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Species Delimitation among Southeastern US Oxyloma (Gastropoda: Succineidae)

Marco A. Martinez Cruz
The University of Texas Rio Grande Valley

SPECIES DELIMITATION AMONG SOUTHEASTERN US OXYLOMA (GASTROPODA: SUCCINEIDAE)

A Thesis

by

MARCO A. MARTINEZ CRUZ

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COMMITTEE MEMBERS

Dr. Kathryn E. Perez
Chair of Committee

Dr. Christopher Vitek
Committee Member

Dr. Erin Schuenzel
Committee Member

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ABSTRACT

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The Succineid genus *Oxyloma* found throughout Canada and United States contains approximately 15 described species whose criterion for differentiation is considered unreliable. As a first step towards understanding the evolutionary history and revising the taxonomy of North American *Oxyloma*, we have sampled four species found in eastern North America (*O. salleana*, *O. subeffusa*, *O. effusa*, and *O. retusa*) from their type localities. We used mitochondrial COI, and nuclear LSU sequences with samples found across their range and members of the family to produce a phylogenetic hypothesis of evolutionary relationships and test species boundaries. Molecular phylogeny and species delimitation analyses using mitochondrial and nuclear data finds three monophyletic groups among the four nominal *Oxyloma* species, confirming doubts concerning the validity of these species.

DEDICATION

This work is dedicated to my ever-supporting mother and brother – Gemma, Luis, this is for you – and to the Light of this life, without whom nothing would be possible: Among these pages are the species that were given to me, and I have named them after their own kind as they appear, according to the rudiments and practices of our time, so that others may know them and see that they exist.

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CHAPTER I

INTRODUCTION

Taxonomy

Succineidae Beck 1837 (Gastropoda: Stylommatophora) is a land snail family with four genera, found in all continents with the exception of Antarctica (Pilsbry 1948), and recognized as the snail family with the most desperate need for revisionary work (Nekola 2014). Of the four succineid genera, *Oxyloma* Westerlund 1885 is the most difficult having been originally described as being at a "makeshift stage" (Pilsbry 1948). *Oxyloma* has ~ 15 described species in North America, although these are ill-defined and with questionable status due to the similarity of the species' morphology (Pilsbry 1948, Miller, Stevens et al. 2000, Stevens, Keim et al. 2001, Nekola 2014). *Oxyloma* can be found in North America, Europe, Asia, and South Africa (Patterson 1971), with the North American taxa described by Pilsbry (1948) as all belonging to the *effusa* group. The *effusa* group contains the northeastern American section with four morphologically similar species: *Oxyloma retusa*, *O. salleana*, *O. effusa*, and *O. subeffusa*. In this study, we examine the phylogenetic relationships and species status of the members of the *effusa* group.

Species Review

Oxyloma retusa (I. Lea 1834), the blunt ambersnail (Figure 1), possesses a thin, translucent shell of yellow coloration resembling an elongated oval, with a short spire, with an

aperture and dilation different from all the other described succineid species at the time of its description (Lea 1834). Its biographical range spans most of the northeastern United States (Figure 9) (Binney 1878, Pilsbry 1948, La Rocque 1953, Franzen 1964, Hubricht 1985) and eastern Canada. The type locality for *O. retusa* was described as: "near Cincinnati, Ohio" (Lea 1834). *O. retusa* is found in habitats associated with still and flowing water, such as marshes, margins of ponds, small streams, and permanent lakes (Lannoo and Bovbjerg 1985, Örstan 2010), and also crawling on mud and on the stems of *Typha latifolia* (cattails), seldom away from the low water (Hubricht 1985, Örstan 2010).

Oxyloma salleana (Pfeiffer 1849), the Louisiana ambersnail (Figure 2), possesses a thin, translucent, striated and relatively depressed shell resembling an elongated oval, with irregular spiral lines, and is of light yellow coloration (Pfeiffer 1849) easily recognized by its minute spire and long aperture (Tryon 1866). Its biogeographic range appears limited to regions along the Mississippi river (Figure 9) (Pilsbry 1948, Patterson 1971, Hubricht 1985, Miller, Stevens et al. 2000, Stevens, Keim et al. 2001). The type locality for *O. salleana* was described as: "Near New Orleans, Louisiana" (Pfeiffer 1849). It can be found in wetland habitats, such as marshes, margins of ponds, small rivers and permanent lakes, crawling on mud and on the stems of *Typha latifolia* (cattails) (Hubricht 1985), and bunches of sedges (Frierson 1900).

Oxyloma effusa (Pfeiffer 1853), the coastal-plain ambersnail (Figure 3), possesses a thin, delicate, depressed shell resembling an elongated oval, of a coloration that resembles dilute straw yellow and dilute cream (Pilsbry 1948) with a singularly short spire (Pfeiffer 1853, Binney 1878). Its biogeographic range encompasses the state of Florida primarily, with a few records reported in Maryland, Virginia, North Carolina, and the District of Columbia (Figure 9) (Binney 1878, Pilsbry 1948, Steury and Pearce 2014). The type locality for *O. effusa* is described as:

"*Florida orientali* [Eastern Florida]" (Pfeiffer 1853). It can be found crawling on the underside of *Sagittaria*, a plant that lives in marshy habitats, and on stems of *Typha*, and rarely on the ground (Hubricht 1985).

Oxyloma subeffusa Pilsbry 1948, the Chesapeake ambersnail (Figure 4), possesses a fragile, thin shell, of a mixture of coloration between dim yellow and dull gray with noticeable growth wrinkles (Pilsbry 1948). *O. subeffusa* can be recognized from all the other *Oxyloma* in that its shell is smaller than the body which makes complete retraction impossible (Pilsbry 1948). Its biogeographic range spans the eastern states of Virginia, Maryland, Pennsylvania, New Jersey, including the District of Columbia (Figure 9) (Pilsbry 1948, Hubricht 1985). The type locality for *O. subeffusa* is described as: "Plum Point, above Riverton, New Jersey" (Pilsbry 1948). It can be found near marshes, rivers, and ponds (Hubricht 1985).

CHAPTER II

SPECIES CONCEPT AND DELIMITATION

The name of a species is a keystone of biological investigations, and therefore the way to correctly define a name is as well. A species name can have extensive practical significance, given that a species' name carries financial, legal, and conservation significance (Hey, Waples et al. 2003). As such, dozens of approaches that attempt to define what a species is, and what it is not, have been formulated, often with acrimonious disagreement among the authors and adherents to different species definitions or concepts. The challenge, as always, is application of idealized, theoretical concepts of what determines a species to the messiness of nature. The task of formulating an evidence-based hypothesis of the boundaries that define a species, to a group of organisms with a complex history and undergoing ongoing evolution by the application of applying a theoretical species concept is species delimitation (De Queiroz 2007).

Currently, more than 24 species concepts can be found in the literature, including those built towards a more generalized approach (Mayden 1997, Wilkins 2009, Hausdorf 2011). From the 24 listed by Mayden (1997), the evolutionary species concept (Simpson 1951) and its descendant the unified species concept (De Queiroz 2007), the biological species concept (Mayr 1942), and the phylogenetic species concept (Eldredge and Cracraft 1980, Nelson and Platnick 1981, Cracraft 1983, Nixon and Wheeler 1990), are most widely used among systematists and taxonomists

The Biological Species Concept

The biological species concept was proposed (Mayr 1947) to fulfill the need left by the inadequacy of a species concept based purely on morphology (typology). It proposed a species as a group of populations that can interbreed but are reproductively isolated from other groups (Dobzhansky 1937, Mayr 1947, Mayr 2000), making interbreeding between different groups the primary criteria for species delimitation – an event that, even if possible under laboratory conditions, does not always translate as true among the fauna. This concept was criticized as misleading in that its descriptions of a species are largely still based on morphology camouflaged by a sense of evolutionary individuality (Blackwelder 1962, Sokal 1962, Sokal and Crovello 1970). It also was found to be highly limited and virtually inapplicable among organisms such as fossils, non-eukaryotes, and those that are exclusively asexual (Ehrlich 1961, Mishler and Donoghue 1982).

The Evolutionary Species Concept

The evolutionary species concept, formulated by Simpson (Simpson 1951, Simpson 1961), was conceived from the idea of species retaining their characters over time, a dimension, which the biological species concept lacked, rejecting definitions purely based on morphology or reproduction and focusing on a species as having its own fate and its own evolutionary history (Simpson 1961, Hennig 1966, Wiley 1978).

The Phylogenetic Species Concept

The phylogenetic species concept (Eldredge and Cracraft 1980, Nelson and Platnick 1981, Cracraft 1983, Nixon and Wheeler 1990) proposed the classification of organisms on the

basis of monophyly and diagnosability (Mishler and Donoghue 1982), and defined a species as the smallest distinguishable unit in which patterns of ancestry and descent can be observed (Eldredge and Cracraft 1980, Mishler and Brandon 1987, Mishler and Theriot 2000).

