laid eggs only at temperatures \geq 22°C, and *L. praerosa* did not begin oviposition until the temperature reached

26°C. The temperature at onset of oviposition did not differ among populations of *L. ampla* and *L. taeniata*. After egg-laying was initiated, all species generally continued to lay eggs for 60-90 days. With the exception of *L. foremani*, which ceased oviposition at 22°C, the temperature at which egg-laying ceased was similar among species and showed no concordance with oviposition pattern.

Clutch-laying species

As a result of culturing difficulties, the environments for all *Leptoxis* species were not completely identical (e.g., size of tank, density of snails, total current), but all clutch laying species were exposed to identical culture regimes. Therefore, statistical comparisons of clutch size are independent of immediate environmental variables.

Leptoxis ampla, *L. taeniata*, *L. foremani*, and *L. picta* all laid eggs in discrete, circular clutches, but the patterns of egg deposition varied among species. *Leptoxis ampla* and *L. taeniata* deposited eggs in concentric rings with one or two central eggs; smaller clutches were triangular (Fig. 4.1A, B). *Leptoxis foremani* and *L. picta* deposited eggs in a spiral pattern (Fig. 4.1D). Clutches of *L. ampla* incorporated large amounts of mucus and external matter (e.g., algae or detritus) to surround each egg, but clutches of *L. taeniata*, *L. foremani* and *L. picta* were held together with much mucus and little or no external matter (Fig. 4.1A, B, D).

Clutch size differed significantly among species (nested-ANOVA, $F_{(\text{among groups})} = 20.1397$, 3 df, $p = 0.0477$; Fig. 4.2) with 26.84% of the variance explained among species. Post-hoc comparisons showed significant differences among all groups except *L. foremani* and *L. ampla*. Clutch size differed significantly among age classes of *L. foremani* (ANOVA, $F = 48.818$, 3 df, $p < 0.001$; Fig. 4.2) with 42.9% of the variance explained among age classes. Post-hoc pairwise comparisons showed significantly higher clutch size for 4 and 5+ yr old individuals

compared to 1 and 2 yr olds. Note the smaller average clutch size depicted for *L. foremani* on Fig. 4.2A compared with Figure 4.2B results from inclusion of smaller clutches laid near the beginning and end of the egg-laying season as opposed to measurements made on a single day during the peak of oviposition.

The average snail SW/AH in the Cahaba River and Little Cahaba River populations of *L. ampla* were 1.05 and 1.03 respectively. The average snail mass of the Cahaba River and Little Cahaba River populations were 0.42 grams and 0.53 grams respectively. There was not a significant difference in SW/AH between *L. ampla* populations (*t*-test, $t = -1.904$, 197 df, $p = 0.587$) but there was a significant difference in snail mass between populations (*t*-test, $t = 3.771$, 197 df, $p < 0.001$). Average clutch size of the *L. ampla* Cahaba River population is 7.3 eggs (N = 536 clutches), and average clutch size of the *L. ampla* Little Cahaba River population is 6.6 eggs (N = 542 clutches). There was a significant difference in clutch size between *L. ampla* populations (*t*-test, $t = -5.370$, $df = 1076$, $p < 0.001$). Average SW/AH for the *L. taeniata* Buxahatchee Creek population and Choccolocco Creek population were 1.18 and 1.06 respectively, and average mass for each population was 0.44 grams and 0.26 grams respectively. Both were significantly different between populations (SW/AH: *t*-test, $t = 12.819$, 197 df, $p < 0.001$; mass: *t*-test, $t = 11.748$, 197 df, $p < 0.001$). Average clutch size of the *L. taeniata* Buxahatchee Creek population is 5.0 eggs (N = 13 clutches), and the average clutch size of the *L. taeniata* Choccolocco Creek population is 3.6 eggs (N = 380 clutches). There was a significant difference in clutch size between *L. taeniata* populations (*t*-test, $t = -4.412$, 391 df, $p < 0.001$).

Single egg-laying species

Leptoxis praerosa, *L. umbilicata*, *L. virgata*, and *L. subglobosa* all laid eggs singly in a random manner. *Leptoxis plicata* also usually laid eggs singly, but occasionally deposited two

eggs in close proximity. *Leptoxis arkansensis* displayed an unusual variant of the single egg-laying strategy. Females held a mass of eggs behind their foot and drug the mass behind them as they deposited single eggs intermittently from the mass (herein termed clutch-dragging; Fig. 4.3). This behavior is only visible if the female is crawling on a clear surface, and would be virtually impossible to observe in the wild. For all single egg-laying species except *L. arkansensis*, strong current, large tanks, and at least 50 individuals per tank were required for captive propagation since they would not oviposit when exposed to other culturing procedures.

Egg-line laying species

Leptoxis compacta, *L. dilatata*, and *L. carinata* all laid eggs in a non-random line pattern (Fig. 4.1C). Individual eggs laid in a line were not connected as a cohesive unit with mucus or other organic matter.

Conchological morphology

Juvenile *Leptoxis* conchological morphologies are roughly similar to adult morphologies in each species with notable exceptions (Figs. 4.4-4.6). *Leptoxis foremani* develops costae (i.e., shallow ribs) as they grow (Fig. 4.4C). *Leptoxis plicata* develops characteristic plicae at the top of the first body whorl as they grow (Fig. 4.5E). *Leptoxis compacta* and *L. carinata* lose juvenile carinae as they grow (Fig. 4.6A,B). *Leptoxis praerosa* juveniles lose their umbilicus as they age but *L. umbilicata* juveniles retain their umbilicus. Snails grown in culture do not undergo decollation (i.e., the erosion of upper shell spires). This absence or reduced decollation in captively reared individuals is maintained after being released in the wild. This is useful for monitoring reintroduced populations of imperiled species since tags are impractical for juveniles (Johnson, 2010). Of note is the similarity of *L. taeniata* juveniles (Fig. 4B) and *L. picta* juveniles (Fig. 4D), which may explain spurious records of *L. taeniata* from the Alabama River (Goodrich,

1922). Overall, phenotypic plasticity does not influence discrete intraspecific shell characters (e.g., a carina in juvenile *L. carinata*, plicae in *L. plicata*, costae in *L. foremani*, a umbilicus in *L. umbilicata*) nor periostracum color since juveniles of different species have different and characteristic morphologies when grown in near identical environments.

Discussion

The diversity of egg-laying behaviors within *Leptoxis* is notable for freshwater snails. Previous analyses of gastropod life history failed to find species-specific difference, and Brown (1983) hypothesized that life history traits may be constrained below the family level. This is clearly not the case in Pleuroceridae. However, *Leptoxis* is likely not monophyletic (Holznagel and Lydeard, 2000; Strong and Köhler, 2009), which obfuscates conclusions about genus-level diversity of egg-laying behaviors. There is clearly a need for comprehensive phylogenetic and taxonomic studies of pleurocerids to fully understand *Leptoxis* taxonomy, diversity, and life history evolution.

Our findings can be used to broadly inform conservation of *Leptoxis* and pleurocerids as a whole. Recent papers have proposed vast synonymizations of pleurocerids based on untested hypotheses of “cryptic phenotypic plasticity”(Dillon, 2011; Dillon, *et al.*, 2013), but our results reject phenotypic plasticity as a cause of conchological difference among currently recognized species—at least for *Leptoxis*. Species synonymizations of imperiled pleurocerids have broad implications for management units and should not be based on untested claims of phenotypic plasticity. Furthermore, when making critical habitat designations, egg-laying behaviors should be taken into consideration. Single egg-laying species seem to reproduce better in fast flowing environments, and clutch-laying *Leptoxis*, are all associated with larger rivers (Burch and Tottenham, 1980; Goodrich, 1921; Goodrich, 1922; Goodrich, 1940). This is the first study to

document a pleurocerid life history that correlates with habitat use, and it may be one reason there were so many Coosa River endemic *Leptoxis* before the river was extensively dammed. We put forth two hypotheses to explain the association of clutch-laying and higher order streams. First, embryos in clutches may need more nutrients in the surrounding water for nutrient exchange through large amounts of mucus. Second, higher order streams have more nutrients available to pleurocerids through foraging, which may provide for the increased energy needed per egg for constructing a clutch surrounded in mucus and other organic matter. Long-term *Leptoxis* survival will be dependent on large river rehabilitation and rivers with clean, fast-flowing waters.

Period of oviposition should inform monitoring techniques as considerable disturbance during egg-laying periods is best avoided for endangered species, and checking for recruits is best done after egg-laying has ceased. Moreover, in light of global climate change, annual temperature profiles of critical habitats should be monitored to predict temporal changes in future egg-laying periods. Little information exists about the upper thermal tolerance of pleurocerids, but species that start laying eggs at higher temperatures that are near potential thermal survival limits (i.e., 26°C for *L. praerosa*) may be especially susceptible to higher average stream temperatures. Considering the link between period of oviposition and water temperature, egg-laying periods will vary with rising average temperatures, and the associated ecosystem wide consequences of these inevitable shifts could be dramatic.

The greater clutch size of older *L. foremani* suggests that *Leptoxis* population dynamics are influenced by demography. However, it cannot be ruled out that younger individuals lay smaller clutches but as many eggs in total as older individuals. Since older pleurocerids are always going to be larger than younger individuals on average (Aldridge, 1982; Huryn, *et al.*,

1994; Miller-Way and Way, 1989), body size, rather than just age, also likely influences larger clutch sizes. A possible explanation for larger clutch sizes in older/larger individuals is that older individuals may allocate more resources for reproduction than younger individuals since they would need to divert fewer resources to building body mass than in previous years. Considering that there was not a significant difference observed between 4 years and 5+ years there may be an upper limit to the average clutch size seen in clutch-laying *Leptoxis* species, but is also likely an upper size limit of female *L. foremani*.

Among clutch-laying *Leptoxis* species, there is a clear difference in average egg clutch size among clutch-laying *Leptoxis* species. Furthermore, egg clutch size was statistically independent of phylogenetic history, which suggests that *Leptoxis* species may rapidly adapt to local environments to optimize clutch size (but see Revell, Harmon and Collar, 2008). For population-level comparisons of *L. ampla* and *L. taeniata*, the actual difference in egg clutch size ($\Delta = 0.7$ and 1.4 respectively) may not represent a biologically significant differences despite being statistically significant. Larger body size may explain the larger clutch size in the *L. taeniata* Buxatchee Creek population since individuals are on average larger there than at the Choccolocco Creek site. Interestingly, the larger *L. ampla* population—Cahaba River—laid smaller clutches.

Clutch-laying species likely expend more energy per egg since there must be costs to the mother associated with laying eggs in organized masses surrounded by mucus. The potential trade-off for this increased parental investment is lower egg mortality since the mucus and organic matter may provide some protection from predation and dislodgement from the substrate. For example, *L. ampla* clutches were virtually impossible to dislodge without applying considerable force, whereas single eggs could be detached from substrates with little effort. On

the other hand single egg individuals may lay more eggs overall to offset the potential greater predation/environmental risk. Long term conservation efforts of AABC support this hypothesis: *L. picta*—a clutch laying species—produces approximately 10 juveniles for every individual in a tank, whereas *L. plicata*—a single egg-laying species—produces nearly 17 juveniles for every individual in a tank (Johnson unpubl. data).

The most unique behavior we observed was *L. arkansensis* clutch-dragging. Typically, pleurocerid eggs leave the pallial oviduct and travel down an egg groove along the right side of a female's foot before being deposited with the aid of an ovipositor (Jones and Branson, 1964). In a process that we did not directly observe, *L. arkansensis* accumulated eggs in mass behind the foot prior to depositing them on a hard substrate. Different *L. arkansensis* females were observed dragging clutches of different sizes, so this was not a rogue behavior of a single female. A potential explanation for this behavior is that it improves locality selection for egg deposition. We are unaware of any other snail that has a clutch-dragging behavior. However, since this behavior is virtually impossible to observe in the wild, it could be more common than currently known.

Both semelparity and iteroparity have been reported in *Leptoxis*, and we find evidence for both lifestyles. *Leptoxis carinata* and *L. dilatata* have been reported to be semelparous biennial species that first lay eggs at 23 months (Aldridge, 1982; Miller-Way and Way, 1989). We randomly sampled *L. carinata* and *L. dilatata* from the wild and likely had both 1 year and two year cohorts in captivity, but most adults died after the egg-laying season indicating semelparity in both these species. Full life cycle data is unavailable for *L. arkansensis*, but this species also had near complete adult mortality after egg-laying and is likely semelparous. On the other hand, it is clear that *L. foremani* reproduces in multiple years as evidenced by the different age classes

analyzed in this study. Furthermore, ongoing propagation projects at AABC have shown that *L. ampla*, *L. taeniata*, *L. picta*, *L. plicata*, *L. praerosa*, and *L. virgata* reproduce for multiple years (Johnson unpubl. data). Interestingly, the three semelparous *Leptoxis* are all from more northern latitudes than other *Leptoxis* species, and semelparity has been reported in other northern ranging pleurocerids (e.g. *Pleurocera acuta* and *Elimia livescens*; Houpe, 1970; Payne, 1979). *Pleurocera acuta* is known to lay clutches (Dazo, 1965), so semelparity and iteroparity are not concordant with egg-laying behaviors. Semelparity may be advantageous for northern species since they are exposed to colder environments and shorter growth periods (Aldridge, 1982). A semelparous life cycle would allow for biomass accumulation in the first year and part of the second for winter survival with death occurring after resources are dedicated to reproduction rather than biomass accumulation for overwintering. Southern species are able to accumulate biomass in the warmer autumn months after reproduction, and they are exposed to milder winters. This possibly makes iteroparity a more viable life history strategy for southern species than for northern species. Given limited conservation resources, iteroparous *Leptoxis* may be better candidates for reintroduction efforts since surviving adults will reproduce for multiple years, which could increase the likelihood of establishing a self-supporting population and reduce the number of yearly releases needed for establishment.

The findings of this study are a critical first step in understanding the life history of an ecologically important and imperiled gastropod group. Qualitative behaviors documented here (i.e., gross egg-laying behaviors or semelparity vs. iteroparity) are unlikely to be different in the wild considering the lack of variation seen within species (e.g., no species characterized as laying single eggs ever laid a rogue clutch). Experimental studies of predation on pleurocerid eggs from crayfish, insects and fish are needed to further assess the potential advantages and

trade-offs of different egg-laying behaviors. Species differences in egg-laying behaviors and clutch size are likely evolutionarily significant (Stearns, 1977), and a full phylogeny of *Leptoxis* with adequate sampling of other pleurocerid genera will be integral to fully understanding the evolution of life history in *Leptoxis* and Pleuroceridae as a whole. Management agencies should be aware of the potential influence of anthropogenically induced habitat change (e.g., rising water temperatures, rapid changes in flow) and its potential to disrupt the natural egg-laying periods of *Leptoxis* and the success of offspring. We hope future studies will continue to make sense of *Leptoxis* diversity and explore how imperiled *Leptoxis* will respond to changing environments.

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Table 4.1: Collection localities, necessary culturing environment to induce egg-laying, egg-laying behavior, and the approximate water temperature that each species started and stopped laying eggs.

Species	Collection locality	Culturing environment	Egg-laying behavior	Approximate Start temperature	Approximate End temperature
<i>L. ampla</i>	Cahaba River: 33.0791°N 87.0678°W	Small tanks, low current	Clutch	14	27
	Little Cahaba River: 33.0537°N 87.0602°W				
<i>L. taeniata</i>	Choccolocco Creek: 33.5445°N 86.0413°W	Small tanks, low current	Clutch	14	27
	Buxahatchee Creek: 33.0727°N 86.6775°W				
<i>L. picta</i>	Alabama River: 32.3207°N 86.8217°W	Small tanks, low current	Clutch	20	27
<i>L. foremani</i>	Oostanula River: 34.4032°N 85.0971°W	Small tanks, low current	Clutch	12	22
<i>L. virgata</i>	Hiwassee River: 35.2195°N 84.5168°W	Large tanks, high current	Single eggs	23	27
<i>L. umbilicata</i>	East Fork of the Stones River: 35.8292°N 86.1784°W	Large tanks, high current	Single eggs	23	27
<i>L. subglobosa</i>	North Fork of the Holston River: 36.9534°N 81.5274°W	Large tanks, high current	Single eggs	23	27
<i>L. arkansensis</i>	Spring Creek: 36.8104°N 92.1474°W	Small tanks, low current	Dragged egg mass, deposited single eggs	13	27
<i>L. praerosa</i>	Limestone Creek: 34.7492°N 86.4414°W	Large tanks, high current	Single eggs	26	30
<i>L. compacta</i>	Cahaba River: 33.1786°N 87.0175°W	Small tanks, little current	Egg line	-	29

<i>L. plicata</i>	Locust Fork: 33.7245°N 86.9823°W	Small tanks, high current	Single eggs	24	29
<i>L. carinata</i>	Roanoke River: 37.2334°N 80.1981°W	Small tanks, high current	Egg line	22	27
<i>L. dilatata</i>	Indian Creek: 37.5151°N 80.7696°W	Small tanks, high current	Egg line	22	27

Figure 4.1: Egg-laying patterns of *Leptoxis*. Single eggs are not figured, but are similar to egg lines but spaced randomly. Scale bars = 0.3 mm A) Small *L. taeniata* egg clutch B) Flipped over *L. ampla* egg clutch showing surface normally attached to substrate C) Egg line D) Spiral egg clutch of *L. picta*. *L. foremani* egg clutches are indistinguishable from those of *L. picta*.