Species Delimitation in Land Snails

Land snails have dramatic intraspecific variation in shell morphology (Vermeij 1995, Schilthuizen 2003), and although these characters have been employed in the past as species delimiters, this variation is also attributable to habitat and environmental factors (Goodfriend 1986, Emberton 1994), which makes their utility uncertain and sometimes unreliable for species delimitation work (Palmer 1985, Bickford, Lohman et al. 2007). These identifying characters have triggered multitudes of disagreements that can further complicate the taxonomy, as illustrated by the case of the terrestrial pulmonate *Trochulus*. A polymorphic genus, *Trochulus* has experienced sharp species numbers fluctuations, such as being reduced from 55 to 3 distinct species at one point in time, even when looking at the same morphological and anatomical evidence (Forcart 1965, Procków 2009, Welter-Schultes 2012, Proćków, Drvotová et al. 2013).

In Succineidae, morphological characters are difficult, unreliable, and even fail to diagnose a level beyond the family name (Kerney, Cameron et al. 1996). Relative sizes and shapes of reproductive system organs have been traditionally used in gastropods as systematic characters (Madec and Guiller 1994), and succineid identification has heavily relied on distal genitalia (Quick 1933, Patterson 1971, Schileyko 2007). But, even if the reproductive parts have been considered as having taxonomic advantages, their variation within a species has rarely been studied (Arnqvist 1997), genitalia environmental dependency remains to be determined (Dépraz, Hausser et al. 2009), and its study requires a high level of specialization due to the dissections

being minute and the sexual size ratio incredibly subtle (Schileyko 1978). Most importantly, reproductive anatomy studies and cross-breeding have failed to mark distinctions between nominal North American snail species (Burch and Ayers 1973, Remigio and Blair 1997). Succineidae identification worldwide remains where it has been stuck for nearly 100 years, with no taxon reliably identifiable below the level of "Succineidae."

Species Delimitation in this Study

In this study, we use the Phylogenetic Species Concept (PSC) as the practical, operational framework for species delineation. We use the PSC because, first, it allows us to diagnose a species by using a unique combination of characters, whether the individuals are sexual or not (Platnick 2000), and decreases delineation errors between species by focusing on novel traits that are particular to a species and its descendants, as in the case of apomorphies (Wheeler 1999). Secondly, other species delimitation concepts do not give us the species resolution the PSC can achieve. They rely on diagnosable traits that are not reliable in Succineidae: Shell characters that give inadequate and unreliable systematic aid, and highly variable genitalia structure between maturity stages that is confusable, because the anatomical features of one species can be matched to that of another (Nekola 2009).

In the case of the Evolutionary Species Concept (ESC), a species is defined as the line pertaining to populations of organisms that maintain their own identity apart from other populations through time (Wiley 1978), which makes the concept highly regarded by evolutionary biologists and difficult to practically apply. However, the ESC is theoretical but hard to apply operationally, so its strength is null if not backed by another concept (Avise and Wollenberg 1997), which makes the concept unhelpful when attempting to delimit a species

because the ESC lacks recognition criteria for species delimitation (Wheeler and Meier 2000); therefore, the ESC alone cannot support a formal succineid delimitation. Contrary to the ESC, the Phylogenetic Species Concept (PSC) can be used as a stand-alone concept. It possesses highly applicable criteria for species delimitation, which have been previously used to recognize species, resolve evolutionary incongruences in gastropod families, and revise classification, including Succineidae (Rundell, Holland et al. 2004, Dayrat, Conrad et al. 2011, Neiber and Hausdorf 2015, Razkin, Gómez-Moliner et al. 2015, Neiber, Sagorny et al. 2016, Bouchet, Rocroi et al. 2017, Neiber, Razkin et al. 2017). Furthermore, the PSC is consistent with the ESC in species recognition, and it performs better than all other concepts given that once descendant species have diverged from an ancestor, it can recognize genetic changes before any subsequent change in morphology or mating behavior (Taylor, Jacobson et al. 2000). As a result, the phylogenetic species concept is the most advantageous species delimitation concept to use in this study of Succineidae.

Molecular Information as a Marker for Evolution

After being established by Linnaeus, taxonomic and species identification work relied for more than two-hundred years on a system highly rooted in morphology (Linneaus 1753, Linnaeus 1758). However, this traditional practice of naming species solely by morphology does not account for the environmental plasticity, genome variability, gender, and life-stages of an organism (Tautz, Arctander et al. 2003), and after the number of taxonomic specialists decreased, the science of taxonomy has suffered, leading to species descriptions bottlenecks (Boero 2001, Tautz, Arctander et al. 2003, Blaxter 2004, Gaston and O'Neill 2004, de Carvalho, Bockmann et al. 2007). With time, an overwhelming issue became apparent: evolutionary history

became threatened to be destroyed before being documented. This became known as the "taxonomic bottleneck" (Wilson 1985, Wheeler and Cracraft 1996) leading to 21st century calls for a new and universal platform for species identification and taxonomy, involving automation and DNA-science taking a more central role in a new "Molecular (or DNA) Taxonomy" (Tautz, Arctander et al. 2002, Hebert, Cywinska et al. 2003, Tautz, Arctander et al. 2003). Criticisms of an exclusively molecular focus led to the development of Integrative Taxonomy, which includes aspects of molecular and traditional taxonomy (Dayrat 2005) by the available data, with the understanding that future data collection and work could revise a the taxonomic hypothesis (Yeates, Seago et al. 2011). In this study, we present a phylogenetic analysis and preliminary taxonomic hypothesis for southern and eastern US *Oxyloma* by applying the phylogenetic species concept to *Oxyloma*.

CHAPTER III

MATERIALS AND METHODS

Selection of Taxa

The taxa included in this study (Table 1) comprise a group of morphologically similar *Oxyloma*, the *effusa* group, from Eastern and Southeastern North America (Pilsbry 1948). We sampled the type localities (these individuals are referred to as topotypes) and when possible, individuals from other parts of the known range of the species. Any material from *Oxyloma* for our target genes that was available on GenBank and could be aligned was also included. We also included representatives from other species from the genera *Succinea, Oxyloma*, and *Hyalimax* in the family Succineidae as outgroups.

Sampling of Topotypes

To allow these data to be used for future taxonomic revision, specimens collected from the type locality of each species, "topotypes" were required. Information on the type locality of each *Oxyloma* species was taken from Pilsbry (1948) as well as the original descriptions of each taxa (Lea 1834, Pfeiffer 1849, Pfeiffer 1853, Pilsbry 1948). In the case of taxa with geographically vague information (e.g. "Habitat in Florida orientali" is listed as the type locality for *O. effusa*), the type locality was determined by reference to the species author's and collector's original materials, or geographic information from their other collections on the collecting expedition when *Oxyloma* material was acquired. Additionally, non-type, target

locations for sampling were taken from the online database records for *Oxyloma* and *Sagittaria* (a common plant that is associated with the same habitat) from the Florida Museum of Natural History and the Field Museum of Natural History. These sites were sampled as possible during two major collection trips focused on sampling type localities.

Specimen Selection

Specimen collection was carried out at our selected locations and performed by hand. At two of our southern locations (Spring Garden Lake, the outflow of Ponce de Leon Springs, Ponce de Leon State Park in Florida; and the intracoastal waterway at Lake Salvador, near Jean Lafitte in Louisiana), the collection was aided by the use of a canoe, wading in those locations was inadvisable due to high alligator abundance. At those sites, *Oxyloma* populations were identified on tussocks, floating mats of vegetation such as water hyacinth and smartweed in the water away from the shore or in cattails emergent near shore. Specimen collections carried out at our two northeastern locations (Greater Miami River, near Shawnee Lookout in Ohio; and, Delaware River, at Plum Point in New Jersey), were approached by foot due to difficulties with river current speed, and canoe safety in highly channelized rivers. The specimens collected were taken from rotten logs and mud in Ohio, and from rotten timber in pooled water in New Jersey, both on the banks of the major rivers. Once collected, all specimens were secured in glass flasks containing 70% molecular grade non-denaturing ethanol solution. After preserving overnight, the ethanol was removed, and the samples stored with fresh 70% molecular-grade non-denaturing ethanol solution.

Sample Preparation and DNA Extraction

We washed our specimens to rid them of soil contaminants using 90% molecular-grade non-denaturing ethanol solution. After the initial wash we cut a piece of tissue from the foot of the snails for total cellular DNA isolation employing a modified procedure based on a hexadecyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof, Soliman et al. 1984): Approximately 100 mg of freshly cut foot tissue was placed in 600μL of extraction buffer consisting of 100mM tris base, 1.4M sodium chloride, 20mM ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA), 2% hexadecyltrimethylammonium bromide (99+%), and 0.2% 2-β-mercaptoethanol. To each extraction reaction, we added 25μL of Proteinase K (100μg/mL), and incubated it at 37°C for 24 hours, occasionally vortexing for 3 seconds each time. At the end of the incubation period we directly applied 600μL of phenol/chloroform/isoamyl alcohol (25:24:1 pH 6.7) to the solution, mixed by inversion for 5 minutes and centrifuged for 10 minutes at 21,130 rcf at 4°C. At the end of the centrifugation period the top aqueous layer was kept, and the phenol layer discarded. The aqueous layer was subsequently mixed with 600μL of chloroform/isoamyl alcohol (24:1), mixed by inversion for 5 minutes and centrifuged for 10 minutes at 21,130 rcf at 4°C. At the end of the centrifugation period the resulting top aqueous layer was again kept, and the chloroform phase discarded. Then, 600μL of ice-cold isopropyl alcohol was incorporated into the solution, and held for 24 hours at -8°C to maximize nucleic acid precipitation. To pellet the precipitated DNA, it was centrifuged for 10 minutes at 21,130 rcf at 4°C, dried for 15 minutes, and resuspended in 50μL of Tris-EDTA-RNase A (10mM: 1mM: 10mg/mL). The resuspended DNA was then purified again using the Gel/PCR DNA fragment extraction kit (IBI Scientific IB47030), following instructions provided by the manufacturer. This secondary extraction step is not always necessary, but it enhances PCR success in snails

with high mucous production which *Oxyloma* displayed. Quality and concentration of the extraction was assessed through electrophoresis in which 0.75g of agarose were incorporated with 6µL of ethidium bromide (10mg/mL) and 75mL of 1X Tris-Borate-EDTA (TBE) buffer resulting in a 1% gel matrix. The gel was run at 120V using 1X TBE as buffer and subsequently visualized under UV light.

Genetic Data

After total cellular DNA isolation and purification, we used the resulting DNA as template to amplify the following gene fragments: The Folmer region of the mitochondrial cytochrome oxidase subunit I (COI), and the LSU region of the ribosomal (R) RNA gene-cluster which is a nuclear region that includes a small section of the 5.8S region, the entire internal transcribed spacer 2 (ITS-2), and part of the large 18S region. Amplification of these genes was carried out in a thermal cycler using primers flanking the 5' and 3' regions of each gene. The primers used can be found in Table 2 and the temperature profiles in Table 3. Amplification of LSU resulted in multiple sized amplicons.

Following initial visualization, the LSU samples were run at 90V using a 1X TBE buffer and a 1% TBE gel using low-melting point agarose, the band at the correct size for the target fragment was excised and extracted using the Gel/PCR DNA fragment extraction kit (IBI Scientific IB47030) prior to sequencing. Sequencing of our amplified gene samples was carried out by Eurofins Genomics (www.eurofinsgenomics.com) using the Sanger method of sequencing, employing fluorescent dye termination labeling and capillary-array electrophoresis, working with our PCR amplification primer pairs as sequencing primers for each corresponding sample. Geneious version 10.2.3 [www.geneious.com, (Kearse, Moir et al. 2012)] was used for

sequence data processing in the following steps: sequences were trimmed, assembled into contigs, checked manually for conflicting base-callings, and consensus sequences created.

Phylogenetic Analyses

Consensus sequences for each individual for COI and LSU were aligned along with selected, available sequences from GenBank (Table 1) using MUSCLE as implemented at Phylogeny.fr (www.phylogeny.lirmm.fr) (Dereeper, Guignon et al. 2008). MUSCLE alignments were refined through Gblocks 0.91b (Castresana 2000, Talavera and Castresana 2007) using the "A la Carte" mode and the least stringent conditions, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. After the consensus sequences were aligned, we obtained phylogenetic trees in two ways by inferring COI and LSU phylogenies separately from each single-gene alignment, and by concatenating COI and LSU gene sequences into a single alignment. The following analysis pipeline was applied to COI and LSU, and COI-LSU concatenated sequences: tree reconstruction was conducted using the free-standing version of IQ-TREE 1.6.1 (www.iqtree.org) (Nguyen, Schmidt et al. 2014) for estimating maximum-likelihood phylogenies combined with Tree Search and ModelFinder (Kalyaanamoorthy, Minh et al. 2017). This procedure allowed us to build a phylogeny faster and with higher likelihoods than RAxML (Stamatakis 2006), and PhyML (Guindon, Dufayard et al. 2010) algorithms, while simultaneously employing less computing power. The model selection approach of ModelFinder granted us the advantage of not being restricted to an arbitrary probability threshold, while being robust with the parameters and predictions when evaluating competing hypotheses (models) for our phylogenetic reconstruction. 1000 ultrafast bootstrap approximation (UFBoot2) replicates (Hoang, Chernomor et al. 2018) were also applied to our tree reconstruction. This procedure

allowed us to assess the clade support in our phylogenetic tree using bootstrapping approximation algorithms that performed better than the computationally intensive standard non-parametric bootstrapping (Felsenstein 1985, Efron 1992), while at the same time reducing computing time, increasing unbiased support, and reducing overestimation of branch support. Ultrafast bootstrapping values followed the unbiased bootstrapping procedure suggested by Mihn (Minh, Nguyen et al. 2013). Clade support of ≥95% was marked on the tree by having a false-positive rate controlled at ≤5%, interpreted as a 0.95 probability of the split being correct. The resulting tree reconstruction was visualized with Dendroscope 3.5.9 (Huson and Scornavacca 2012) using *Hyalimax perlucida* to root the tree. The exception to the previous was the COI-LSU maximum likelihood analysis on IQ-TREE included 10,000 bootstrap replicates instead of 1,000, and was combined with a resampled partitioning analysis (each gene modeled separately) in order to reduce false positives (Gadagkar, Rosenberg et al. 2005, Chernomor, von Haeseler et al. 2016).

Species Delimitation Analyses

Most species delimitation analyses are computationally intensive to the point where they will not proceed to calculate p-values with too many individuals in a tree (>12 per clade). To allow us to compare the results of several species delimitation approaches, we used the same pruned tree for each analysis. To assign the organisms to hypothetical species, the COI phylogenetic tree was pruned to include a maximum of 12 representative individuals from each clade (Figure 14). These individuals were selected to include topotypes as well as all the populations and distinct lineages present in the tree. We used only COI for these analyses as this

is the most widely-used DNA barcoding locus for animals and allows comparison of results with other taxa.

The selected sequences were first analyzed using the automated barcode gap discovery (ABGD) graphic web version (wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) (Puillandre, Lambert et al. 2012) under the default parameters: P_{min}=0.001, P_{max}=0.1, steps=10, X (relative gap width)=1.5, Nb bins (for distance distribution)=20, and the Jukes-Cantor (JC69) molecular model, which is a Markov model of evolution for base substitution that can also help us derive distances between sequences (barcoding gap). ABGD automates finding of the "barcoding gap," rather than more simplistic methods that use a set p-distance which can vary among taxa. This analytical method infers the barcode gap from the data (and model) and partitions the dataset, going on to apply this method to all sequences in the tree. This allows the genetic distance (barcode gap) to infer species boundaries to vary across all taxa in the tree, arranging the sequences into putative species based on this distance (Puillandre, Lambert et al. 2012).

We assessed delimitation for the resulting hypothetical species-level groups using the Species Delimitation plug-in (SDP) within Geneious (Masters, Fan et al. 2011) using the ABGD-generated species-clade assignment output as our a priori group assignment (required for this analysis). This procedure tests the monophyly of each species-labeled group by examining if the grouping is likely to have occurred by chance using the probability of reciprocal monophyly under a random coalescent model (Masters, Fan et al. 2011). We also used the COI phylogenetic tree produced by IQ-TREE as input data to conduct a multi-rate Poisson tree process (mPTP) model (Kapli, Lutteropp et al. 2017) using the Exelixis Lab phylogenetic post-analysis web server (www.exelixis-lab.org), due to the fact that mPTP is faster and more accurate for species delimitations by outperforming distance-based methods and single-rate PTP, because it allows

each species to have its own evolutionary rate instead of assuming rate homogeneity across all branches.

CHAPTER IV

RESULTS

DNA Amplification and Sequence Analyses

PCR carried out using the universal CO1 primer pair LCO1490 and HCO2198 resulted in a single product of approximately 714 base pairs (bp). PCR performed with primer pair LSU1 and LSU3 for the LSU region resulted approximately 882 nucleotide sites in size which represented our fragment of interest. Sanger sequencing produced 294 data reads for CO1, and 188 for LSU, from which 147 contiguous overlapping sequences were assembled for CO1, and 94 for LSU.

Molecular Phylogeny Reconstruction

Maximum likelihood analyses of 147 COI and 94 LSU consensus sequences belonging to our succineid specimens (Table 1) yielded two corresponding phylogenetic trees with similar topology (COI log likelihood = -5316.097, Figure 5; LSU tree log likelihood = -2561.428, Figure 6), and with well supported species-level clades, as shown by the ultrafast bootstrap approximation values. We find the southeastern US *Oxyloma* fall into three well-supported clades, on both trees. Maximum likelihood analysis of 186 COI-LSU concatenated sequences (1576 nucleotide sites) yielded a tree (log likelihood = -8541.123, Figure 7) with topology similar to that of our single COI and LSU trees, and with well-supported species-level clades, including the three eastern and southern US *Oxyloma* clades. In our pruned tree for species

delimitation analyses, a total of 94 COI sequences yielded a phylogenetic tree with topology similar to our LSU, COI, and COI-LSU trees. In addition to that, *Succinea* was seen to be intermixed with *Oxyloma* in all the phylogenetic trees. Two unidentified *Oxyloma* clades from the Wakulla River, Florida, were also present in all the trees.