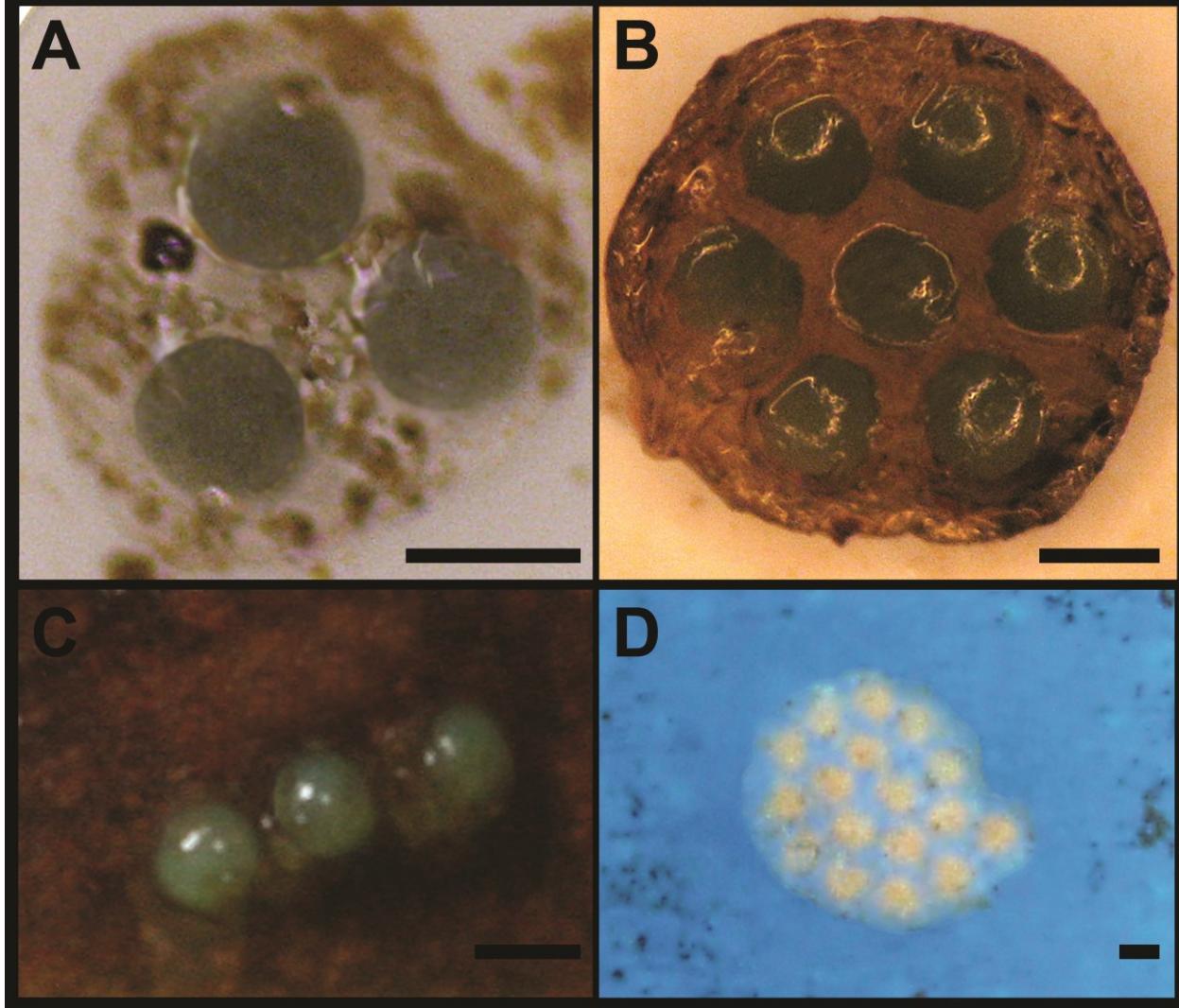


Figure 4.2: Bar graphs of log-transformed clutch size. Error bars are 95% confidence intervals. Bars with different letters above them had significantly different post-hoc pairwise comparisons. A) Bar plot for clutch laying species with *L. ampla* and *L. taeniata* split by populations. B) Bar plot for *L. foremani* age classes.

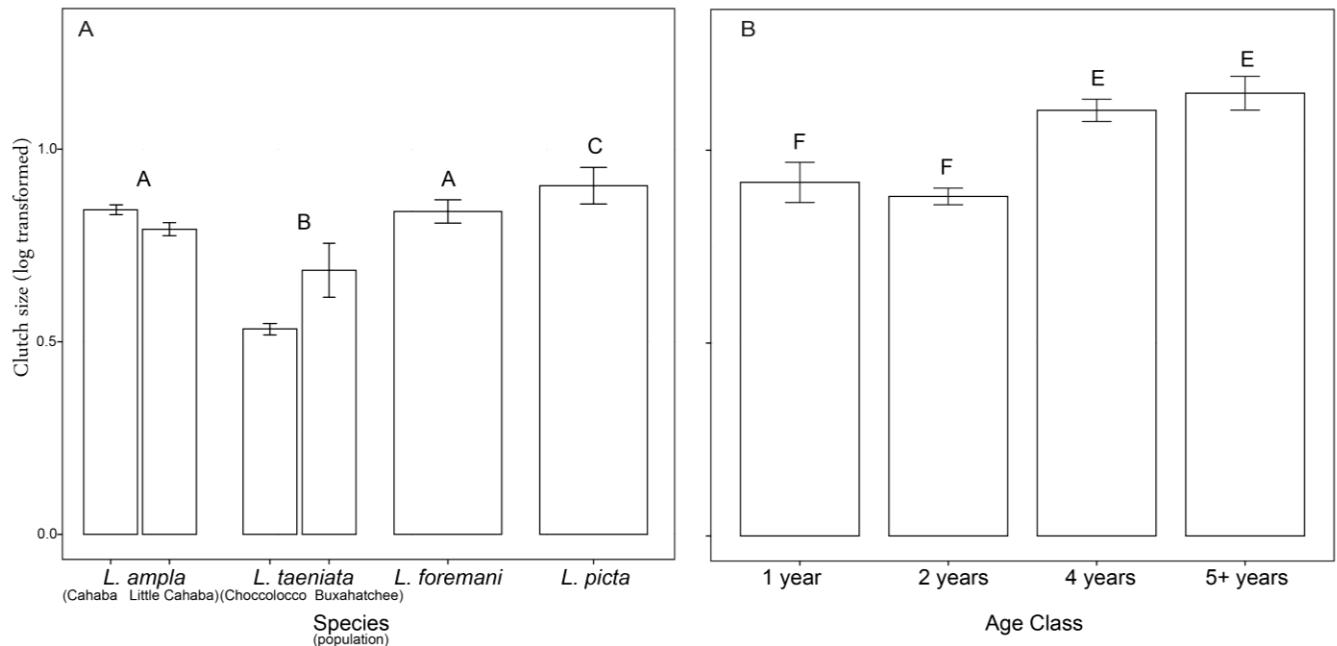


Figure 4.3: *Leptoxis arkansensis* female dragging group of eggs behind its foot.



Figure 4.4: Conchological growth series of clutch claying species. A) *L. ampla* B) *L. taeniata* C) *L. foremani* D) *L. picta*. Scale bar = 5 mm.



Figure 4.5: Conchological growth series of single egg-laying species. A) *L. virgata* B) *L. praerrosa* C) *L. subglobosa* (all wild collected individuals) D) *L. umbilicata* E) *L. plicata* F) *L. arkansensis*. Scale bar = 5 mm.

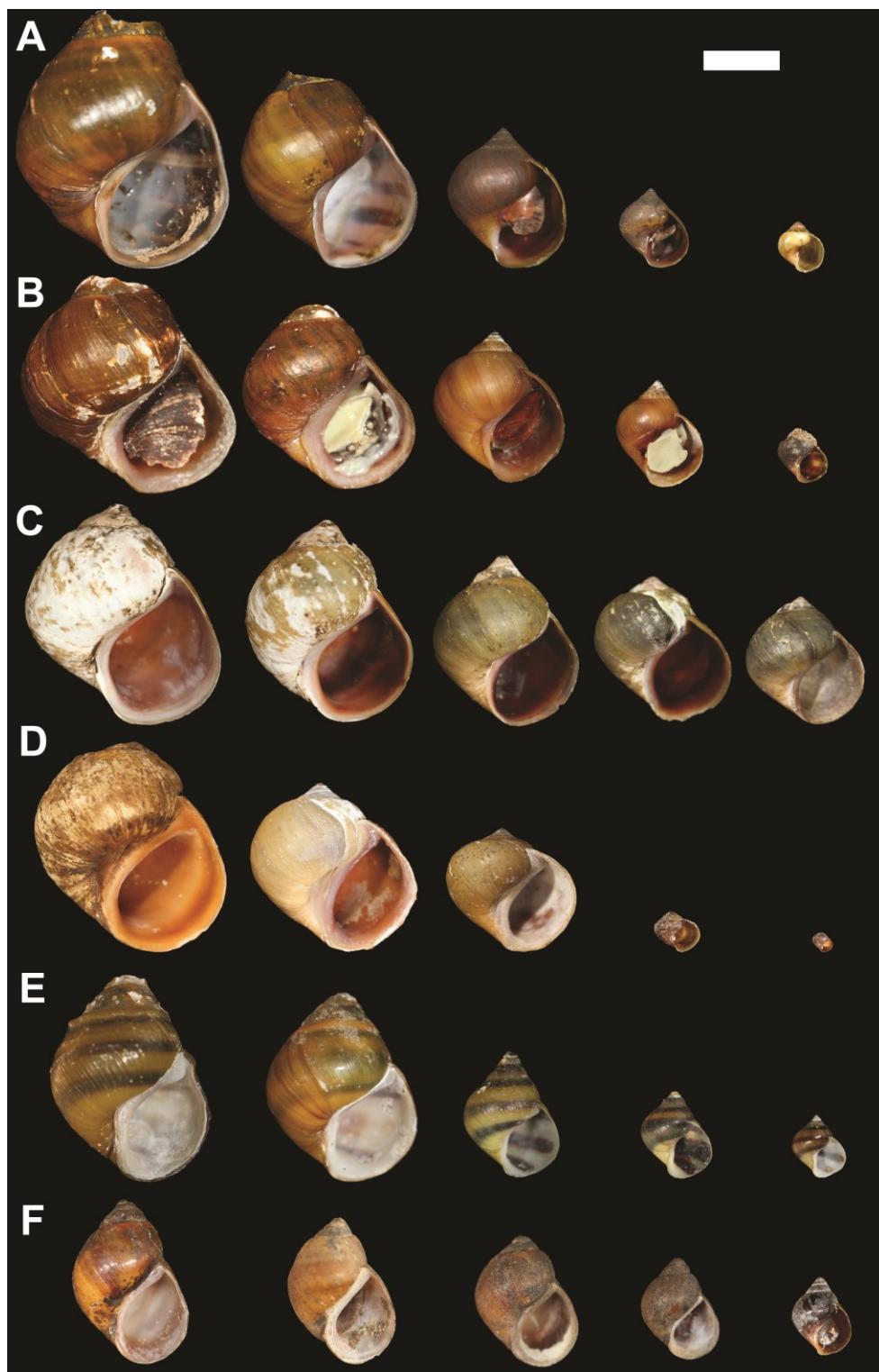
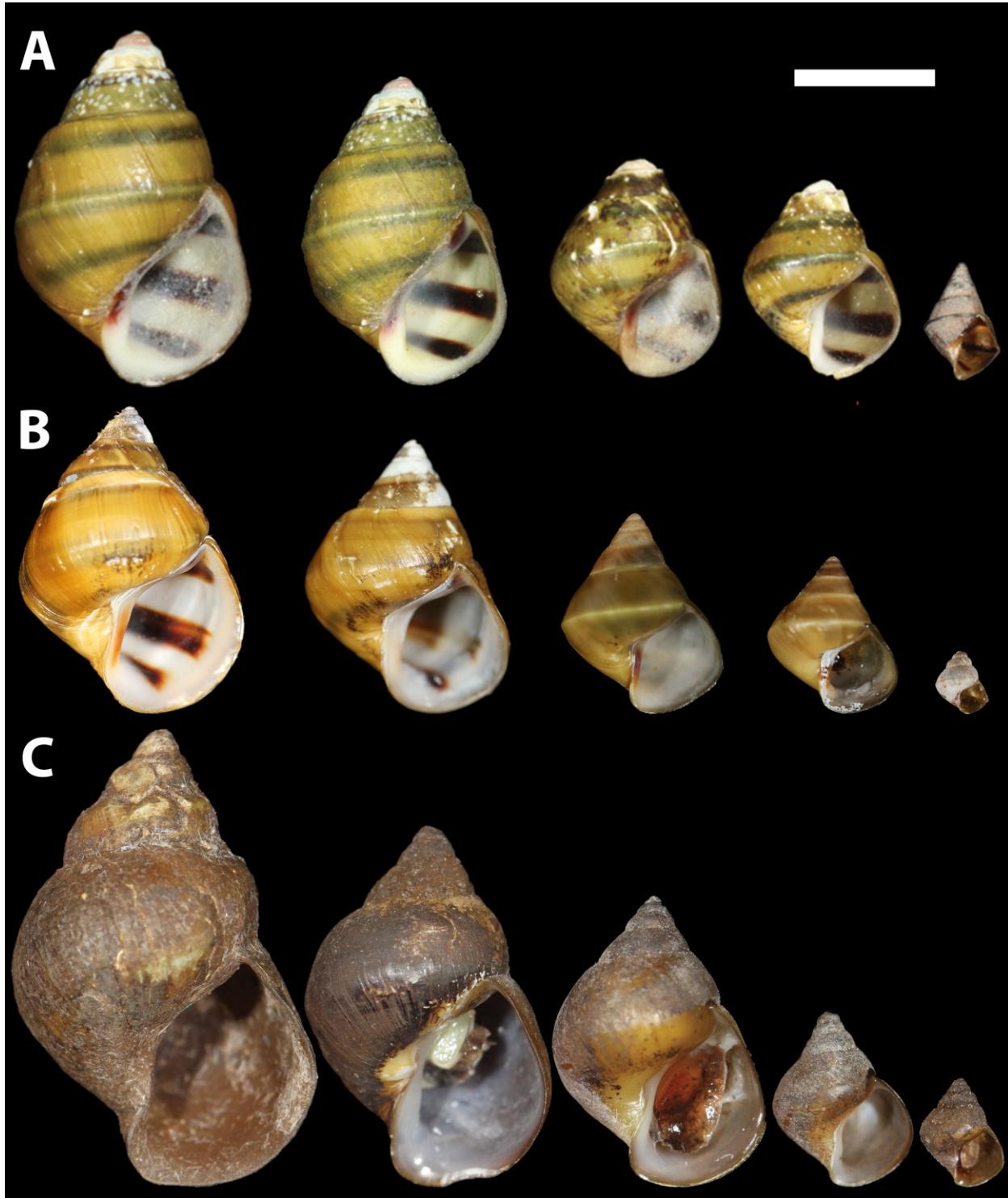


Figure 4.6: Figure 6: Conchological growth series of egg line laying species. A) *L. compacta* (adopted from Whelan *et al.* 2012) B) *L. dilatata* C) *L. carinata* (all wild collected individuals). Scale bar = 5 mm.