The first of the three major clades of *Oxyloma* (Figure 5, green clade) is composed of all representatives of *Oxyloma subeffusa* from the type locality of Plum Point, NJ. The second major clade of *Oxyloma* (Figure 5, red clade) is composed of all representatives of *Oxyloma retusa* from the type locality of Shawnee Lookout, OH, and a single *O. retusa* taken from the NJ locality. The third major clade of *Oxyloma* (Figure 5, yellow clade; Figure 11), is composed of all representatives of *Oxyloma salleana* from the type locality of intracoastal waterway near Jean Lafitte, LA, *Oxyloma effusa* from the type locality of Spring Garden Lake, FL, and *Oxyloma* taken from Washington DC, and eastern Canada, and a single *Succinea* from Wyoming.

Species Delimitation

The results of all species delimitation analyses are congruent for the southeastern US *Oxyloma*. ABGD analysis of the COI pruned sequences resulted in 12 different species-level groups (Figure 8), including lumping the four southeastern and eastern US *Oxyloma* taxa into three clades, and revealed two unidentified *Oxyloma* species-level groups. Assessment of the 12 ABGD groups through SDP in Geneious also found monophyly in all groups, including the three southeastern and eastern US *Oxyloma* clades. Values from this analysis for strict (PS) and liberal (PL) probabilities, along with Rosenberg's $P_{(AB)}$ results, are recorded in Table 4. Monophyly assigned by the multi-rate Poisson tree process analysis differed from that of the ABGD analysis

by rejecting monophyly in few non-target *Succinea* groups. However, it identified the same three southern and eastern US *Oxyloma* clades as monophyletic (Table 4).

CHAPTER V

DISCUSSION

The identification of Succineidae and *Oxyloma* have been impossible for more than seven decades due to unreliable characteristics used to delineate species (Bickford, Lohman et al. 2007). These erroneous species-delimitation practices have compromised *Oxyloma* and Succineidea by not accounting for their morphological variability, therefore placing them under the burden of urgent revisionary work (Nekola 2014). We have found four nominal southern and eastern US *Oxyloma* species forming three species-level groups in our phylogeny reconstructions, supported by all species delimitation methods applied.

Our data supports the application of the name *Oxyloma retusa* to the species represented by the *Oxyloma retusa* topotypes from Shawnee Lookout, OH. These individuals form a species-level clade in all our phylogeny reconstructions, with strong clade support of ≥95% given by ultrafast bootstrapping. All three species delimitation analyses also support the species-level status of this clade. However, it is worth noting that within the *O. retusa* clade in our reconstructions a single specimen taken from another locality (New Jersey) can be observed (Figure 13). We hypothesize *O. retusa* to have been introduced to the New Jersey locality by human activity or natural dispersal, given that we have seen other *Oxyloma* species away from their geographic regions of origin. In addition to that, during one of our collection trips we

observed *Oxyloma* hitchhiking by sticking to the sides of our canoe, which adds to the suspicion that they are easily transported by humans. *Oxyloma* are also commonly found on birds such as doves and likely disperse readily that way.

Our data supports the application of the name *Oxyloma subeffusa* to the species represented by the *Oxyloma subeffusa* topotypes from Plum Point, NJ. These individuals form a species-level clade all of our phylogenetic reconstructions, with a strong ultrafast bootstrapping clade support of ≥95%. All three species delimitation analyses also support the species-level status of this clade.

Our data supports the application of the name *Oxyloma salleana* to the species represented by the topotypes *Oxyloma salleana* from the intracoastal waterway near Jean Lafitte, LA, and *Oxyloma effusa* from Spring Garden Lake, FL, along with *Oxyloma* individuals from a wide geographic range including Eastern Canada and Maryland are seen forming one species-level clade with ≥95% ultrafast bootstrapping clade support instead of two independent clades. This contradicts the previous classification as two separate species. All three species delimitation analyses also support a single species-level status of this clade. We propose the clade be recognized as *Oxyloma salleana*, described in 1849, under the taxonomic principle of priority, and *Oxyloma effusa,* described in 1853, to be reduced to a junior synonym of *O. salleana*. An updated map of localities can be seen in figure 12, as well as an updated distribution map for Southeastern *Oxyloma* in figure 13. A collapsed maximum likelihood tree of COI-LSU concatenated sequences with proposed species-level clade assignations can be found in figure 10.

In our phylogenetic reconstructions, we observe *Succinea* intermixed with *Oxyloma*. Genera are required to form natural groups for them to be independent taxonomic ranks, and their intermixing proves otherwise. These data support indicate that along with species-level

revisions, the genera *Succinea* and *Oxyloma* also require revision. This analysis does not include a comprehensive survey of either genus or the type species of each genus so it is unclear if these genera should be unified, or if species should be transferred. However, it is clear that both genera must be revised.

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APPENDIX

APPENDIX

TABLES AND FIGURES

Table 1. Table of Specimens Used in this Study.

List of specimens used in our study, including their original sampling localities. Localities marked with asterisks (*) denote the locality as a type locality from where topotypes of the species were selected. Alphanumeric identifiers correspond to GenBank accession numbers.

Taxon	Locality	Identifying Number	Latitude	Longitude
Oxyloma				
Oxyloma cf. *effusa*	Dyke Marsh, Fairfax County, State of Virginia, United States of America	2623, 2624, 2626, 2628, 2629, 2630,2631, 2632, 2633, 2634, 2635, 2637	38.768431 N	-77.052959 W
Oxyloma cf. *effusa*	Little Hunting Creek, Fairfax County, State of Virginia, United States of America	2640, 2641, 2643, 2644, 2645, 2646, 2648, 2649, 2651, 2654, 2655, 2656	38.714118 N	-77.073971 W
Oxyloma cf. *subeffusa*	Theodore Roosevelt Island, Washington DC, United States of America	2658, 2659, 2660, 2661, 2664, 2665, 2668, 2669, 2672, 2673, 2674, 2675, 2676, 2678, 2679, 2680, 2681,2682, 2683, 2684, 2686, 2687, 2688, 2689, 2690, 2691, 2692, 2694, 2695, 2697, 2698, 2699, 2700, 2701	38.892906 N	-77.060203 W
Oxyloma effusa	*Spring Garden Lake, Volusia County, State of Florida, United States of America	2737, 2738, 2739, 2740, 2741, 2742, 2743, 2744, 2745, 2746, 2747, 2748, 2749, 2750, 2751, 2752, 2753, 2754, 2755, 2756, 2757	29.136254 N	-81.36917 W
Oxyloma sp.	Wakulla River, crossing HW98 1.5mi upstream of river crossing on east side of island west of channel, Wakulla County, State of Florida, United States of America	2758, 2759, 2760, 2761, 2762, 2762, 2763, 2764, 2765, 2766, 2767, 2768, 2769, 2770, 2771, 2772, 2773	30.189881 46 N	-84.26086465 W

Oxyloma salleana	*Lake Salvador, intracoastal waterway, Jean Lafitte, Jefferson Parish, State of Louisiana, United States of America	2778, 2779, 2780, 2781, 2782, 2783, 2784, 2785, 2786, 2787, 2788, 2789, 2790, 2791, 2792, 2793, 2794, 2795, 2796, 2797, 2798, 2799, 2800, 2801, 2802, 2803	29.741947 N	-90.141741 W
Oxyloma retusa	*Shawnee Lookout, 20ft from ramp, Hamilton County, State of Ohio, United States of America	2817, 2818, 2819, 2821, 2822, 2823, 2824, 2825, 2826, 2827, 2828, 2829, 2830, 2831, 2832, 2833, 2834, 2835, 2836, 2837	39.132174 N	-84.799354 W
Oxyloma subeffusa	*Plum Point, above Riverton, Burlington County, State of New Jersey, United States of America	2862, 2863, 2864, 2865, 2866, 2867, 2868, 2869, 2870, 2871, 2872, 2873, 2874, 2875, 2876, 2877, 2878, 2879, 2880, 2881, 2883, 2884	40.031707 N	-74.991203 W
Oxyloma elegans	Zuid-Holland, Leiden, Netherlands	2928	–	–
Oxyloma patentissima	Kwazulu-Natal, South Africa	2929	–	–
Oxyloma elegans	Breclav, Czech Republic	2952	–	–
Oxyloma hirasei	Japan	AY150084	–	–
Oxyloma sp.	Tawayik Lake are trails, Elk Island National Park, Alberta, Canada	KM611855	–	–
Oxyloma sp.	Corral Creek old road, Banff National Park, Alberta, Canada	KM611886	–	–
Oxyloma sp.	Point Peele National Park, Ontario, Canada	KM611985	–	–
Oxyloma sp.	Corral Creek old road, Banff National Park, Alberta, Canada	KM612050	–	–
Succinea				
Succinea luteola	Edinburg, Hidalgo County, State of Texas, United States of America	2708, 2710, 2711	26.306024 N	-98.172288 W
Succinea putris	Leiden, Netherlands	2927	–	–
Succinea sp.	Cuba	2930	–	–
Succinea putris	Bukovec, Moravia, Czech Republic	2932	–	–
Succinea floridana	Jacksonville, Duval County, State of Florida, United States of America	2934	–	–
Succinea sp.		2942	–	–
Succinea sp.		2944	–	–
Succinea campestris	Amelia Island, Nassau County, State of Florida, United States of America	2949	–	–

Table 1. Table of Specimens Used in this Study (Continued).