Appendix

Table 4.A1: Species, localities, GenBank numbers and voucher catalogue numbers of individuals used for phylogenetic inference.

Species	Locality	16S GenBank	COI GenBank	H3 GenBank	USNM Catalogue #
<i>L. ampla</i>	Cahaba River Little, Cahaba River	To be assigned	To be assigned	To be assigned	To be assigned
<i>L. taeniata</i>	Choccolocco Creek, Buxahatchee Creek	To be assigned	To be assigned	To be assigned	To be assigned
<i>L. picta</i>	Alabama River	To be assigned	To be assigned	To be assigned	To be assigned
<i>L. foremani</i>	Bred in captivity, brood stock from Oostanula River	To be assigned	To be assigned	To be assigned	To be assigned

Table 4.A2: Species and associated catalogue numbers for shells used in growth series figures.

Species	USNM catalogue # [^]
<i>Leptoxis ampla</i>	TBA
<i>Leptoxis arkansensis</i>	TBA
<i>Leptoxis carinata</i>	TBA
<i>Leptoxis compacta</i>	TBA
<i>Leptoxis dilatata</i>	TBA
<i>Leptoxis foremani</i>	TBA
<i>Leptoxis picta</i>	TBA
<i>Leptoxis plicata</i>	TBA
<i>Leptoxis praerosa</i>	TBA
<i>Leptoxis subglobosa</i>	TBA
<i>Leptoxis taeniata</i>	TBA
<i>Leptoxis umbilicata</i>	TBA
<i>Leptoxis virgata</i>	TBA

[^]To be assigned.

CHAPTER 5

SYSTEMATICS OF *LEPTOXIS* RAFINESQUE 1819 (GASTROPODA: CERITHIOIDEA: PLEUROCERIDAE)

Abstract

The freshwater gastropod family Pleuroceridae is one of the most imperiled groups of gastropods in North America. However, their chaotic taxonomy, which has been shown to not reflect phylogeny, prevents effective communication about the family and obscures efforts to robustly define management units. Currently, the systematics of pleurocerids is based on variable shell characters and outdated perspective on species diversity that rely heavily on geographic ranges rather than shared evolutionary history. Previous attempts to refine pleurocerid taxonomy have been rife with problems including the use of inappropriate data sets (e.g. allozymes), employing paralogous genes in phylogenetic inference, poor taxon sampling, and single locus approaches. Of particular interest is the genus *Leptoxis*, which has a nearly 91% imperilment rate. We employed a four gene molecular phylogenetic approach to explore species boundaries in *Leptoxis* and test the monophyly of the genus. We resolve *Leptoxis* as non-monophyletic. Furthermore we find strong support for resurrecting *L. subglobosa*, which is currently synonymized with *L. praerosa*. Three major *Leptoxis* clades are resolved, and we elevate one genus (*Alleghenya*) and describe a new genus (*Alatoxis*). This study is an important first step in establishing a base-line framework for future studies of pleurocerid systematics.

Introduction

Ground zero of the biodiversity crisis in North America is the rivers of the southeastern United States. The Coosa River alone—a hotspot of freshwater biodiversity—has seen at least ten mussel extinctions and 37 snail extinctions as a result of damming and other anthropogenic habitat destruction (Bogan, Pierson and Hartfield, 1995; Johnson, *et al.*, 2013; Lydeard and Mayden, 1995). This river is an extreme example, but the majority of extant freshwater fish and mollusk species across the southeastern US currently suffer from historical range declines and population fragmentation (Jelks, *et al.*, 2008; Johnson, *et al.*, 2013; Lydeard and Mayden, 1995; Neves, *et al.*, 1997; Williams, Bogan and Garner, 2008; Williams, *et al.*, 1993).

Pleuroceridae Fisher, 1885—the second most species rich family of gastropods in North America—is no exception. This family is most abundant in the southeastern United States, but its range extends throughout eastern North America (Johnson, *et al.*, 2013; Strong and Köhler, 2009). Pleurocerids suffer an imperilment rate of over 79%, and an entire genus—*Gyrotoma* Shuttleworth, 1845—has gone extinct in the past 70 years (Johnson, *et al.*, 2013). However, pleurocerids are integral to basic ecosystem processes (e.g. nutrient cycling and predator prey dynamics; Huryn, Benke and Ward, 1995; Richardson, Scheiring and Brown, 1988), and their decline has had cascading effects that are detrimental to other freshwater resources (Brown and Johnson, 2004; Brown, Lang and Perez, 2008). As such, they are of high conservation concern, but pleurocerid generic taxonomy is not concordant with phylogeny (Holznagel and Lydeard, 2000; Minton and Lydeard, 2003; Strong and Köhler, 2009), and species—the currency of conservation plans—are poorly defined (MRB Mollsuk Recovery Committee, 2010; Lysne, *et al.*, 2008; Perez and Minton, 2008).

Pleurocerid taxonomy is a 150+ year old problem resulting from vaguely and overdescribed species. There are over 800 nominal pleurocerids (Graf, 2001; Garner et. al., unpubl. data), but only 162 species are currently considered valid (Johnson, *et al.*, 2013). Complicating matters are vague type localities (e.g. *L. ampla* (Anthony, 1855): Alabama), type localities in drainages from which the species likely never occurred (e.g. *L. plicata* (Conrad, 1834): tributaries of the Tennessee River; *L. taeniata* (Conrad, 1834): Alabama River near Claiborne), or type localities from which the species is extirpated (e.g. *L. praerosa* (Say, 1821): the falls of the Ohio River). The first thorough systematic treatment of pleurocerids was completed by Tryon (1873), whom synonymized many of the species described by early malacologists. Tryon (1873) condensed some invalid generic names, but conjointly used junior synonyms (e.g. *Anculosae* Say, 1821 for *Leptoxis* Rafinesque, 1819 and *Goniobasis* Lea, 1862 for *Elimia* H. and A. Adams, 1854) or unavailable names (e.g. *Schizostoma* Lea, 1842 for *Gyrotoma*). The next extensive taxonomic treatments were those of Goodrich. Goodrich published over 35 papers on pleurocerids, but his synonymizations were based only on variable shell morphology and geography. Goodrich also frequently used junior synonyms (e.g. *Goniobasis* for *Elimia*) and unavailable generic names (e.g. *Nitocris* H. and A. Adams, 1854 for some *Leptoxis* spp.). In general, Goodrich's taxonomy reflects outdated perspectives of species and generic boundaries that do not conform with phylogeny (Holznagel and Lydeard, 2000; Minton and Lydeard, 2003).

After Goodrich and until the 1980s, only a handful of other pleurocerid systematic studies that had a narrow focus and limited impact were published (e.g. Clench, 1965; Clench and Boss, 1967; Clench and Turner, 1956). Burch and Tottenham (1980) published the basis of modern pleurocerid taxonomy: a list of species and the ranges of all North American freshwater snails. However, this work was never meant as a thorough systematic treatment, and it did not include a

phylogenetic hypothesis for pleurocerids. Even recently, authors continue to use junior synonyms (e.g. *Goniobasis* for *Elimia*; Dillon, 2000; Dillon and Robinson, 2009) and some have used untested hypotheses of phenotypic plasticity and similarities at allozyme loci to justify overreaching and absurd generic- and species-level taxonomic revisions (Dillon, 2011; Dillon, Jacquemin and Pyron, 2013). Species have even been described from paralogous nuclear copies of mitochondrial genes (NUMTs; Lopez, *et al.*, 1994) that were confused as mitochondrial homologs (Chapter 3; Minton, 2013; Minton, Savarese and Campbell, 2005). Clearly, pleurocerid taxonomy is in a sorry state, and a robust phylogenetic hypothesis for the family is needed.

The pleurocerid genus *Leptoxis* is in special need of attention since ten of 22 species have gone extinct in the past 70 years, and four are listed under the US Endangered Species Act (ESA; Johnson, *et al.*, 2013). Of the limited phylogenetic studies to date, none have resolved *Leptoxis* as monophyletic (Holznagel and Lydeard, 2000; Lydeard, *et al.*, 1997; Minton and Lydeard, 2003; Strong and Köhler, 2009), and species boundaries have also been questioned (Dillon and Lydeard, 1998). However, the conclusions that can be drawn from previous studies are limited since they did not include all *Leptoxis* species or adequate outgroup sampling to test the putative non-monophyly of *Leptoxis*. Furthermore, no previous molecular phylogenetic analysis has utilized more than one locus. Many studies have also included NUMTs as mitochondrial orthologs (Chapter 3). *Leptoxis* conservation plans would greatly benefit from a stable and natural taxonomy (Johnson, *et al.*, 2013; Lysne, *et al.*, 2008; Mace, 2004; Perez and Minton, 2008).

The current pleurocerid taxonomy undoubtedly results in misused conservation resources since it is impossible to effectively conserve species if the true extent of diversity is not

understood or taxonomy is ambiguous (MRB Mollusk Recovery Committee, 2010; Lysne, *et al.*, 2008; Mace, 2004; Perez and Minton, 2008). As one troubling example, consider the taxonomic status of *L. subglobosa* (Say, 1825). The Virginia Department of Conservation and Recreation considers *L. subglobosa* to be a synonym of *L. virgata* (Lea, 1841), whereas the Tennessee Department of Environment and Conservation considers *L. subglobosa* to be a valid species and *L. umbilicata* (Wetherby, 1876) a subspecies of *L. subglobosa*. Moreover, the published species lists of Johnson *et al.* (2013) and Burch and Tottenham (1980) consider *L. subglobosa* a synonym of *L. praerrosa* (Say, 1821) and *L. umbilicata* a valid species. If two states that share many watersheds cannot agree on a single taxonomy, what hope is there for communicating pleurocerid conservation at the national level or to the public?

In order to provide the first thorough phylogenetic hypothesis of *Leptoxis* and make necessary taxonomic revisions, we sampled all extant *Leptoxis* species and other pleurocerids that were previously considered in the genus. Species from other extant pleurocerid genera are included to test the putative non-monophyly of *Leptoxis*. This thorough sampling, coupled with sequencing of multiple loci, allows us to test the putative non-monophyly of *Leptoxis*, and explore *Leptoxis* species boundaries. A stable and natural *Leptoxis* taxonomy will enhance conservation efforts, and a robust phylogeny of *Leptoxis* will enhance other studies on this ecologically important genus.

Materials and Methods

Taxon sampling

We examined records at the Florida Museum of Natural History (FLMNH) in Gainesville, Florida, the National Museum of Natural History (USNM) in Washington D.C., and the Natural History Museum in London (NHMUK) to clarify historical species ranges and

determine sampling localities. When possible, we emphasized collecting *Leptoxis* and type species of other pleurocerid genera from their type localities. However, for reasons noted above, this was often not possible. Snails were identified by making comparisons to original species descriptions and type specimens (Table 5.1); we followed the taxonomy of Johnson et al. (2013).

We sampled at least five individuals of each *Leptoxis* species (Table 5.2). *Leptoxis* species listed as endangered under the ESA were sampled from experimental populations in captivity at the Alabama Aquatic Biodiversity Center in Marion, Alabama (i.e. *L. foremani* (Lea, 1843) and *L. plicata* (Conrad, 1834)). Species with comparatively large ranges (e.g. *L. praerosa*) had a larger number of individuals and populations sampled than species with small ranges. Species that were once considered members of *Leptoxis* (i.e. *Atheurnia anthonyi* (Redfield, 1854), *Lithasia geniculata pinguis* (Lea, 1852), *Elimia melanoides* (Conrad, 1834)) were also collected. The type species of each genus, except *Io* Say, 1825, plus additional non-*Leptoxis* pleurocerids to test the putative non-monophyly of *Leptoxis* were also sampled (Holznagel and Lydeard, 2000; Minton, Garner and Lydeard, 2003; Strong and Köhler, 2009). *Io* sequences were retrieved from GenBank (AF100999, EF586915, DQ311134). *Juga plicifera* (Lea, 1838) (Semisulcospiridae Morrison, 1952) and *Cleopatra mwerensis* (Smith, 1893) (Paludomidae Stoliczka, 1868) were sampled as non-pleurocerid outgroups (Strong, *et al.*, 2011). Shell vouchers were deposited at USNM.

Character Sampling

Whole genomic DNA was extracted from snail foot tissue using a Qiagen DNEasy plant mini kit. A plant kit was used since freshwater gastropods have large amounts of mucus polysaccharides in their tissues, (Palumbi, 1996; Winnepenninckx, Backeljau and Dewachter,

1993), and plant DNA extraction kits are designed to purify DNA from tissue with high amounts of polysaccharides (e.g. cell walls).

Two partial nuclear (Histone H3, 28S rRNA) and two partial mitochondrial (16S rRNA, Cytochrome *c* Oxidase I; COI) gene fragments were amplified for all individuals we collected. The primers LCO1490/HCO2198 (Folmer, *et al.*, 1994) and D23F/D6R (Park and Foighil, 2000) and PCR parameters described by Whelan *et al.* (2011) were used to amplify COI and 28S respectively. Problems with sequencing 28S, ostensibly associated with secondary structure, necessitated cloning for *L. ampla* (Anthony, 1854), *L. picta* (Conrad, 1834), *L. foremani* and *L. taeniata* (Conrad, 1834). 28S PCR products for these species were cloned into pCR4-TOPO cloning vector (Invitrogen), and five clones per individual were sequenced. The primers 16Sar-L-myT/16Sbr-H-myT (Mulvey, *et al.*, 1997) and H3aF/H3aR (Colgan, *et al.*, 1998) were used to amplify 16S and H3, respectively, following PCR protocols of Chapter 3. Amplified PCR products were purified with polyethelyne glycol precipitation (Rosenthal, Coutelle and Craxton, 1993) or Qiagen PCR purification kits. Purified PCR products were directly sequenced on an ABI 3100 at the University of Alabama following manufacturer's protocols. Chromatograms were visualized and edited as needed in Geneious Pro 6.1 (Biomatters).

We took high resolution photographs of live pleurocerids to document species coloration and external, non-conchological characters. At least one individual of each *Leptoxis* species and some outgroups species, were also removed from their shells following Fukuda *et al.* (2008), and the structure of the hypobranchial gland was documented under a Leica 12.5 dissecting microscope.

Phylogenetics

NUMTs, which are easily sequenced in pleurocerids, were identified following Chapter 3 and removed from analyses (Table 5.2). 28S sequences were aligned using the E-INS-I algorithm in MAFFT (Katoh and Standley, 2013); other loci were aligned with default parameters in MUSCLE (Edgar, 2004). Partitioning schemes for each gene and the most likely model of molecular evolution for each partition were determined with Partition Finder 1.0 (Lanfear, *et al.*, 2012) using Bayesian information criteria (Table 5.3). Bayesian gene trees were inferred in MrBayes 3.1.2 for each nuclear gene separately and the two mitochondrial genes they both share a single evolutionary history (Funk and Omland, 2003). A Bayesian phylogeny was also inferred with all genes concatenated. For every MrBayes analysis, four independent runs with six chains each were run for 4×10^7 Markov chain Monte Carlo (MCMC) generations. Convergence and adequate mixing was assessed in Tracer 1.4 (Rambaut and Drummond, 2007) and AWTY (Nylander, *et al.*, 2008). A 50% majority rule consensus tree for each analysis was calculated from the posterior distribution of trees minus a 25% burn-in. Nodal support was measured by posterior probabilities (PP).

We utilized *BEAST (Heled and Drummond, 2010), which resolves gene tree conflict with the multispecies coalescent model (Rannala and Yang, 2003), to infer a species trees. *BEAST requires *a priori* species/OTU designations, and they were assigned to each sequence based on the combined mitochondrial gene tree and morphological identifications (Fig. 5.1; Table 5.2). Gene partitions and models of molecular evolution were inferred with PartitionFinder, except that 28S and H3 were not combined (Table 5.3). We used three gene tree models (i.e. one for each nuclear gene and a single tree for both mitochondrial genes), an uncorrelated lognormal relaxed clock model for each gene partition, and a Yule process species tree prior. Four independent *BEAST analyses were run for 4×10^8 MCMC generations.

Adequate MCMC mixing and convergence was assessed in Tracer. We combined the four runs, minus 25% burn-in, and compiled a maximum clade credibility in TreeAnnotator (Drummond, *et al.*, 2012).