Succinea putris	Spisake Vlachy, Slovakia	2951	–	–
Succinea striata	Kwazulu-Natal, South Africa	2953	–	–
Succinea sp.	Catstail swamp at canyon floor, Tensleep Canyon, State of Wyoming, United States of America	2955	–	–
Succinea canella	Molokai, Hawaii, United States of America	AY148572	–	–
Succinea caduca	Kalanianaole, Hawaii, United States of America	DQ658537	–	–
Succinea putris	Cambridge, Ontario, Canada	KT708385	–	–
Succinea striata	Mambassa Hu, Natal, South Africa	AY841295	–	–
Succinea putris	Boksitogorsk, Russia	MF148308	–	–
Hyalimax				
Hyalimax perlucida	Grand Bassin, Mauritius	2931	–	–

Table 1. Table of Specimens Used in this Study (Continued).

Table 2. PCR Primer Pairs

Amplification primer pairs used for LSU and COI PCR reactions.

Target gene	*Primer*	*Sequence 5' – 3'*	*Reference*
LSU	LSU1	CTAGCTGCGAGAATTAATGTGA	(Wade and Mordan 2000)
	LSU3	ACTTTCCCTCACGGTACTTG	(Wade and Mordan 2000)
COI	LCO1490	GGTCAACAAATCATAAAGATATTG	(Folmer, Black et al. 1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAAATCA	(Folmer, Black et al. 1994)

Table 3. PCR Temperature Profiles

PCR reaction temperature profiles used to amplify the LSU and COI region fragments.

Target	*Initial Denaturation*	*Step 2 Denaturation*	*Primer Annealing*	*Primer Extension*	*Go To Step 2*	*Final Extension*	*Hold*
LSU	94°C, 0:30	94°C, 0:10	51.2°C, 0:30	72°C, 1:30	X35	72°C, 10:00	12°C, ∞
COI	92°C, 2:00	92°C, 0:40	41.9°C, 0:40	72°C, 1:30	X30	72°C, 5:00	8°C, ∞

Table 4. Species Delimitation Analyses

The labeled clade column indicates the species-level group number assigned to the pruned COI tree by the ABGD analysis. PS=Strict Probability, PL=Liberal Probability, and $P_{(AB)}$=Rosenberg's $P_{(AB)}$ method assigned by the Species Delimitation Plug-in in Geneious. mPTP=Multi-rate Poisson Tree Process clade support for monophyly.

Labeled Clade	*PS*	*PL*	*$P_{(AB)}$*	*mPTP*
1	0	0.96	0.00022	Yes
2	0.57	0.82	0.00082	No
3	0	0.96	0.00202	Yes
4	0.54	0.93	0.0001	Yes
5	0.93	0.98	0.00092	Yes
6	0	0.96	0.00092	Yes
7	0.96	0.99	4.70E-10	Yes
8	0.97	0.99	5.20E-17	Yes
9	0	0.96	0.05	No
10	0	0.96	0.03	No
11	0.93	1.00	0.03	No
12	0.93	0.98	0.05	No

Figure 1. Apertural and Reverse View of Shell of *Oxyloma retusa*.

Photograph of *Oxyloma retusa* shell taken from the type locality near Cincinnati, Ohio. Shell is 9.72 mm total height.

Figure 2. Apertural and Reverse View of Shell of *Oxyloma salleana*.

Photograph of *Oxyloma salleana* shell taken from the type locality near New Orleans, Louisiana. Shell is 13.11 mm total height.

Figure 3. Apertural and Reverse View of Shell of *Oxyloma effusa*.

Photograph of *Oxyloma effusa* shell taken from the type locality in eastern Florida. Shell is 11.28 mm total height.

Figure 4. Apertural and Reverse View of Shell of *Oxyloma subeffusa.*

Photograph of *Oxyloma subeffusa* taken from the type locality in Plum Point, New Jersey. Shell is 18.38 mm total height.

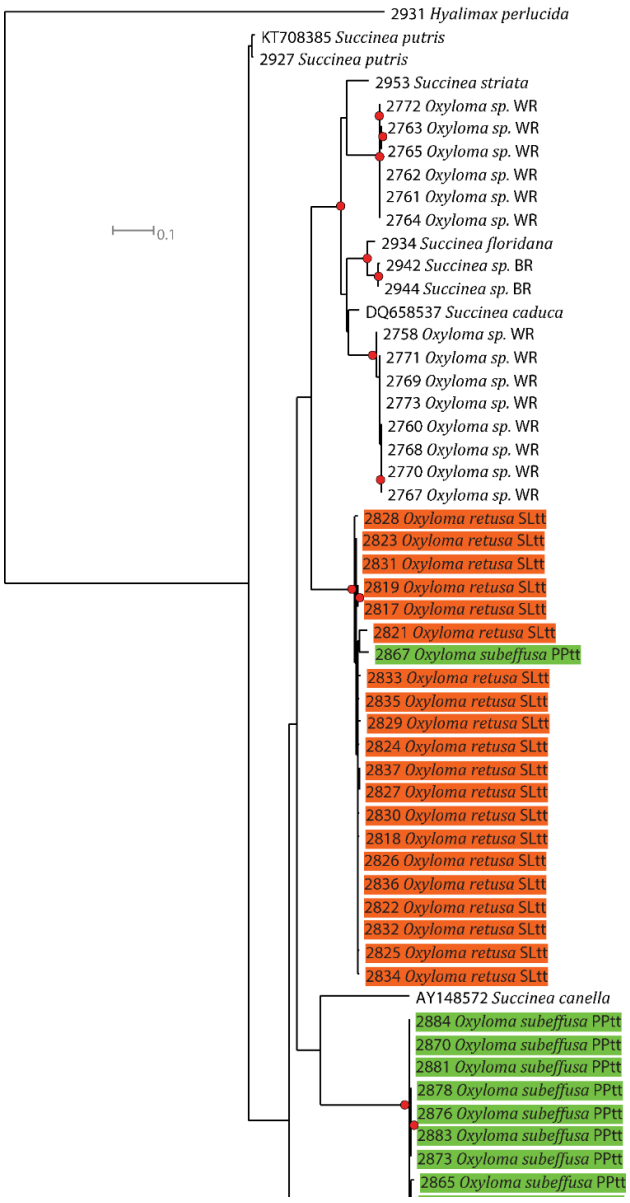

Figure 5. Maximum Likelihood Phylogeny Based on 714bp of COI Mitochondrial Sequences of 147 Individuals.

Red dots at nodes indicate ≥95% support by ultrafast bootstrap approximation. Terminals are labeled based on the presumed identification based on current taxonomy. Individuals labeled with "tt" were collected from the type locality. Abbreviations for each locality are as follows: SGL=Spring Garden Lake, FL, PP=Plum Point, NJ, SL=Shawnee Lookout, OH, JL=intracoastal waterway near Jean Lafitte, LA. Tree is rooted with *Hyalimax perlucida.*

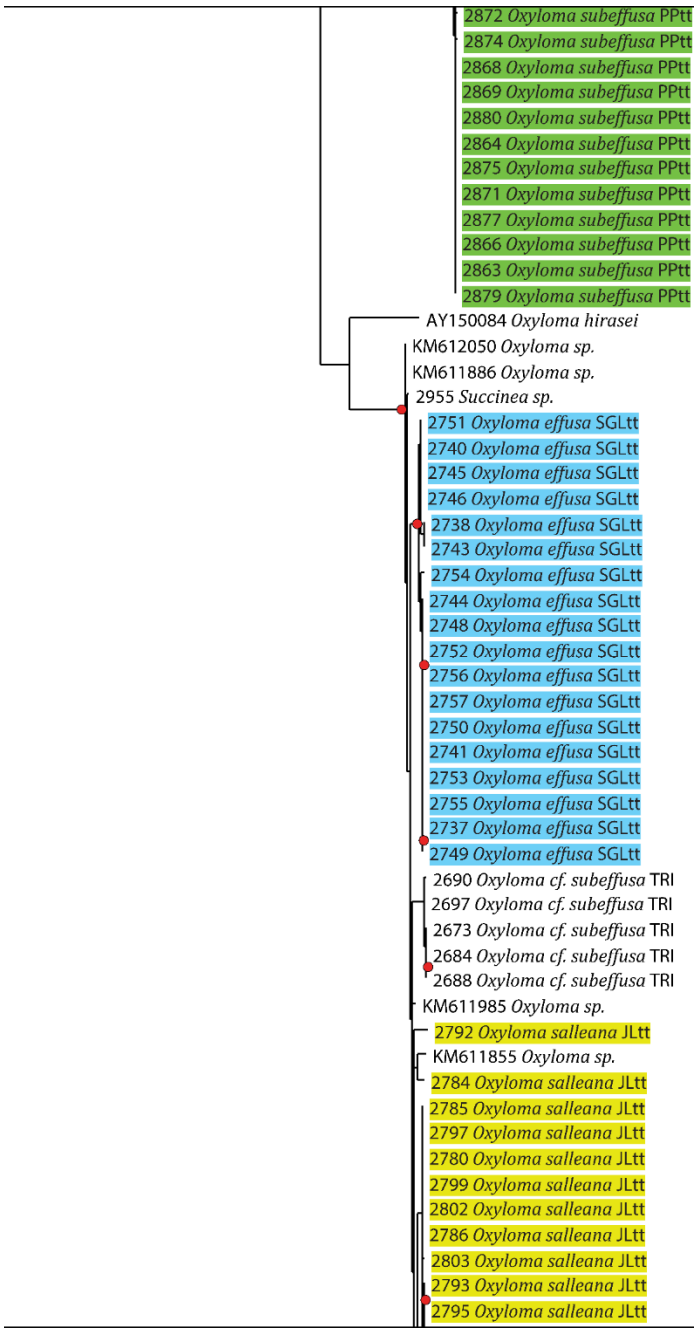

Figure 5. Maximum Likelihood Phylogeny Based on 714bp of COI Mitochondrial Sequences of 147 Individuals (Continued).