Results

Taxon sampling and Leptoxis species' ranges

In the Mobile River basin, most extant *Leptoxis* species have undergone drastic range reductions (MRB Recovery Committee 2010; Richardson and Selby, 2009; Whelan, Johnson and Harris, 2012), and our field work reflected this reality. *Leptoxis plicata* is found in the Locust Fork in Blount County Alabama, but since *L. plicata* is federally endangered we sampled captively reared juveniles from Locust Fork broodstock. We also only sampled *L. foremani* individuals bred in captivity since it is currently known only from one location in the Oostanula River in Georgia (Table 5.2). *Leptoxis compacta* (Anthony, 1855) was sampled from its only known extant population, the Cahaba River in Shelby County Alabama (Whelan, *et al.*, 2012). We found *L. ampla* to be widespread throughout the upper Cahaba River and its tributaries, but the type locality is the state of Alabama (Anthony, 1855), so it impossible to know if we collected *L. ampla* from where it was originally described. Museum and published records of *L. ampla* from the Coosa River drainage (e.g. FLMNH 416820, 416845, 416903; Goodrich, 1922) were determined to be misidentifications of the morphologically similar *L. taeniata*. We sampled *L. taeniata* from its three remaining, but fragmented, populations in the Coosa River basin (Table 5.2). *Leptoxis picta* (Conrad, 1834) was sampled from its type locality—"Alabama River near Claiborne" (Conrad, 1834 p 62).

We sampled *Leptoxis* throughout the Tennessee, Cumberland, Ohio River drainages, but several diving expeditions at historical *Leptoxis* collection sites in the Cumberland and Ohio

Rivers failed to yield live *Leptoxis*. We found *L. praerosa*—the *Leptoxis* type species—in two direct tributaries of the Ohio River (Table 5.2), which are the two extant populations closest the type locality of *L. praerosa*: the Falls of the Ohio River in Louisville. *Leptoxis praerosa* was also sampled from tributaries of the Tennessee and Cumberland Rivers (Table 5.2). We found snails that possessed the distinct umbilicus of *L. umbilicata* in the East Fork of the Stones River and Smith Branch in Tennessee. Species initially identified as *L. virgata* or *L. praerosa* were sampled from the Paint Rock River and from the Tennessee River and its tributaries upstream of South Pittsburg, Tennessee. We also found a species that resembles other *Leptoxis* species (i.e. it has a large aperture with a reduced spire) from the Collins River in Warren County, Tennessee (Table 5.2).

We collected *L. dilatata* (Conrad, 1835) from its type locality in Indian Creek, Monroe County, West Virginia, and from the North and Middle Forks of the Holston River in Virginia. *Leptoxis carinata* (Bruguière, 1792) is located in drainages of the Atlantic slope from Virginia to New York (Aldridge, 1982; Burch and Tottenham, 1980), but we were only able to sample *L. carinata* at its type locality, the Roanoke River at Lafayette, Virginia (Clench and Boss, 1967; Tryon, 1873).

Atheurnia anthonyi and *Lithasia geniculata pinguis* were each sampled from two localities (Table 5.2), but we could not find these species at their type locality. *Elimia melanoides* was only sampled from its neotype locality in the Little Warrior River in Blount County, Alabama (Minton, *et al.*, 2003). We sampled the type species of *Lithasia* Haldeman, 1840 (*Lithasia geniculata geniculata* Haldeman, 1840) and *Elimia* (*Elimia clavaeformis* (Lea, 1841)) from their type localities. The *E. clavaeformis* we sampled have the shell shape of the *nomen, acutocarinata* (Lea, 1841), which would be the type for *Elimia* had it not been

synonymized with *E. clavaeformis* (see Burch, 1989). We failed in our attempts to sample the type of *Pleurocera* Rafinesque, 1818 (*Pleurocera acuta* Rafinesque, 1831) from its type locality in Lake Erie. Instead, we sampled *P. acuta* from the Salt River in Kentucky. We also sampled an odd *Pleurocera* shell form with carina that we putatively identified as *P. acuta* following Burch and Tottenham (1980) from the North Fork of the White River in Douglas County, Missouri. Information on other non-*Leptoxis* species that we sampled can be found in Table 5.2.

Phylogenetic Character Sampling

Twenty-eight individuals were removed from phylogenetic analyses because they produced NUMT sequences using standard sequencing protocols for mitochondrial genes (Table 5.2; see Chapter 3 for more information). The aligned data matrix consisted of 150 individuals and 2,218 alignment positions with limited missing data (< 1% of entire matrix).

Gene Trees

Relationships are poorly resolved on the 28S (Fig. 5.2) and H3 gene trees (Fig. 5.3). However, on both nuclear gene trees a clade of all extant *Leptoxis* species from the Alabama River Basin, minus *L. compacta*, is the most basal pleurocerid lineage, and *Leptoxis* is non-monophyletic. Many Tennessee and Ohio River *Leptoxis* basin species are not monophyletic on either nuclear gene tree, but are instead part of a large polytomy that encompasses the majority of each tree.

On the mitochondrial gene tree (Fig. 5.1) there are five well supported *Leptoxis* (*sensu lato*) clades (PP = 1). The most basal clade includes *L. picta*, *L. foremani*, *L. ampla*, and *L. taeniata*. *Leptoxis picta* is paraphyletic with *L. foremani*, albeit with poor nodal support. The most specious *Leptoxis* clade includes species from the Tennessee, Cumberland, and lower Ohio River basins, including *L. praerosa*—the type species of *Leptoxis*—and *A. athearnia*. Within this

large *Leptoxis* clade, a clade of populations currently considered *L. praerosa* is sister to *A. Athearnia*, rather than sister to other *L. praerosa* populations. This clade includes two individuals from the type locality of the *nomen subglobosa*. A third clade contains *Leptoxis carinata* and *L. dilatata*, but *L. dilatata* from the Tennessee River drainage and *L. dilatata* from the New River drainage are not monophyletic. *Leptoxis compacta* and *L. plicata* are resolved as sister but part of a larger clade containing *Elimia* species and *Pleurocera foremani*. *Leptoxis arkansensis* is sister to *E. potosiensis* (Lea, 1841), but the sister relationship of this clade is not resolved. Neither *E. melanoides* nor *Lithasia geniculata pinguis* are closely related to other *Leptoxis*. The potentially undescribed, “*Leptoxis*-like,” species from the Collins River is sister to *Lithasia geniculata pinguis*. Of note, the type species for *Elimia*, *E. clavaeformis*, is sister to *Li. obovata* rather than other *Elimia*. All pleurocerid genera except the two monotypic genera, *Io* and *Athearnia*, are resolved as non-monophyletic.

Concatenated and species trees

The topology of the concatenated phylogeny (Fig. 5.4) is almost identical to that of the concatenated mitochondrial gene tree (Fig. 5.1), and the major clades described for the concatenated mitochondrial gene tree are well supported by the *BEAST analysis. However, among-clade relationships on the *BEAST tree have poor nodal support (PP < 90), and relationships among major clades differ considerably between the concatenated phylogeny and the *BEAST tree (Fig. 5.6). First, the clade of *E. potosiensis* and *L. arkansensis* is resolved as a relatively basal offshoot in the *BEAST phylogeny, rather than related to *Elimia* species as on the concatenated tree. Furthermore, *L. dilatata* and *L. carinata* are sister to the large clade of Tennessee, Cumberland and lower Ohio River basin *Leptoxis* on the *BEAST phylogeny. On both trees, the Alabama River basin *Leptoxis* clade is the most basal clade, and *A. anthonyi* is

nested within the clade of *Leptoxis* from the Tennessee, Cumberland, and lower Ohio River basins. *Leptoxis compacta* and *L. plicata* are more closely related to *Elimia* spp. than other *Leptoxis* on both phylogenies.

Morphological features of Leptoxis

The body pigmentation of *Leptoxis* varies among species, sometimes populations and even individuals within populations (Fig. 5.6). For example, *L. ampla*, *L. plicata*, and *L. compacta* are all generally yellow with black mottling and a single horizontal black band in the middle of the snout (Fig. 5.6A), but some *L. ampla* we collected from the Cahaba River were orange with black mottling. *Leptoxis foremani*, *L. picta* and *L. taeniata* are orange with black mottled across the body and mantle (Fig. 5.6B). *Leptoxis* species from the Tennessee and Cumberland drainages are either orange or dark red with black mottled across the body (Fig. 5.6C). *Leptoxis arkansensis* is dark orange with black mottled across the body and a light blue horizontal band around the eyes (Fig. 5.6D). *Leptoxis carinata* and *L. dilatata*, from its type locality in Indian Creek, are dark yellow with black covering most of the head and mottled across the foot (Fig. 5.6E). However, *L. dilatata* from the North and Middle Forks of the Holston River is blue with orange mottled across the body and head (Fig. 5.6F).

Atheurnia anthonyi and *Leptoxis* species from the Tennessee, Cumberland and Ohio River basins, except for *L. dilatata*, have a pronounced ocular peduncle (Fig. 5.6C). All other species have either weakly formed ocular peduncles or they are absent. *Leptoxis dilatata* has a cephalic tissue extension above the eye on both sides of the head (Fig. 5.7E, F). *Leptoxis picta*, *L. foremani*, *L. ampla* and *L. taeniata* have translucent, broadly triangulate hypobranchial glands that comprise over 1/3 of the mantle cavity. All other pleurocerid species analyzed and *Juga*

possess an opaque, narrow hypobranchial gland that is adjacent to the intestine and spans only a small portion of the mantle cavity.

Discussion

Our findings contribute to a growing body of evidence that current pleurocerid taxonomy does not reflect the realized phylogeny. Only the two monotypic pleurocerid genera, *Io* and *Atheurnia*, were resolved as monophyletic, but *Atheurnia* is nested within a clade of *Leptoxis*. Although *Leptoxis* was the focus of this study, it is clear that other genera are in need of a thorough systematic analysis. However, the limited *Elimia* and *Pleurocera* species sampling in the present study limits our ability to clarify pleurocerid taxonomy and relationships as a whole. Nevertheless, *Leptoxis* is clearly non-monophyletic and some *Leptoxis* populations currently recognized as *L. praerosa* deserve recognition as distinct species.

There are notable differences among the inferred phylogenies, which is not surprising given the different inference methods and phylogenetic signal among genes. The two nuclear gene trees are poorly resolved and many species are non-monophyletic. This is likely a result of limited phylogenetic signal and/or incomplete lineage sorting in the nuclear loci rather than being indicative of the true evolutionary history of these animals. Additionally, in the concatenated analysis, the two mitochondrial genes appear to swamp out any signal given by the nuclear genes considering the similarities between the concatenated phylogeny and mitochondrial gene trees. In contrast, the *BEAST tree differs considerably from the mitochondrial gene trees. Since *BEAST infers individual gene trees for each locus and then resolves gene tree conflict with the multi-species coalescent model to infer a species tree, the two nuclear genes likely contribute more to the overall topology of the *BEAST tree than in the concatenated analysis.

Body and mantle coloration varies within species and populations, and species with similar coloration (e.g. *L. compacta*, *L. plicata*, and *L. ampla*) are not always closely related. Therefore, coloration is probably a poor character for systematic analyses of pleurocerds. However, other non-conchological morphological characters we documented are an underexplored suite of diagnostic features that can be used as diagnosable characters for genera and species. For example, *L. dilatata* could easily be misidentified as *L. praerosa/subglobosa*, in locations where the species are sympatric (e.g. North Fork of the Holston River) because of conchological similarities (Chapter 4), but *L. dilatata* individuals can be distinguished by an autapomorphic cephalic tissue extension on the side of their head above the eye (Fig. 5.6E, F). Exploring whether similar characters exist in other sympatric pleurocerids that are difficult to identify by shell alone (e.g. *E. clara* and *E. variata* in the Cahaba River) may reveal consistent methods for species identification.

Species taxonomy and ranges

Leptoxis praerosa, was resolved as non-monophyletic with high nodal support on the concatenated phylogeny, the mitochondrial gene trees, and the *BEAST tree (Figs. 5.1, 5.4, 5.5). Populations of *L. praerosa* collected in tributaries of the Holston River and the Nolichucky River in Tennessee and Virginia are sister to *Atheurnia anthonyi*. These populations of *L. praerosa* are morphologically distinct from *A. anthonyi* (Chapter 4; Dillon and Ahlstedt, 1997), and they lay eggs singly (Chapter 4), whereas *A. anthonyi* lays eggs in a clutch formation (Whelan and Johnson, unpublished data). Under the general lineage species concept (de Queiroz, 2007), these populations are a distinct species. The population from the North Fork of the Holston River, which is in the clade sister to *A. anthonyi*, is the type locality of the *nomen subglobosa*. Furthermore, the original description of *subglobosa* matches the morphology of these

populations (Say, 1825). As such, we resurrect *L. subglobosa* to include *Leptoxis* species from the Forks of the Holston, the Clinch, Powell, and Nolichucky Rivers in Tennessee and Virginia. Of note, is that *L. virgata* and *L. subglobosa* are sympatric in the Nolichucky River. Although the shells of these species are slightly different (Chapter 4), they are not easily identified in the field where algae often grow on shells. Furthermore, additional regional sampling is needed to clarify the ranges of *L. subglobosa* and *L. virgata* in Tennessee River tributaries of eastern Tennessee, North Carolina and Virginia that we were unable to sample.

Populations of *L. praerosa* other than those discussed above and *L. umbilicata* are part of a species complex (Fig. 5.1, 5.4, 5.5) with few or no distinct morphological characters aside from the umbilicus of *L. umbilicata* (Chapter 4, Burch and Tottenham 1980). *Leptoxis umbilicata* was historically found throughout the Stones River drainage and in the Red River in Tennessee, but after considerable efforts, we only found snails with the distinct umbilicus of *L. umbilicata* in a small stretch of the East Fork of the Stones River drainage and in Smith Branch (Table 5.2); Smith Branch is outside the known range of *L. umbilicata* (Burch and Tottenham, 1980). Interestingly, populations from the Flint and Duck Rivers in Tennessee that do not have an umbilicus are more closely related to *L. umbilicata* than other *L. praerosa* (Figs. 5.1, 5.4, 5.5). At this time, we are hesitant to modify the species-level taxonomy of this complex, aside from emphasizing that *L. umbilicata* is found outside the Stones River drainage and that *L. subglobosa* is clearly not a subspecies of *L. umbilicata* (see Introduction). Finer scale phylogeographic studies should help clarify the relationships and taxonomy of this species complex.

Since *Leptoxis* is extirpated from much of the main stem Tennessee River it may be difficult to determine the precise geographical break separating the ranges of *L. praerosa* and *L. virgata*, especially since *L. virgata* is found below Walden's Ridge—a historical biogeographic

barrier for freshwater organisms (Etnier and Starnes, 1994; Starnes and Etnier, 1986). In the mean time, all *Leptoxis* “*praerosa*,” not including the *L. subglobosa* population, lacking an umbilicus as adults should be considered *L. praerosa*, and researchers should take care when identifying *L. praerosa*, *L. subglobosa*, *L. virgata*, *L. umbilicata* since each species’ conchological morphology is strikingly similar (Chapter 4).

The discovery of *L. dilatata* in the upper Tennessee River basin (Table 5.2) considerably extends its range (Burch and Tottenham, 1980), but more molecular data is needed to resolve relationships among *L. carinata* and *L. dilatata*. That is, *L. dilatata* was resolved as paraphyletic with *L. carinata* on the mitochondrial and concatenated phylogenies (Figs. 5.1, 5.4). However, all *L. dilatata* were monophyletic on the 28S gene tree (Fig. 5.2), and *L. dilatata* has a morphological autapomorphy—cephalic tissue extensions—that distinguishes it from *L. carinata*. For these reasons, *L. dilatata* and *L. carinata* were coded as separate OTUs in the *BEAST analysis (Table 5.2), and we continue to recognize *L. carinata* and *L. dilatata* as separate species.

Leptoxis picta was paraphyletic with *L. foremani* in the concatenated and mitochondrial trees, and they share many morphological and egg-laying behaviors (Chapter 4; Goodrich, 1922). Although they were each monophyletic on the *BEAST tree (Fig. 5.5), a different outcome was impossible since both were coded as separate OTUs. These species may deserve to be synonymized or they may represent a very recent speciation event. Since *L. foremani* and *L. picta* are conservation priorities, the remaining populations are allopatric, and temporal and fecundity differences in life history strategies have been observed between the two species (Chapter 4), we continue to recognize both taxa as valid species. Additional nuclear loci should be sequenced for *L. picta* and *L. foremani* so objective species delimitation methods like

spedeSTEM (Ence and Carstens, 2011) or BPP (Yang and Rannala, 2010) can be used (Fujita, *et al.*, 2012; Whelan, 2011).