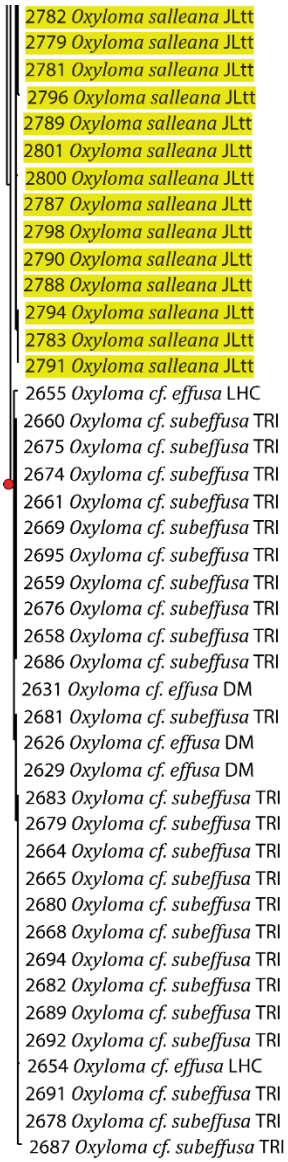

Figure 5. Maximum Likelihood Phylogeny Based on 714bp of COI Mitochondrial Sequences of 147 Individuals (Continued).

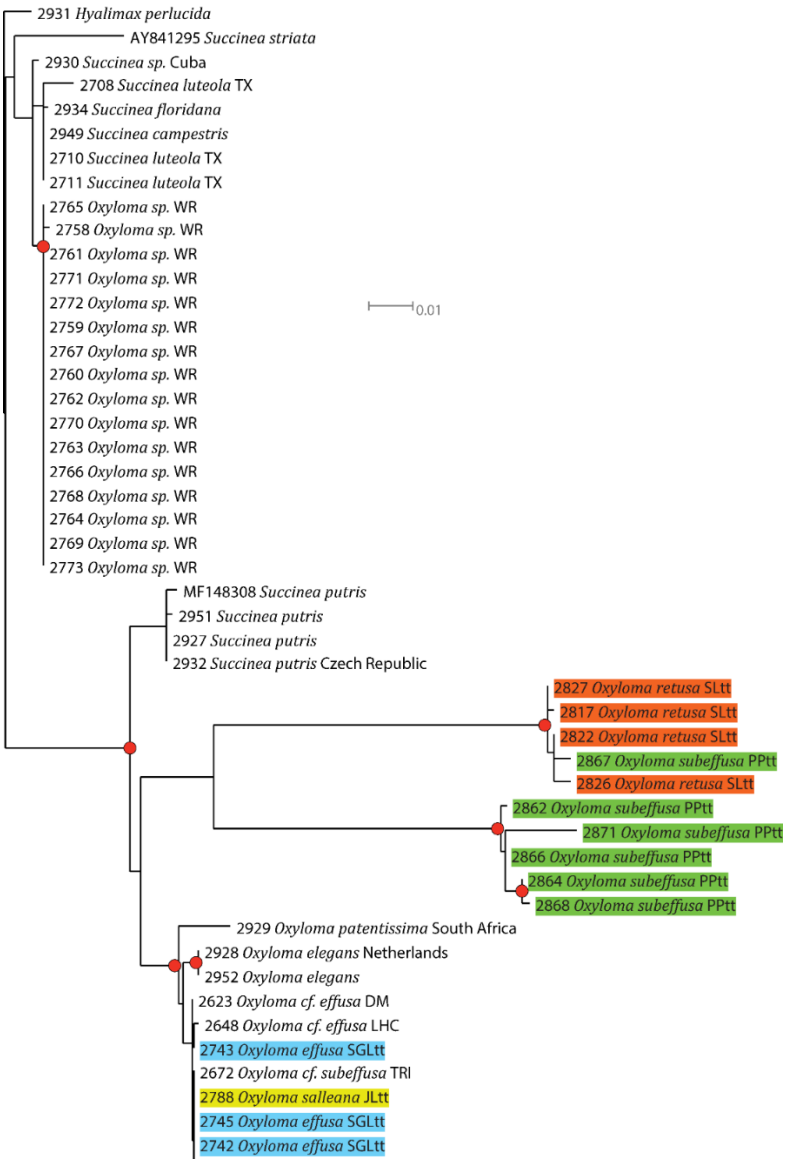

Figure 6. Maximum Likelihood Phylogeny Based on 882bp of LSU Nuclear Sequences of 94 Individuals.

Red dots at nodes indicate ≥95% support by ultrafast bootstrap approximation. Terminals are labeled based on the presumed identification based on current taxonomy. Individuals labeled with "tt" were collected from the type locality. Abbreviations for each locality are as follows: SGL=Spring Garden Lake, FL, PP=Plum Point, NJ, SL=Shawnee Lookout, OH, JL=intracoastal waterway near Jean Lafitte, LA. Tree is rooted with *Hyalimax perlucida.*

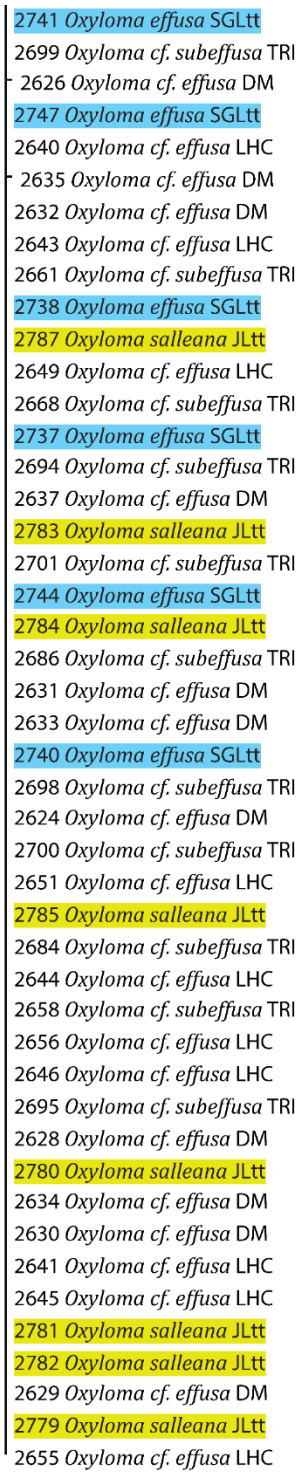

Figure 6. Maximum Likelihood Phylogeny Based on 882bp of LSU Nuclear Sequences of 94 Individuals (Continued).

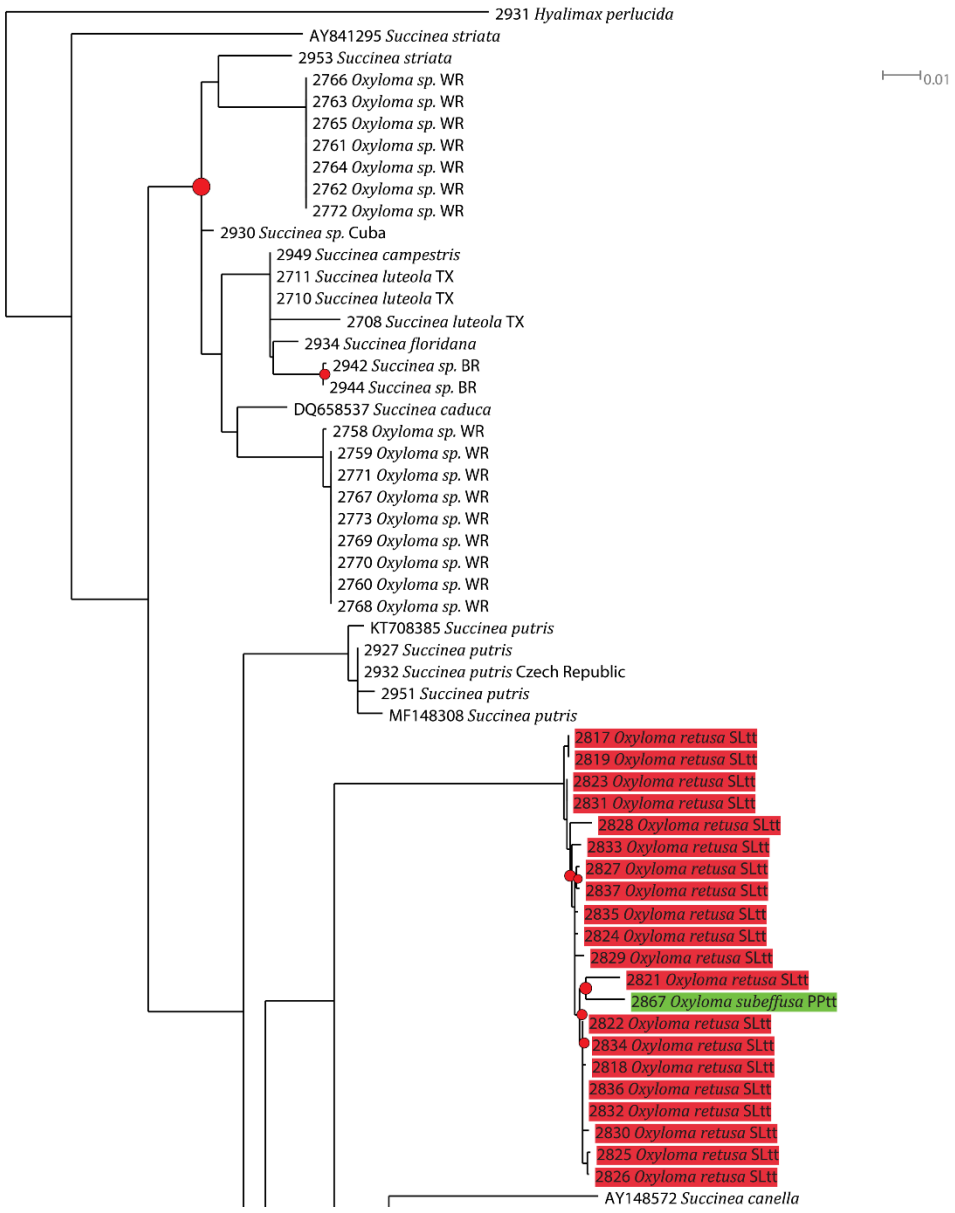

Figure 7. Maximum Likelihood Phylogeny Based on COI-LSU Concatenated Sequences. Red dots at nodes indicate ≥95% support by ultrafast bootstrap approximation. Terminals are labeled based on the presumed identification based on current taxonomy. Individuals labeled with "tt" were collected from the type locality. Abbreviations for each locality are as follows: SL=Spring Garden Lake, FL, PP=Plum Point, NJ, SL=Shawnee Lookout, OH, JL=intracoastal waterway near Jean Lafitte, LA. Tree is rooted with *Hyalimax perlucida.*

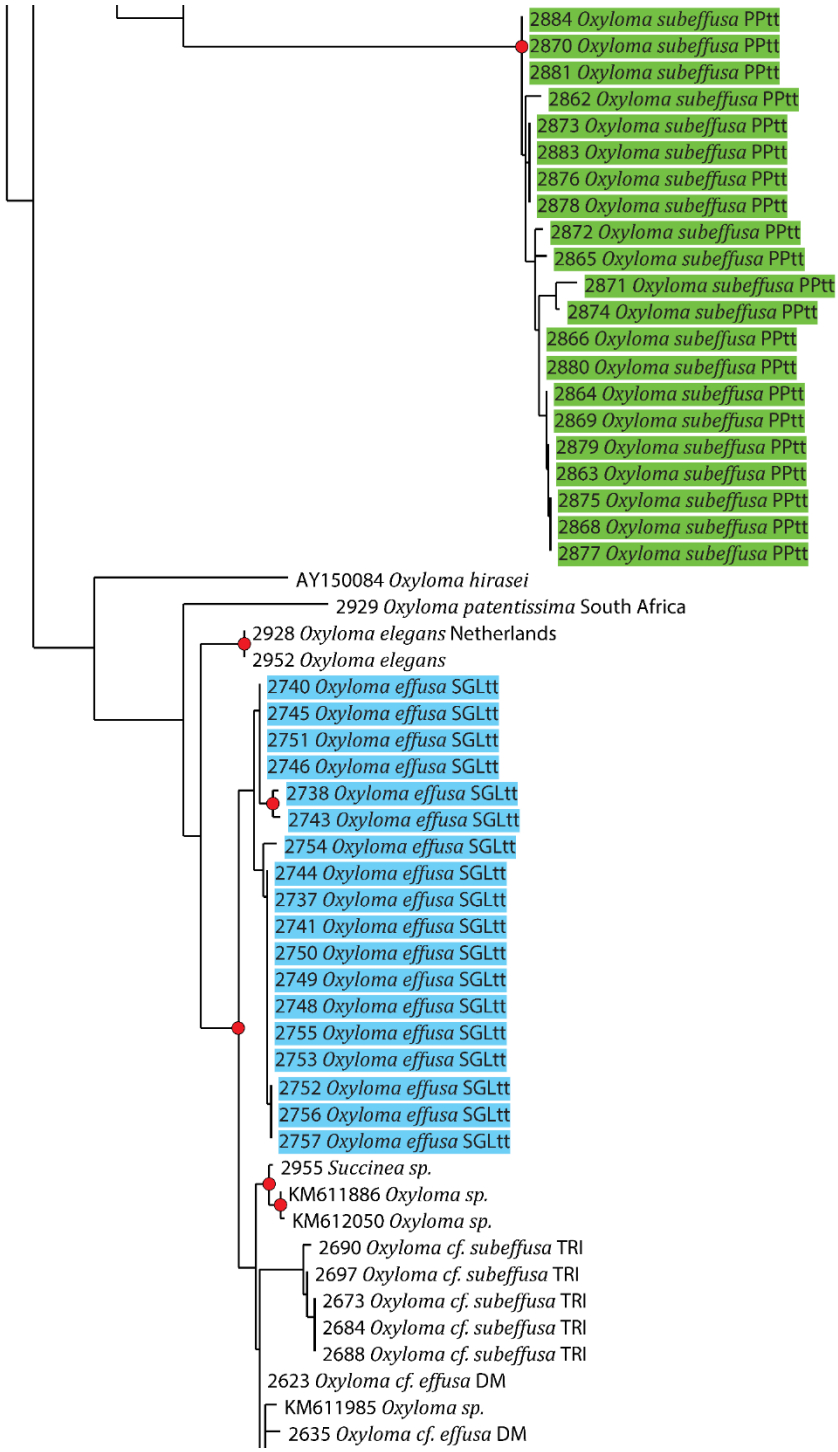

Figure 7. Maximum Likelihood Phylogeny Based on COI-LSU Concatenated Sequences (Continued).

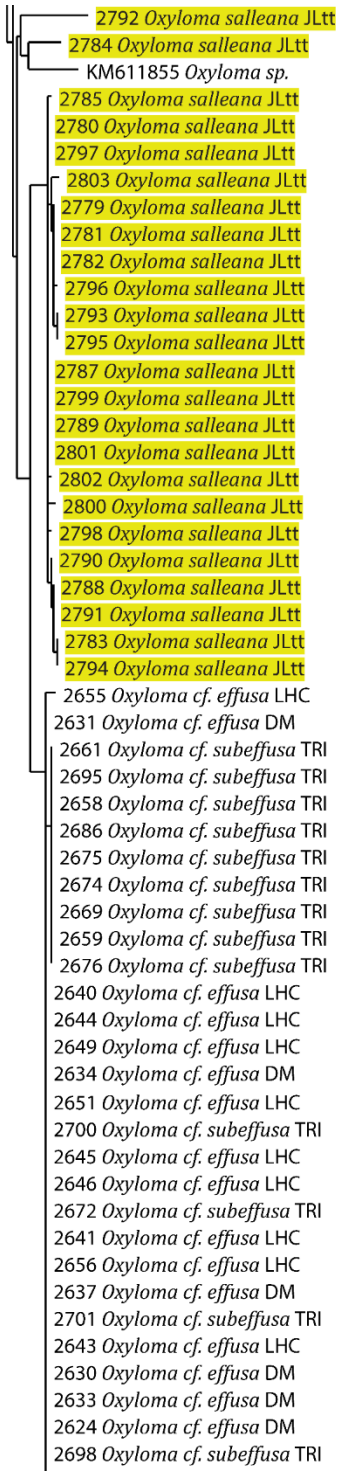

Figure 7. Maximum Likelihood Phylogeny Based on COI-LSU Concatenated Sequences (Continued).