Leptoxis ampla, *L. taeniata*, *L. compacta*, *L. plicata*, and *L. arkansensis* are all distinct lineages based on phylogeny (Figs. 5.1, 5.4, 5.5), morphology and life history (Chapter 4). Except for on the H3 gene tree, all five species are each reciprocally monophyletic. *Leptoxis taeniata*, *L. compacta*, *L. plicata*, and *L. arkansensis* all suffer from severe population fragmentation and range contraction compared to their historical ranges. Interestingly, even though *L. ampla* is listed as threatened under the US Endangered Species Act, we found it to be common throughout the Cahaba River upstream of Centerville, Alabama (see Chapter 3 for more detailed *L. ampla* sampling), and there was no evidence of considerable physical population fragmentation or dispersal barriers throughout its current range.

Generic Relationships and Taxonomy

Genera other than *Leptoxis* are clearly non-monophyletic, but as in the discussion of species-level taxonomy above, we focus primarily on *Leptoxis*. Nevertheless, three issues should be noted concerning outgroup genera. First, the type for *Elimia*, *Elimia clavaeformis*, is not related to other *Elimia* species (Figs. 5.1, 5.4, 5.5). *Elimia* has long served as a dumping ground for non-descript pleurocerids lacking adequate study, and a more thorough analysis than the one presented here will be needed to clarify the generic taxonomy of non-*Leptoxis* (*s.l.*)pleurocerids. However, *Pleurocera* and *Elimia* are conclusively not a monophyletic clade, and contrary to the claims of Dillon (2011) they should not be treated as a single genus. Second, our results corroborate the findings of Sides (2005) that *Pleurocera foremani* is more closely related to Mobile River Basin *Elimia* than *Pleurocera* species (Figs 5.1, 5.4, 5.5). Finally, *Mudalia* Haldeman, 1840 has been considered a subgenus of *Leptoxis* (Burch and Tottenham, 1980), but

our findings do not support this taxonomic hypothesis since *M. turgida* Haldeman, 1840, is the type of *Mudalia*, but it is a junior synonym of *E. melanoides* (see Figs. 5.1, 5.4, 5.5).

Nevertheless, further study may determine *Mudalia* is a valid generic name for some *Elimia* species considering the phylogenetic placement of *E. clavaeformis*.

Leptoxis species from the Tennessee, Cumberland, and Ohio River basins minus *L. dilatata* form a well supported monophyletic clade (Figs. 5.1, 5.4, 5.5). *Atheurnia anthonyi* is also resolved within this clade. Since this clade includes *L. praerosa* and the name *Leptoxis* has priority over *Atheurnia*, *Atheurnia* is not a valid genus (Appendix 1). The species in this clade all possess a pronounced ocular peduncle that has not been observed in other pleurocerids or in *Juga* (Figure 5.7; Strong and Frest, 2007). Furthermore, all the species except *A. anthonyi* lay eggs singly (Chapter 3). The clutch egg-laying behavior of *A. anthonyi* (Whelan and Johnson unpubl. data), is an autapomorphic behavior in this clade.

Leptoxis ampla, *L. taeniata*, *L. foremani*, and *L. picta* comprise the most basal, monophyletic, pleurocerid clade in every phylogenetic analysis (Figs. 5.1-5.5). The consistently basal placement of this clade in multiple analyses suggests that pleurocerids first diversified in the Mobile River basin. All species in this clade lay eggs in a clutch formation (Chapter 4), and they all have a large, translucent hypobranchial gland that is different from the hypobranchial gland morphology of other pleurocerids and *Juga* (Strong and Frest, 2007). There are eight extinct *Leptoxis* from the Coosa River in Alabama that are morphologically similar to the species in this clade (Goodrich, 1922; Johnson, *et al.*, 2013), and they are likely members of this clade. We elevate this clade to generic status under the new name *Alatoxis* (Appendix I)

Leptoxis carinata and *L. dilatata* are resolved in a monophyletic clade in every phylogeny (Figs. 5.1-5.5). The sister group to this clade is unresolved in the two nuclear gene

trees (Figs. 5.2, 5.3), the second most basal pleurocerid clade in the mitochondrial and concatenated data sets (Figs 5.1, 5.4), or it is resolved as sister to the *Leptoxis* clade with *L. praerosa* in the *BEAST tree (Fig. 5.5), albeit with limited nodal support (<90% P.P.). These species do not have a pronounced ocular peduncle like the *L. praerosa* clade, and unlike the species in the *L. praerosa* clade, they lay eggs in a single line (Chapter 4). As such, we consider this clade a distinct genus. Previously, Clench and Boss (1967) elevated these two species to generic status with the name *Allegheny*, but it was not widely adopted. We resurrect this genus (Appendix I), but note that future studies with additional non-*Leptoxis* (*s.l.*) taxon sampling may reveal that additional species belong in this genus.

The phylogenetic placement of *Leptoxis compacta* and *L. plicata* is either nested within a clade of *Elimia* from the Mobile River Basin or unresolved depending on the inference methods (Figs. 5.1-5.5), but they are not resolved as in a clade with other *Leptoxis* (*s.l.*). There are problems associated with *Elimia* from a nomenclatural perspective (see above), and resolving these issues are outside the scope of this study. However, continuing the use the *nomen Leptoxis* to describe these species will only serve to confuse the taxonomic revisions put forth here. As such we propose *L. plicata* and *L. compacta* be considered “*Elimia*” species until further study suggests otherwise.

Leptoxis arkansensis poses a similar problem. It is sister to *Elimia potosiensis*, but this clade’s sister relationships differ among phylogenetic hypotheses and it is never resolved with high nodal support. Unlike for other taxonomic changes that we have put forth here there is no clear morphological or life history characters that support *L. arkansensis* + *E. potosiensis* as a new genus. More research with additional pleurocerid sampling from west of the Mississippi

River is needed, but until then we suggest *Leptoxis arkansensis* be considered a “*Leptoxis*” with the explicit understanding that this species is not in the *Leptoxis* (*s.s.*) clade.

Conclusions

Pleurocerid taxonomy has long been a source of confusion, and until recently relationships among species and genera have been obscured by the inclusion of NUMTs as homologous mitochondrial sequences in phylogenetic inference (Chapter 3). We chose to focus on *Leptoxis* since almost half the genus is extinct, and most other species are critically imperiled (Johnson, *et al.*, 2013). Of broad interest, is that the newly described genus *Alatoxis* was consistently resolved as the most basal pleurocerid clade, which indicates pleurocerids first diversified in the Mobile River Basin—a global hotspot of freshwater diversity. The chaotic pleurocerid taxonomy has hindered and discouraged research on an important and evolutionarily interesting group, but we hope that our findings and initial taxonomic clarifications will excite researchers about the possibility of studying pleurocerids as a model for freshwater invertebrate diversification in a comparative concept.

Challenges remain for elucidating the evolutionary history of all pleurocerids. Much broader taxon and geographic sampling will be necessary for other pleurocerid genera, and a combination of phylogenetic and biogeographic analyses will likely be necessary to clarify generic and species boundaries. High throughput sequencing should aid in this endeavor. We expect the genera described here to remain valid after future studies, but we acknowledge that additional species, not sampled here, may fall within these genera. Time is of the essence for studying pleurocerid diversity as many species are under immediate threat of extinction, and global climate change may exacerbate their habitat degradation and population fragmentation.

Appendix

(a) *Atheurnia* is phylogenetically resolved within *Leptoxis* (*s.s.*), and we designate it as junior synonym of *Leptoxis*.

***Leptoxis* Rafinesque 1819**

Anculosa Say, 1821

Eurycaelon Lea, 1864

Atheurnia Morrison, 1971

Type species: *Leptoxis praerosa* (Say, 1821). The type specimen is presumed lost (Garner, JT et al. personal communication)

Diagnosis: Shells subglobular and often decollated with a suboval to subtriangulate aperture.

Shells with or without umbilicus, which is more prominent in juveniles if present. Eggs laid singly (Chapter 4) or as a triangulate clutch for *L. anthonyi* (Whelan & Johnson unpubl. data). Prominent ocular peduncle that extends past the eye. Found in the Ohio River drainage as far east as the Licking River and throughout the Cumberland and Tennessee River basins. Phylogenetically, the least inclusive clade containing *L. praerosa*, *L. subglobosa*, *L. umbilicata*, *L. virgata*, *L. anthonyi*.

Remarks: Species of this genus are difficult to identify by shell alone, especially compared to *Alatoxis* (described below), but they generally have a wider aperture and reduced spire compared to other pleurocerid genera. The sole morphological synapomorphy is a large ocular peduncle. This clade includes the *Leptoxis* type species—*L. praerosa*—so it retains the name *Leptoxis*. The clade is well resolved on the mitochondrial gene tree, the concatenated phylogeny, and the *BEAST tree. Future study with additional nuclear loci is still needed to resolve the *L. praerosa*/*L. umbilicata* species complex, but *L.*

subglobosa is a valid species. *Atheurnia* is not a valid genus, but rather a junior synonym of *Leptoxis* (s.s.). *Atheurnia crassa* is extinct and was not included in this study, but considering its similarities in shell morphology with *A. crassa*, it is almost certainly a *Leptoxis*.

***Leptoxis anthonyi* (Redfield, 1854)**

Anculosa anthonyi Redfield, 1854: 130, pl. 1, fig. 6.

Eurycaelon anthonyi Tryon, 1873: 347-348 fig. 659.

Atheurnia anthonyi Morrison, 1971: 110-111.

Material examined: Neotype (here designated) USNM “to be assigned” collected from Sequatchie River, Marion County, Tennessee 34.7130°N 86.8684°W. Limestone Creek, Limestone County, Alabama 35.0605°N 85.6067°W. Extirpated from original type locality: Holston River near Knoxville Tennessee. Neotype designated following ICZN Code Article 75 to clarify the taxonomic status of this species. A holotype type was not designated and the original collection sent to Redfield is presumed lost (Garner, JT et al., unpublished data). Furthermore, the possible syntype, MCZ 47795, as mentioned by Graf (2001), is from Chattanooga, Tennessee, and there was no mention of this locality in the original description (Garner, JT et al., unpublished data). The morphology of *L. anthonyi* we collected from the Sequatchie River matches that of the original description, and since the species is extirpated from the original type locality, the neotype is instead from the Sequatchie River.

Diagnosis: Shell rhomboidly ovate with a single prominent carina in younger individuals and sutures that are more pronounced in larger individuals. Aperture narrower and taller than congeners. Some individuals possess a deep and prominent groove or lip at the base of

the aperture that is distinct from the columella. Eggs are laid in small triangular clutches of up to three eggs.

Current distribution: Restricted to three populations: Limestone Creek in Limestone County Alabama, the Sequatchie River near its mouth in Marion County Tennessee, and in the Tennessee River in Jackson County, Alabama.

(b) The species *L. dilatata* and *L. carinata* are not in the *Leptoxis* (*s.s.*) clade and the genus *Alleghenya* is herein resurrected.

***Alleghenya* Clench and Boss 1967**

Nitocris H. and A. Adams, 1854 p. 308. *non Nitocris* Rafinesque 1815 (Hymenoptera)

Type Species: *Alleghenya carinata* (Bruguière, 1792). Type specimen is presumed lost (Garner JT, et al. unpublished data)

Diagnosis: Medium spired shells (4 body whorls) with a sharp angle of approximately 110 degrees at the midpoint of the columella/aperture wall and broadly ovate outside aperture wall. Light to pronounced columella lip. Lays eggs in lines. Least inclusive phylogenetic clade of *Alleghenya carinata* and *Alleghenya dilatata*.

Remarks: The shells of this genus can vary considerably in the shape of the aperture base and presence or absence of carinae (Chapter 4). Outside of Tennessee River tributaries (i.e. the Atlantic slope and upper Ohio River tributaries), the genus is distinguished from sympatric pleurocerids by a large aperture with an ovate outside aperture wall. In tributaries of the Tennessee, the genus can be distinguished by the characteristic columella/apertural wall angle and four body whorls (Chapter 4). *Leptoxis carinata* and *L. dilatata* both lay eggs in a line (Chapter 4). Burch and Tottenham (1980) placed *L.*

carinata and *L. dilatata* in the subgenus *Mudalia*, but the nomen *turgida*, which is a synonym of *E. melanoides*, is the type for *Mudalia* (Clench and Boss, 1967; Haldeman, 1840). *Elimia melanoides* is not in the *Alleghenya* clade (Figs. 5.1, 5.2, 5.4, 5.5) so *Mudalia* is not an available generic name for *A. carinata* and *A. dilatata*.

(c) *Leptoxis ampla*, *L. foremani*, *L. picta*, and *L. taeniata* are in a monophyletic and well supported clade, and have synapomorphic hypobranchial gland anatomy. A generic name is not available for these species so we propose *Alatoxis*.

***Alatoxis* gen. nov.**

Type Species: *Alatoxis picta* (Conrad, 1834) since it is the first described extant species in the genus. Type is presumed lost (Garner, JT et al., unpubl. data).

Etymology: A combination of “Alabama” and “*Leptoxis*. ” All species are found in the Alabama River basin, and were once considered *Leptoxis*.

Diagnosis: Ovate shells with 2-3 body whorls. First body whorl is particularly large with the second and third body whorls being less than 1/8 of total spire height. Wide circular or ovate apertures that are over 1/2 the shell size in younger individuals and approximately or slightly less than 1/2 in large adults. No columella indentation or other notable features. Translucent hypobranchial gland comprising over 1/3 of the mantel cavity. Eggs laid in circular clutches.

Remarks: This genus is distinguished from sympatric pleurocerids by their wide apertures and the small number of their body whorls (i.e. ≤ 3). Species of this genus are found in larger rivers, and no records are known from headwaters or springs. Based upon conchological similarities, it is likely that recently extinct *Leptoxis* (*s.l.*) from the Coosa River basin are

also members of this genus even though they have not been included in any phylogenetic hypotheses to date. All four extant species of *Alatoxis* lay eggs in circular clutches (Chapter 4) and they all possess a translucent hypobranchial gland that comprises approximately 1/3 of the mantel cavity—much larger than other pleurocerids analyzed and the semisulcospirid *Juga* (Strong and Frest, 2007). All four species are considered imperiled (Johnson, *et al.*, 2013), and all but *L. picta* are listed under the ESA. *Alatoxis* is the most basal pleurocerid genus.

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Table 5.1: *Leptoxis* species or species once assigned to *Leptoxis* and information associated with their original description

Species	Type catalogue number	Original description	Figure^	Type Locality
<i>Atheastaria anthonyi</i>	Presumed lost	Redfield, 1854	pl. 2, figs. 12a-b	Holston River near Knoxville, Tennessee
<i>Elimia melanoides</i>	FMNH 301993	Conrad, 1834	pl. 8, fig. 19	Little Warrior River, Blount County, Alabama
<i>Leptoxis ampla</i>	MCZ 161735	Anthony, 1854	pl. 2, fig. 12	Alabama
<i>Leptoxis arkansensis</i>	USNM 271764	Hinkley, 1915	pl. 78, fig. 3	North Fork White River in Arkansas
<i>Leptoxis carinata</i>	Presumed lost	Bruguière, 1792	-	Roanoke River at Lafayette, Virginia
<i>Leptoxis compacta</i>	MCZ 72063	Anthony, 1854	pl. 3, fig. 22	Alabama
<i>Leptoxis dilatata</i>	Presumed lost	Conrad, 1835	pl. 9, fig. 5	Indian Creek, Monroe County, West Virginia
<i>Leptoxis foremani</i>	USNM 121259	Lea, 1843	-	Alabama
<i>Leptoxis picta</i>	Presumed lost	Conrad, 1834	pl. 1, fig. 15	Alabama River near Claiborn
<i>Leptoxis plicata</i>	Presumed lost	Conrad, 1834	pl. 8, fig. 18	Tributaries of Tennessee River in North Alabama
<i>Leptoxis praerrosa</i>	Presumed lost	Say, 1821	-	Falls of the Ohio River
<i>Leptoxis subglobosa</i>	Presumed lost	Say, 1825	-	North Fork Holston River
<i>Leptoxis taeniata</i>	Presumed lost	Conrad, 1834	-	Alabama River near Claiborn
<i>Leptoxis umbilicata</i>	Presumed lost	Wetherby, 1876	pl. 1, fig. 4	Stones River, Rutherford County, Tennessee
<i>Lithasia geniculata pinguis</i>	USNM 121219	Lea, 1852	pl. 30, fig. 11	Lebanon, Wilson County, Tennessee

[^]Figure from the original species description

Table 5.2: Species sampled and collection localities.

Species	Locality	County	State	Country	Latitude	Longitude	# sequenced	# NUMTs [‡]	*BEAST OTU [^]
<i>Atheurnia anthonyi</i>	Limestone Creek	Limestone	Alabama	USA	34.7130	-86.8684	2	-	<i>A. anthonyi</i>
<i>Atheurnia anthonyi</i>	Sequatchie River	Marion	Tennessee	USA	35.0605	-85.6067	2	-	<i>A. anthonyi</i>
<i>Cleopatra mwerensis</i>	Kalungwishi River	-	-	Zambia	-9.5424	29.3867	2	-	<i>C. mwerensis</i>
<i>Elimia claeveformis</i>				USA			4	-	<i>E. claeveformis</i>
<i>Elimia crenatella</i>	Cheaha Creek	Talladega	Alabama	USA	33.5321	-86.0414	1	-	<i>E. crenatella</i>
<i>Elimia hydei</i>	Locust Fork	Blount	Alabama	USA	35.8920	-86.6928	1	-	<i>E. hydeii</i>
<i>Elimia melanoides</i>	Little Warrior River	Blount	Alabama	USA	33.8803	-86.5806	5	-	<i>E. melanoides</i>
<i>Elimia potosiensis</i>	Unnamed trib Bates Creek	Washington	Missouri	USA	37.9286	-90.7923	4	-	<i>E. potosiensis</i>
<i>Io fluvialis</i>	From GenBank	-	-	-	-	-	-	-	<i>I. fluvialis</i>
<i>Juga plicifera</i>	Willamette River	Benton	Oregon	USA	44.5519	-123.252	4	-	<i>J. plicifera</i>
<i>Leptoxis ampla</i>	Cahaba River	Bibb	Alabama	USA	33.0799	-87.0668	3	2	<i>L. ampla</i>
<i>Leptoxis ampla</i>	Little Cahaba River	Bibb	Alabama	USA	33.0537	-87.0602	2	-	<i>L. ampla</i>
<i>Leptoxis arkansensis</i>	Spring Creek	Douglas	Missouri	USA	36.8104	-92.1462	5	1	<i>L. arkansensis</i>
<i>Leptoxis carinata</i>	Roanoke River	Montgomery	Virginia	USA	37.2332	-80.1982	5	-	<i>L. carinata</i>
<i>Leptoxis compacta</i>	Cahaba River	Shelby	Alabama	USA	33.1786	-87.0175	5	-	<i>L. compacta</i>
<i>Leptoxis dilatata</i>	Indian Creek	Monroe	West Virginia	USA	37.5150	-80.7696	5	3	<i>L. dilatata</i>
<i>Leptoxis dilatata</i>	North Fork Holston River	Smyth	Virginia	USA	36.9534	-81.5272	6	3	<i>L. dilatata</i>
<i>Leptoxis foremani</i>	Oostanula River	Gordon	Georgia	USA	34.4032	-85.0971	5	-	<i>L. foremani</i>
<i>Leptoxis picta</i>	Alabama River	Wilcox	Alabama	USA	32.0676	-87.4020	5	1	<i>L. picta</i>
<i>Leptoxis plicata</i>	Locust Fork	Jefferson	Alabama	USA	33.7248	-86.9823	5	3	<i>L. plicata</i>
<i>Leptoxis praerosa</i>	Licking River	Pendleton	Kentucky	USA	38.7897	-84.3680	2	-	<i>L. praerosa</i> 1
<i>Leptoxis praerosa</i>	Duck River	Hickman	Tennessee	USA	35.8262	-87.6656	4	1	<i>L. praerosa</i> 1
<i>Leptoxis praerosa</i>	Limestone Creek	Limestone	Alabama	USA	34.7492	-86.8243	4	-	<i>L. praerosa</i> 2
<i>Leptoxis praerosa</i>	Flint River	Madison	Alabama	USA	34.7412	-86.4414	3	-	<i>L. praerosa</i> 2

<i>Leptoxis praerosa</i>	Elk River	Lincoln	Tennessee	USA	35.1397	-86.6590	2	-	<i>L. praerosa</i> 2
<i>Leptoxis praerosa</i>	Shoal Creek	Lauderdale	Alabama	USA	34.9522	-87.5932	3	2	<i>L. praerosa</i> 2
<i>Leptoxis praerosa</i>	Blue River	Harrison	Indiana	USA	38.2303	-86.2534	3	-	<i>L. praerosa</i> 2
<i>Leptoxis praerosa</i>	Bear Creek	Colbert	Alabama	USA	34.6761	-88.0860	2	-	<i>L. praerosa</i> 2
<i>Leptoxis praerosa</i>	Harperth River	Davidson	Tennessee	USA	36.0548	-87.0888	5	3	<i>L. praerosa</i> 3
<i>Leptoxis praerosa</i>	Clinch River	Tazewell	Virginia	USA	37.0883	-81.7681	1	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Clinch River	Russell	Virginia	USA	36.9631	-82.1205	1	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Clinch River	Hancock	Tennessee	USA	36.5306	-83.1509	1	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Clinch River	Scott	Virginia	USA	36.7369	-82.6200	1	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Middle Fork Holston River	Smyth	Virginia	USA	36.7961	-81.6813	2	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Powell River	Lee	Virginia	USA	36.6211	-83.2848	5	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	Nolichucky	Greene	Tennessee	USA	36.0979	-83.0515	1	-	<i>L. subglobosa</i>
<i>Leptoxis praerosa</i>	North Fork Holston River	Smyth	Virginia	USA	36.9275	-81.6734	2	-	<i>L. subglobosa</i>
<i>Leptoxis taeniata</i>	Coosa River	Shelby	Alabama	USA	33.3744	-86.3550	2	-	<i>L. taeniata</i>
<i>Leptoxis taeniata</i>	Choccolocco Creek	Taladega	Alabama	USA	33.5445	-86.0414	2	-	<i>L. taeniata</i>
<i>Leptoxis taeniata</i>	Buxahatchee Creek	Shelby	Alabama	USA	33.0727	-86.6775	2	-	<i>L. taeniata</i>
<i>Leptoxis umbilicata</i>	Smith Fork	Dekalb	Tennessee	USA	36.0851	-85.9089	3	-	<i>L. umbilicata</i>
<i>Leptoxis umbilicata</i>	East Fork Stones River	Cannon	Tennessee	USA	35.8293	-86.1784	5	-	<i>L. umbilicata</i>
<i>Leptoxis virgata</i>	Paint Rock River	Jackson	Alabama	USA	34.6873	-86.3102	4	-	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Sequatchie River	Sequatchie	Tennessee	USA	35.2465	-82.3759	4	1	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Holston River	Knox	Tennessee	USA	36.0087	-83.8249	6	3	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Hiwassee River	Polk	Tennessee	USA	35.2195	-84.5168	3	-	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Dry Creek	Dekalb	Alabama	USA	34.6958	-85.5592	2	-	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Tennessee River	Marion	Tennessee	USA	35.0359	-85.6537	2	-	<i>L. virgata</i>
<i>Leptoxis virgata</i>	Nolichucky	Greene	Tennessee	USA	36.0979	-83.0515	4	-	<i>L. virgata</i>
<i>Lithasia armigera</i>	Ohio River	Hardin	Illinios	USA	37.4710	-88.1410	1	-	<i>Li. armigera</i>
<i>Lithasia geniculata fuliginosa</i>	East Fork Stones River	Rutherford	Tennessee	USA	35.9417	86.3780	1	-	<i>Li. g. fuliginosa</i>
<i>Lithasia geniculata geniculata</i>	Duck River	Hickman	Tennessee	USA	35.8262	-87.6656	1	-	<i>Li. g. geniculata</i>
<i>Lithasia geniculata pinguis</i>	Collins River	Warren	Tennessee	USA	35.6752	-85.7917	4	2	<i>Li. g. pinguis</i> 1

<i>Lithasia geniculata pinguis</i>	Little Duck River	Coffee	Tennessee	USA	35.4864	-86.0913	4	3	<i>Li. g. pinguis</i> 2
<i>Lithasia obovata</i>	Ohio River	Crawford	Indiana	USA	38.1942	-86.3446	3	-	<i>Li. obovata</i>
<i>Lithasia verrucosa</i>	Ohio River	Massac	Illinios	USA	37.1822	-88.7912	1	-	<i>Li. verrucosa</i>
<i>Pleurocera acuta</i>	Salt River	Bullitt	Kentucky	USA	34.9853	-85.7158	1	-	<i>P. acuta</i> (Salt River)
<i>Pleurocera acuta</i>	North Fork White River	Ozark	Missouri	USA	36.6432	-92.2258	2	-	<i>P. acuta</i> (N. Fork White River)
<i>Pleurocera foremani</i>	Yellowleaf Creek	Shelby	Alabama	USA	33.2592	-86.4508	1	-	<i>P. foremani</i>
<i>Pleurocera prasinata</i>	Alabama River	Wilcox	Alabama	USA	32.0676	-87.4020	2	-	<i>P. prasinata</i>
<i>Pleurocera pyrenella</i>	Limestone Creek	Limestone	Alabama	USA	34.6314	-86.8864	1	-	<i>P. pyrenella</i>
<i>Pleurocera unciale</i>	North Fork Holston River	Smyth	Tennessee	USA	36.9534	-81.5273	1	-	<i>P. unciale</i>
Undescribed pleurocerid	Collins River	Warren	Tennessee	USA	35.6752	-85.7917	3	-	Undescribed

† These are the number of individuals that were removed from analyses after sequencing of NUMTS

^ OTU designations corresponding to Figure 5.6

Table 5.3: Partitioning schemes for MrBayes and *BEAST phylogenetic inference

<u>MrBayes</u>		
Gene	Partition scheme	Model(s)
COI	by codon	SYM+G, F81+I, GTR+G
16S	-	GTR+G
28S + H3	combined nuclear genes	GTR+G

<u>*BEAST</u>		
Gene	Partition scheme	Model(s)
COI	by codon	TrNef+G, F81 +I, GTR +G
16S	-	TVM+G
H3	-	TIM+G
28S	-	TIM+G

Figure 5.1: Bayesian phylogram inferred with mitochondrial genes with *Cleopatra* dropped for visualization purposes. Numbers in front of nodes are posterior probabilities for each node.

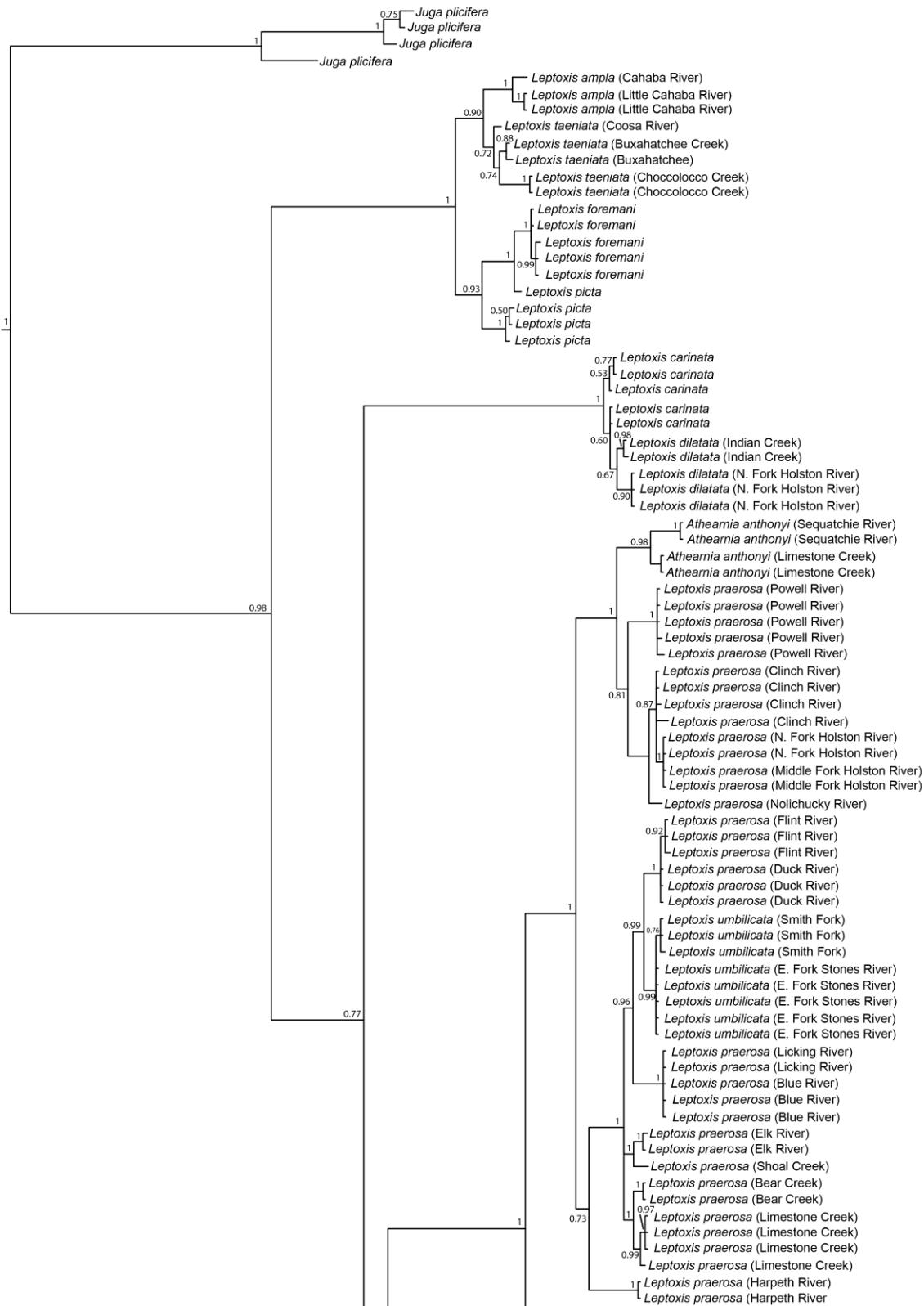


Figure 5.1 (continued)

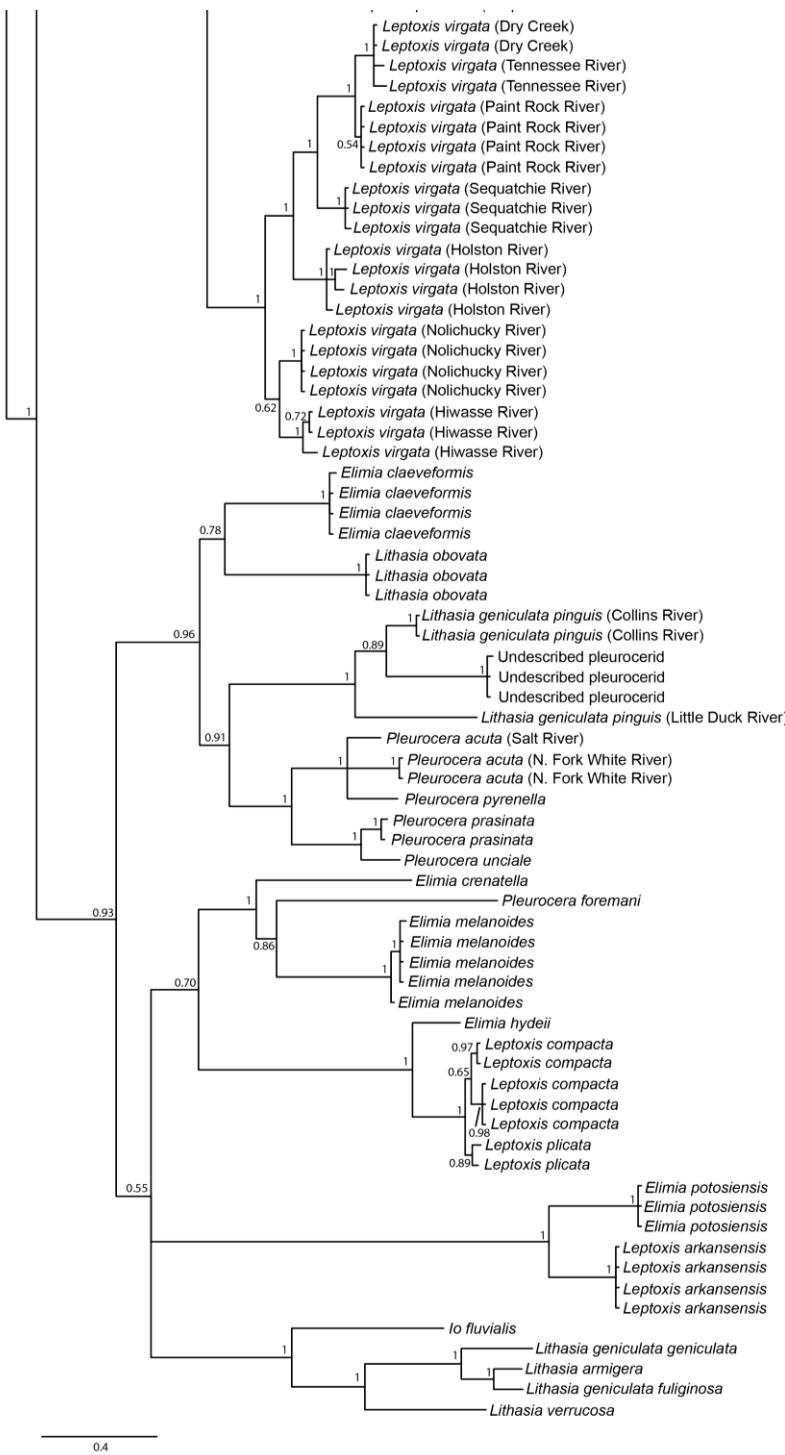


Figure 5.2: Bayesian phylogram inferred with 28S with *Cleopatra* dropped for visualization purposes. Numbers in front of nodes are posterior probabilities for each node.

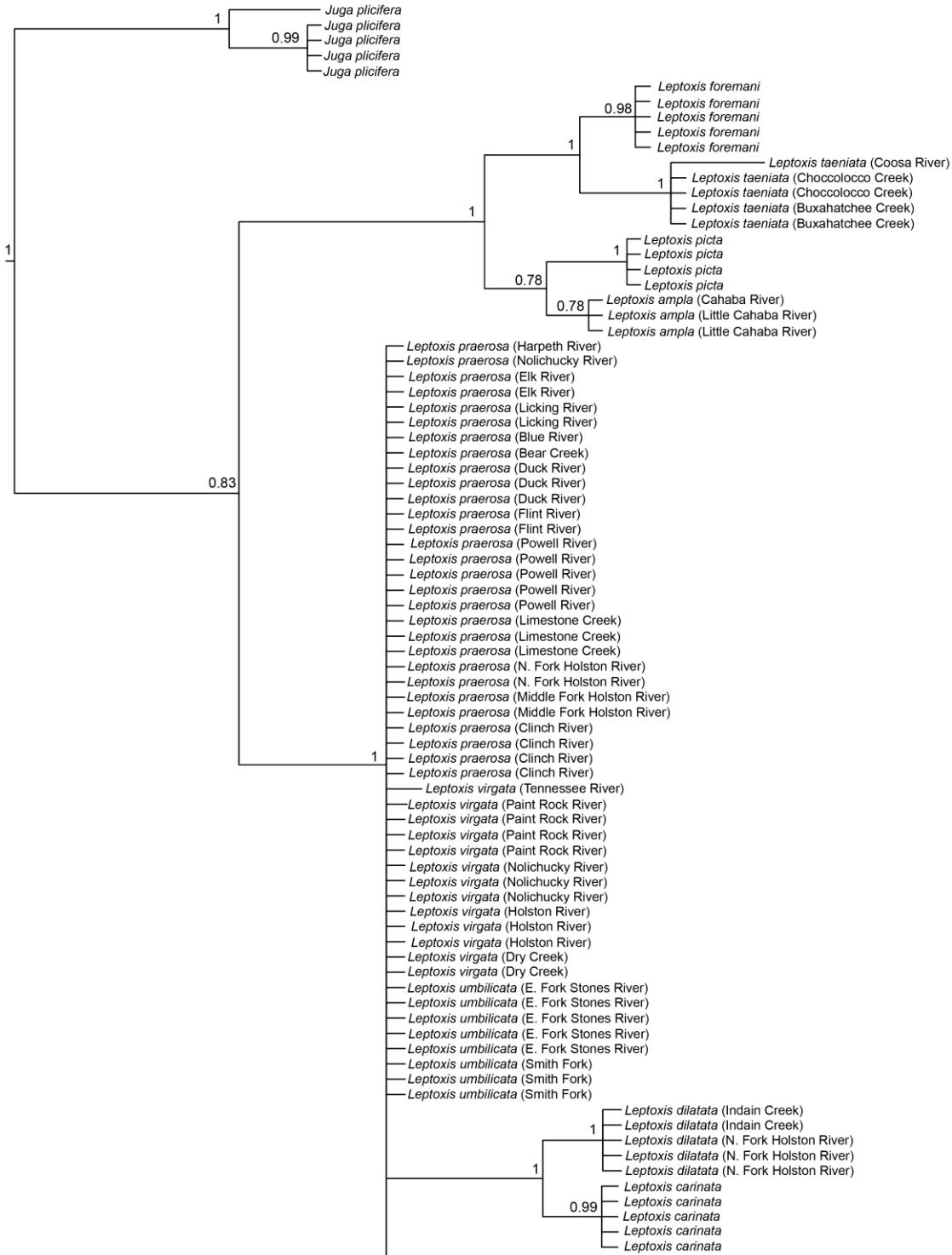


Figure 5.2 (continued)

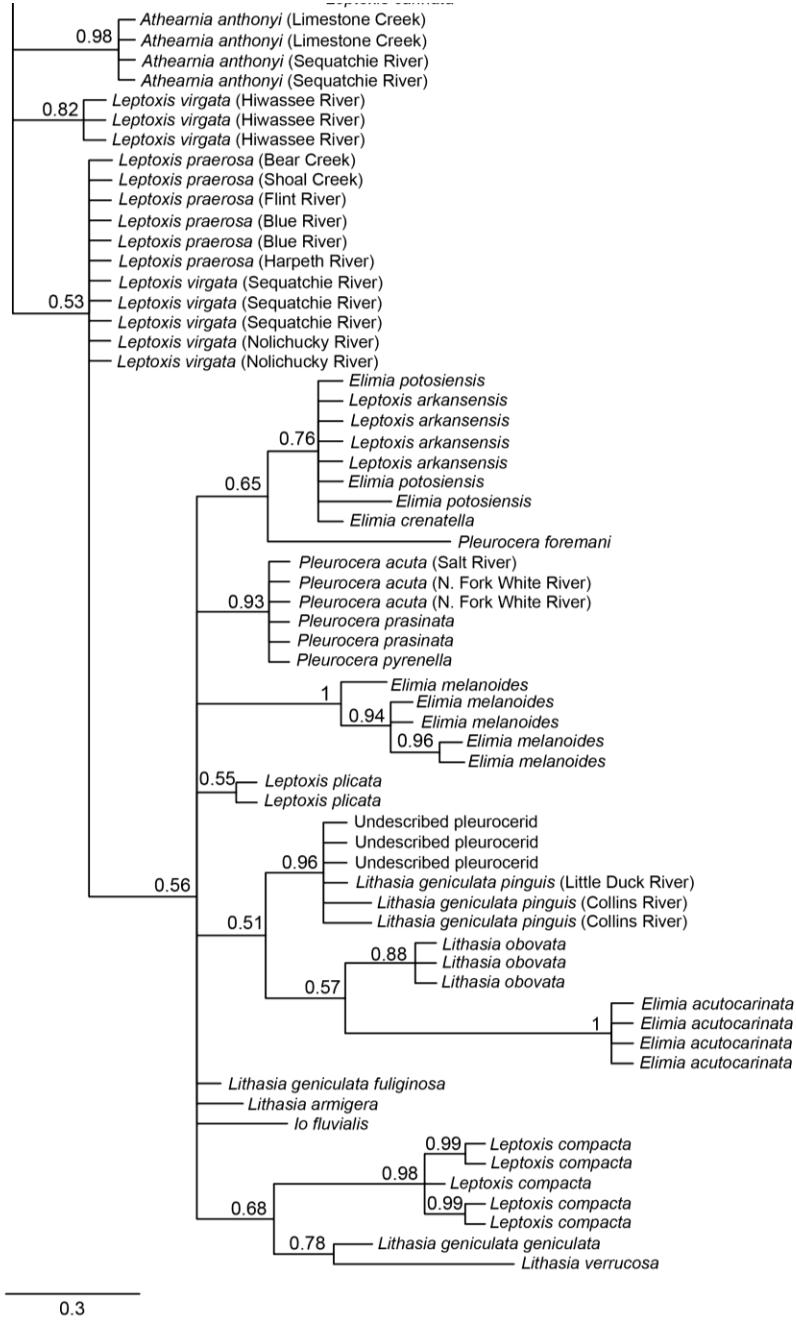


Figure 5.3: Bayesian phylogram inferred with H3 with *Cleopatra* dropped for visualization purposes. Numbers in front of nodes are posterior probabilities for each node.

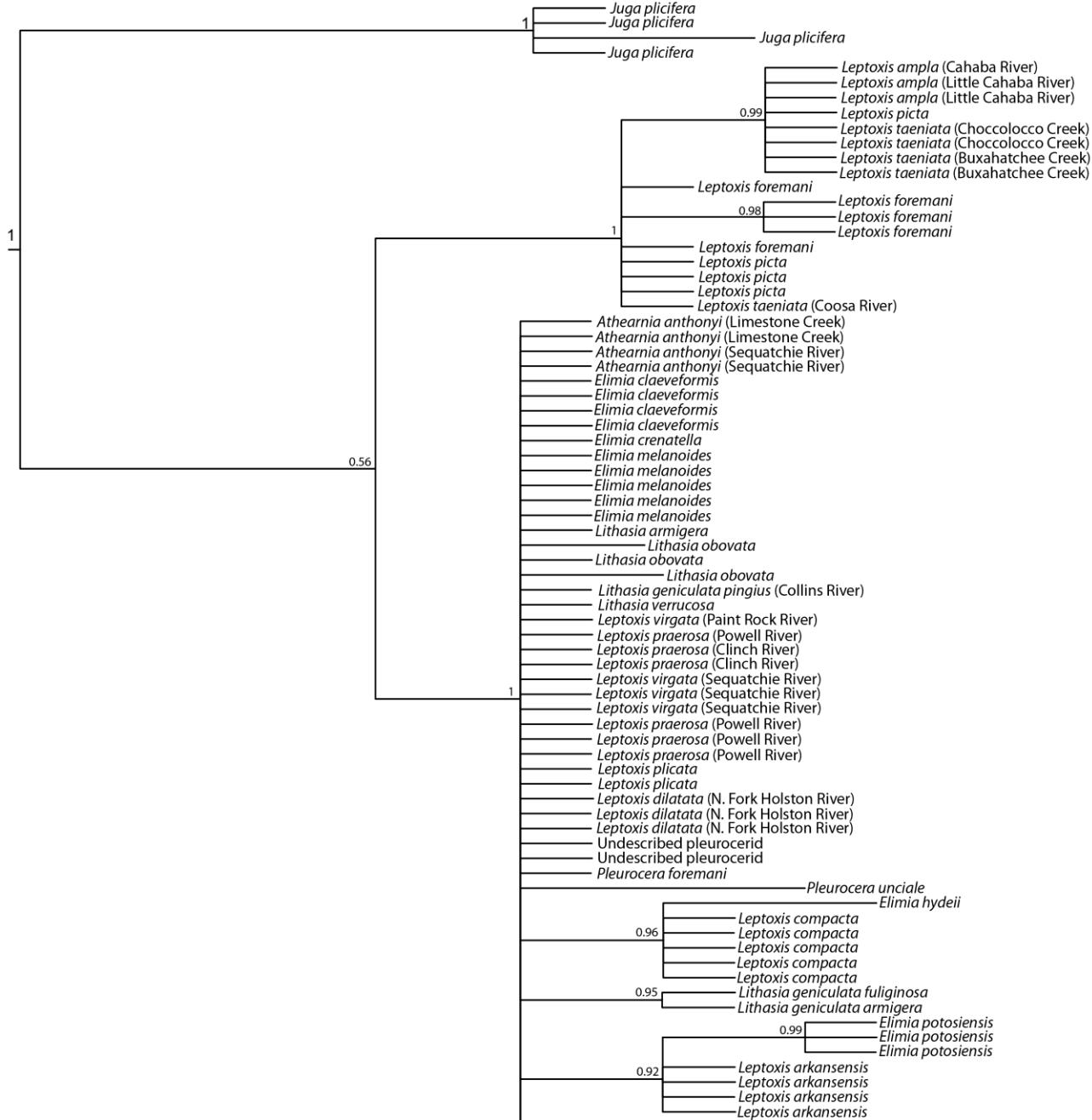


Figure 5.3 (continued)

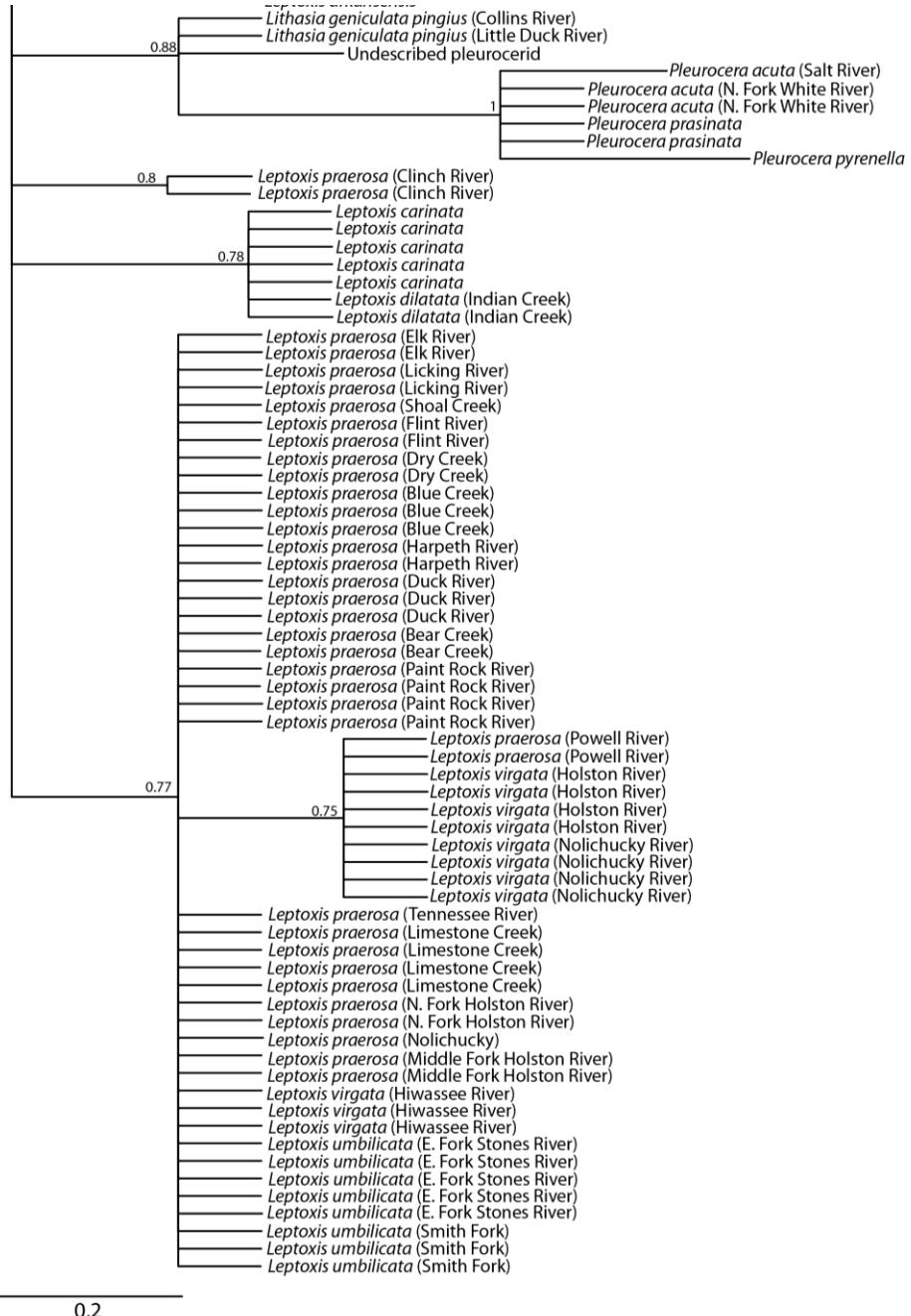


Figure 5.4: Bayesian phylogram inferred with the concatenated dataset with *Cleopatra* dropped for visualization purposes. Numbers in front of nodes are posterior probabilities for each node.

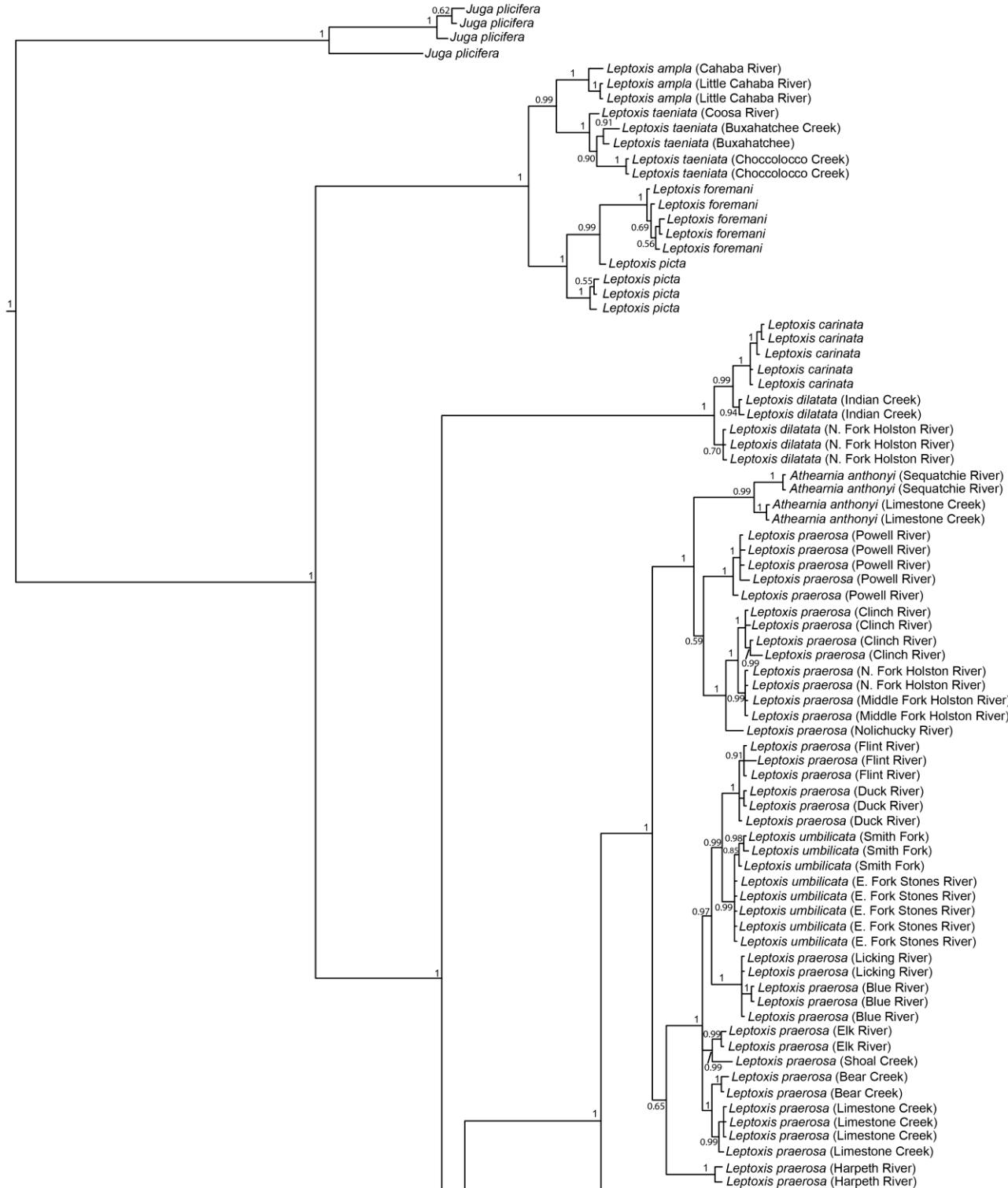


Figure 5.4 (continued)

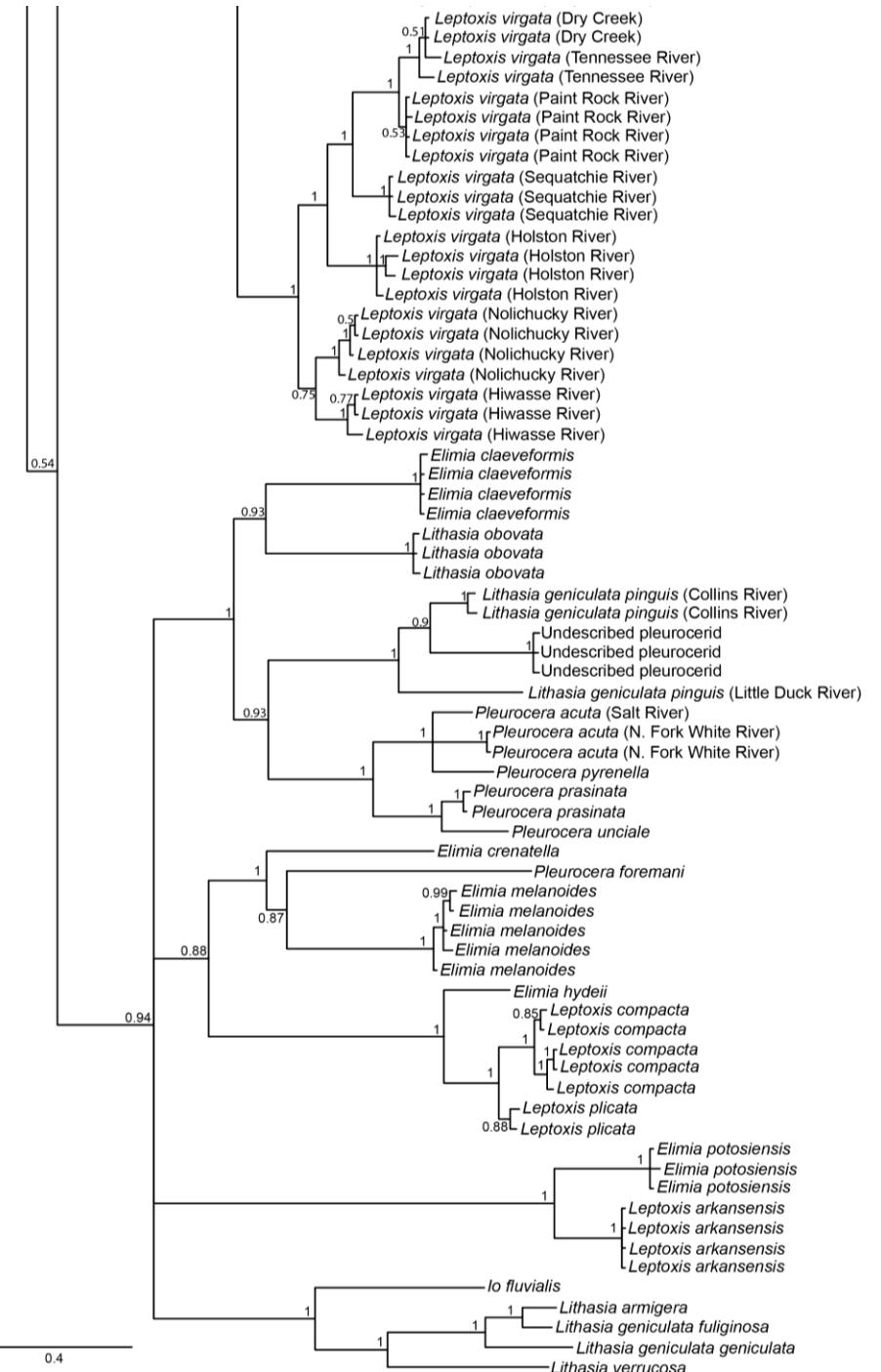


Figure 5.5: *BEAST species tree. Labels in front of nodes are posterior probability support values. The three horizontal bars are next to the *Leptoxis* (s.s.) clade and the two clades with elevated or new genus names.

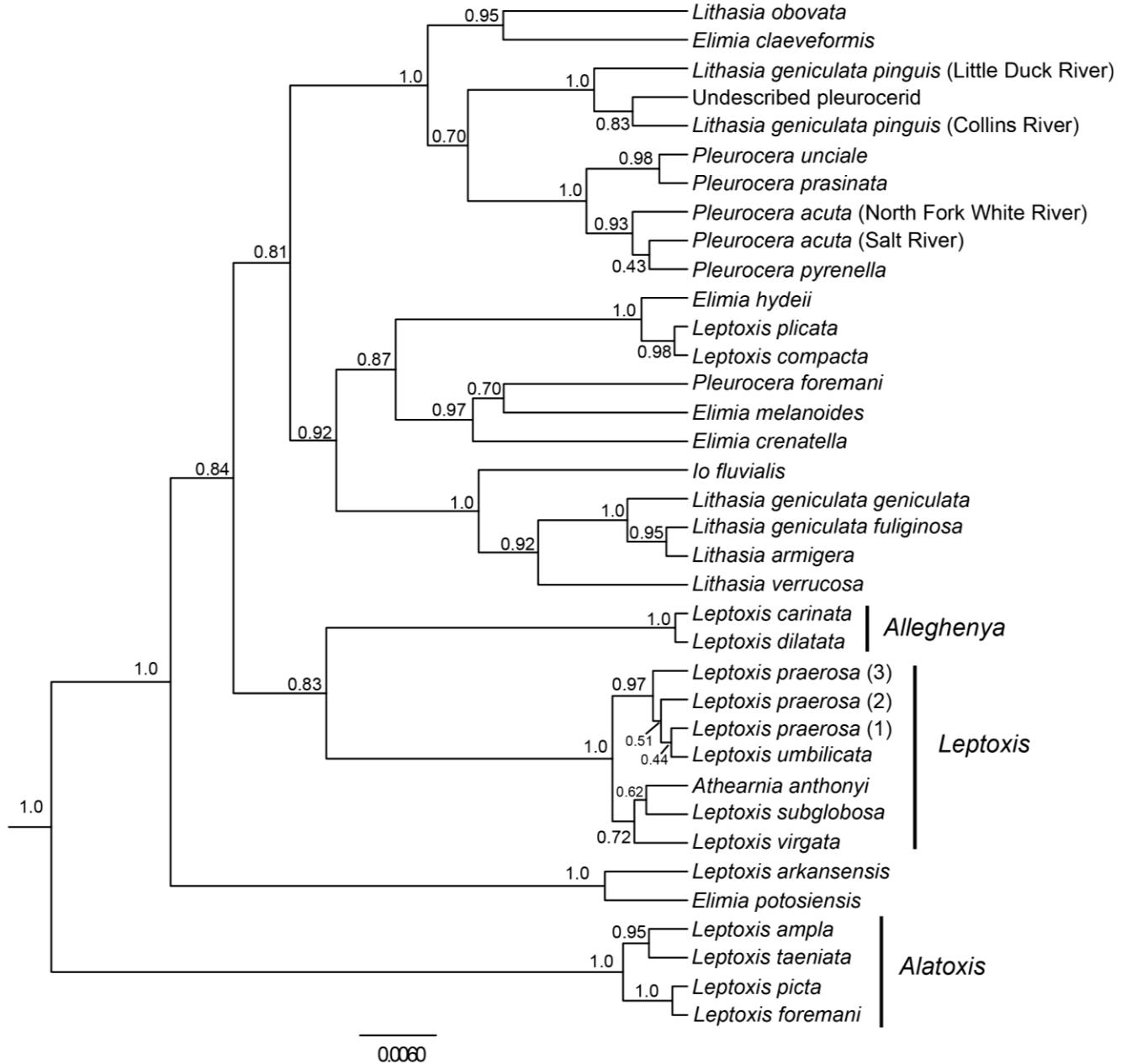


Figure 5.6: Photographs of live *Leptoxis*. A) *L. ampla* B) *L. picta* C) *L. virgata* from Dry Creek D) *L. arkansensis* E) *L. dilatata* from Indian Creek F) *L. dilatata* from the North Fork of the Holston River.



CHAPTER 6

OVERALL CONCLUSIONS

The ecological importance of pleurocerid gastropods and their high levels of imperilment make understanding their diversity and unique genetic patterns not only an interesting scientific endeavor, but it also one of considerable practical application. For example, details concerning the life history of *Leptoxis* will allow for better captive propagation of imperiled species. Moreover, the conclusions of this dissertation on pleurocerid and *Leptoxis* systematics will enable more effective communication about pleurocerids, and it has revealed that NUMTs are easily sequences in pleurocerids and that they have been confused as mitochondrial homologs for over a decade. Overall, the findings presented here represent a significant advancement in the study of freshwater biodiversity in eastern North America.

The positive identification of *L. compacta* in the wild is a welcome finding at a time when conservation stories are often negative. However, *L. compacta* still faces a long road to recovery. Land use in the Cahaba River watershed is increasing at a surprising rate as the population of the Birmingham metropolitan area increases. As such, urban run-off and siltation from construction and land clearing threatens the sole remaining *L. compacta* population. A captive propagation plan is in place at the Alabama Aquatic Biodiversity Center so a second population of *L. compacta* can be established within the species' historical range. Hopefully, the rediscovery of this enigmatic snail will ensure its long-term survival.

Of course, before pleurocerid conservation plans can be effective the true extent of the family's diversity must be recognized since unrecognized species inherently cannot be the focus

of management plans. In chapter three, I explored the cause of chronic species-level paraphyly on mitochondrial genomes in pleurocerids and semisulcospirids. The only hypothesis not rejected by our analyses is that highly “divergent” haplotypes are NUMTs. These findings should invigorate pleurocerid systematic studies, and it reveals that mitochondrial genes can and will be useful in inferring pleurocerids phylogeny.

The diversity of egg laying strategies within *Leptoxis* is striking. Species have evolved the behavior of either laying single eggs, eggs in a line, or circular clutches. Future experimental studies have the potential to reveal the selective advantages of these different behaviors, but I hypothesize that clutches may provide some level of protection from dislodgement from the substrate and/or protection from predators. Perhaps most interesting is the significant differences in egg clutch size observed in all three comparisons. First, the average egg clutch size among clutch laying *Leptoxis* species is significantly different. Second, average egg clutch size was also significantly different between populations of the same species, which indicates species and populations quickly evolve optimal clutch sizes as a response to their natural environment. Finally, as individuals age, the average clutch size increases. This has been a long held hypothesis, and corroborating it suggests that laying larger clutch sizes is a strategy that helps off balance natural death and fewer females at older life stages. The findings of this chapter have important implications for understanding population dynamics, and provides concrete data on cues for egg laying in captivity—important information for management agencies.

Finally, chapter five demonstrates conclusively that *Leptoxis* is not a monophyletic lineage. The results of this chapter represent the culmination of a multi-year effort to explore the systematics of *Leptoxis*. The data gathered for chapter five is the most extensive taxon and character sampling in an evolutionary study of pleurocerids to date. *Leptoxis* (*s.l.*) is now split

into three genera, and *L. plicata* *L. compacta* have been transferred to *Elimia*. *Athearnia* is not a valid genus but rather a junior synonym of *Leptoxis*. We confirm *L. subglobosa* as a valid species, and our data suggest that *L. umbilicata* is also a valid species but more work is needed on the *L. praerosa*/*L. umbilicata* species complex. Even though this study is the most extensive systematic analysis of pleurocerids thus far, much research is needed to clarify relationships among genera and species. Genomic resources and more extensive sampling of *Pleurocera* and *Elimia* will be integral to finally solving the problem of pleurocerid taxonomy.

Every chapter in this dissertation will serve as a resource for the conservation of imperiled *Leptoxis*. The threats to biodiversity in the rivers of the eastern United States are clear, and many species, including 33 pleurocerids are already extinct. However, conservation successes like the discovery of *L. compacta* and current efforts to extend the species' range provide hope that the health of our freshwater resources will improve. The advancements this dissertation has made towards understanding pleurocerid diversity will provide a foundation for future systematic and evolutionary studies in this important and interesting family of freshwater gastropods.

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