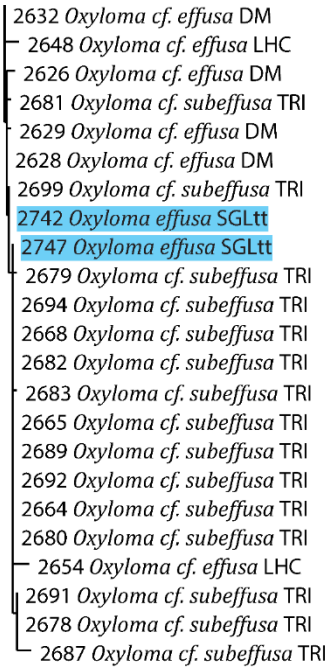

Figure 7. Maximum Likelihood Phylogeny Based on COI-LSU Concatenated Sequences (Continued).

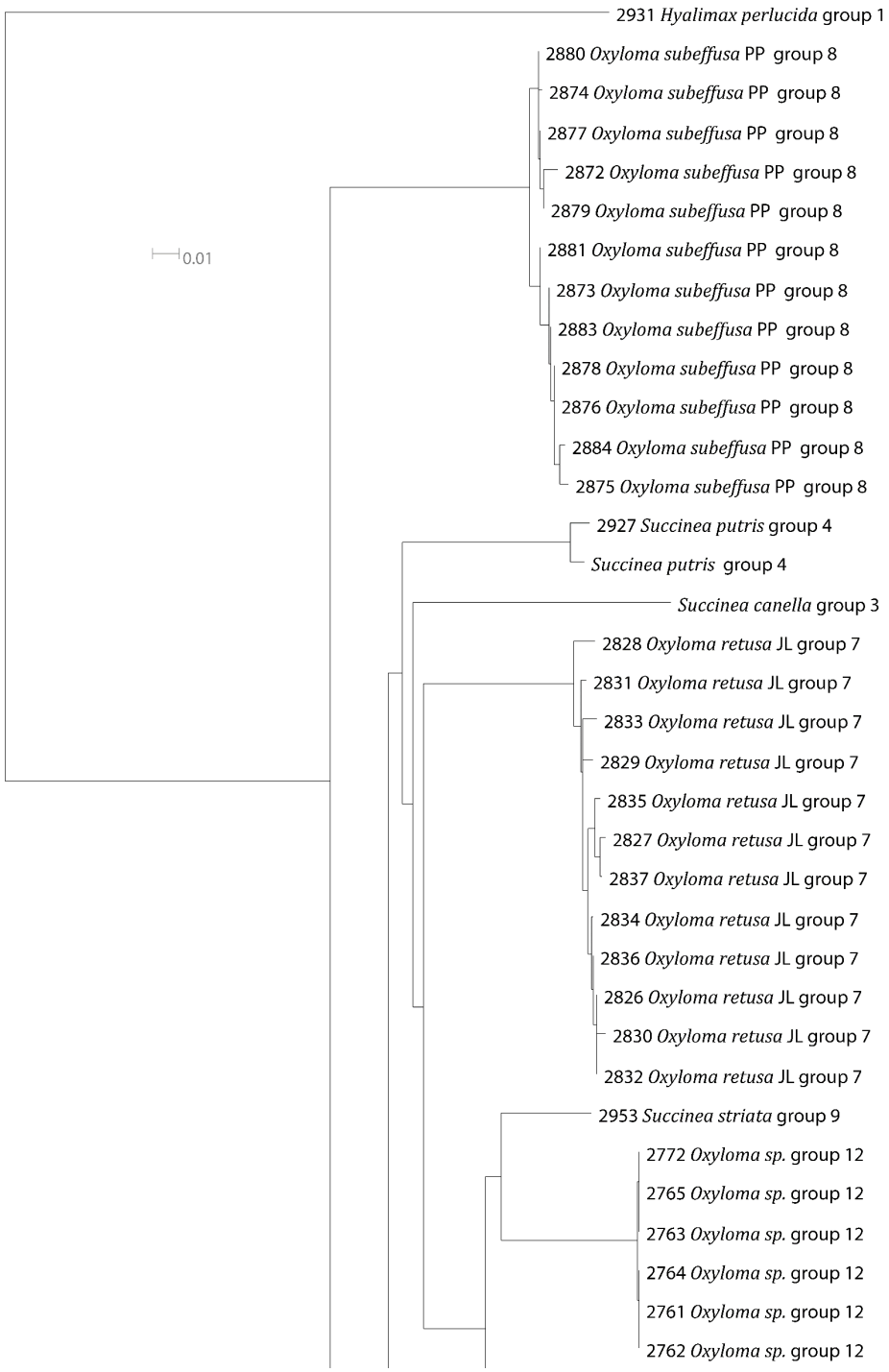

Figure 8. ABGD Group Assignment Tree.

The Groups assigned by ABGD are appended to the end of the terminal label. These group assignments were then also tested by the Species Delimitation Analysis.

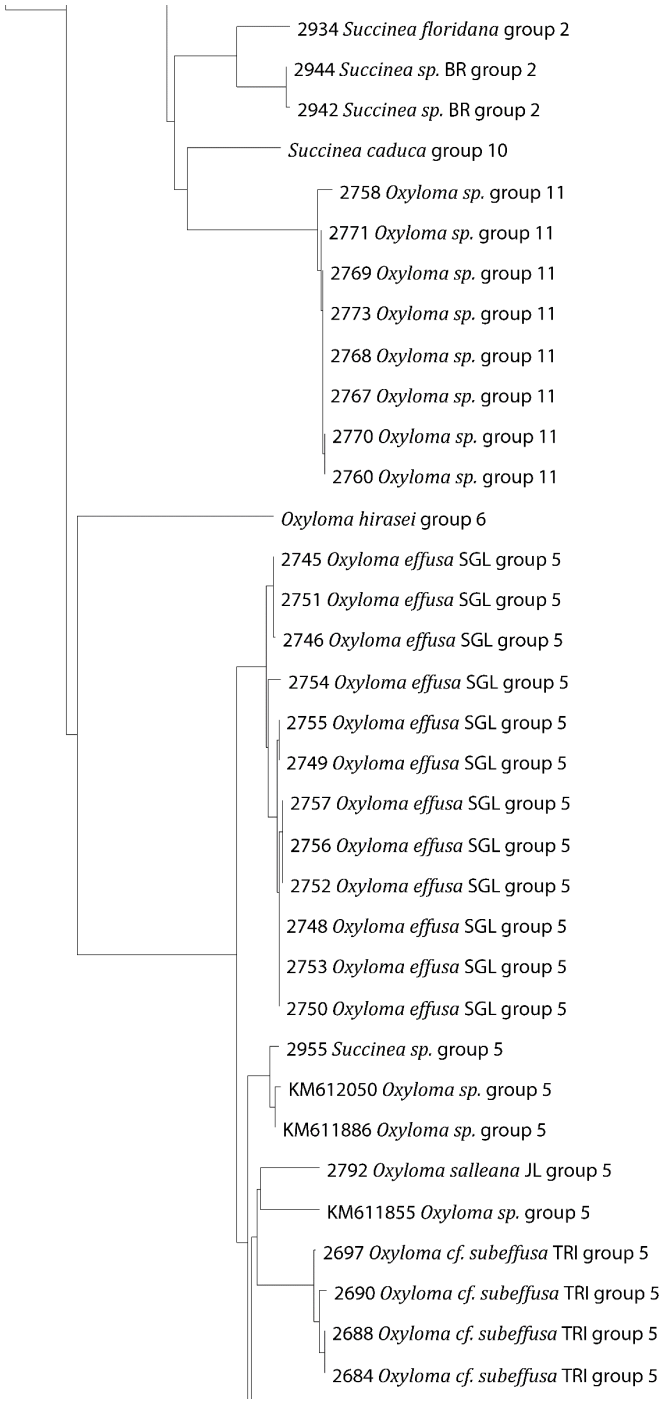

Figure 8. ABGD Group Assignment Tree (Continued).

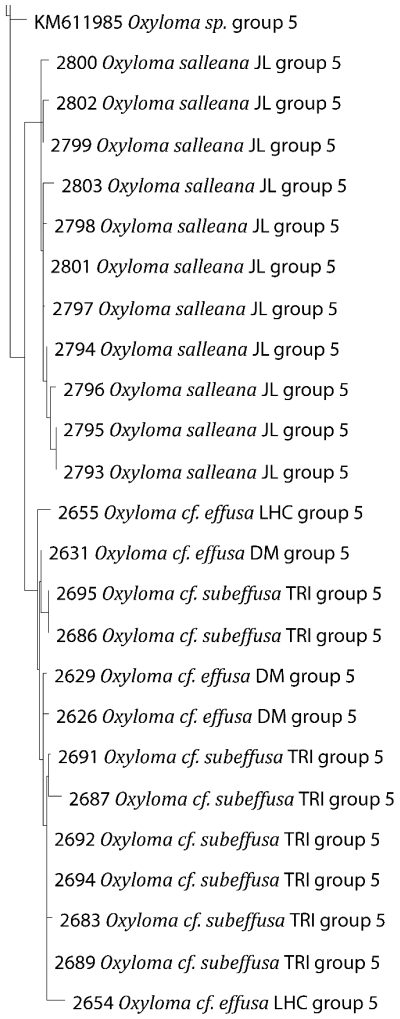

Figure 8. ABGD Group Assignment Tree (Continued).

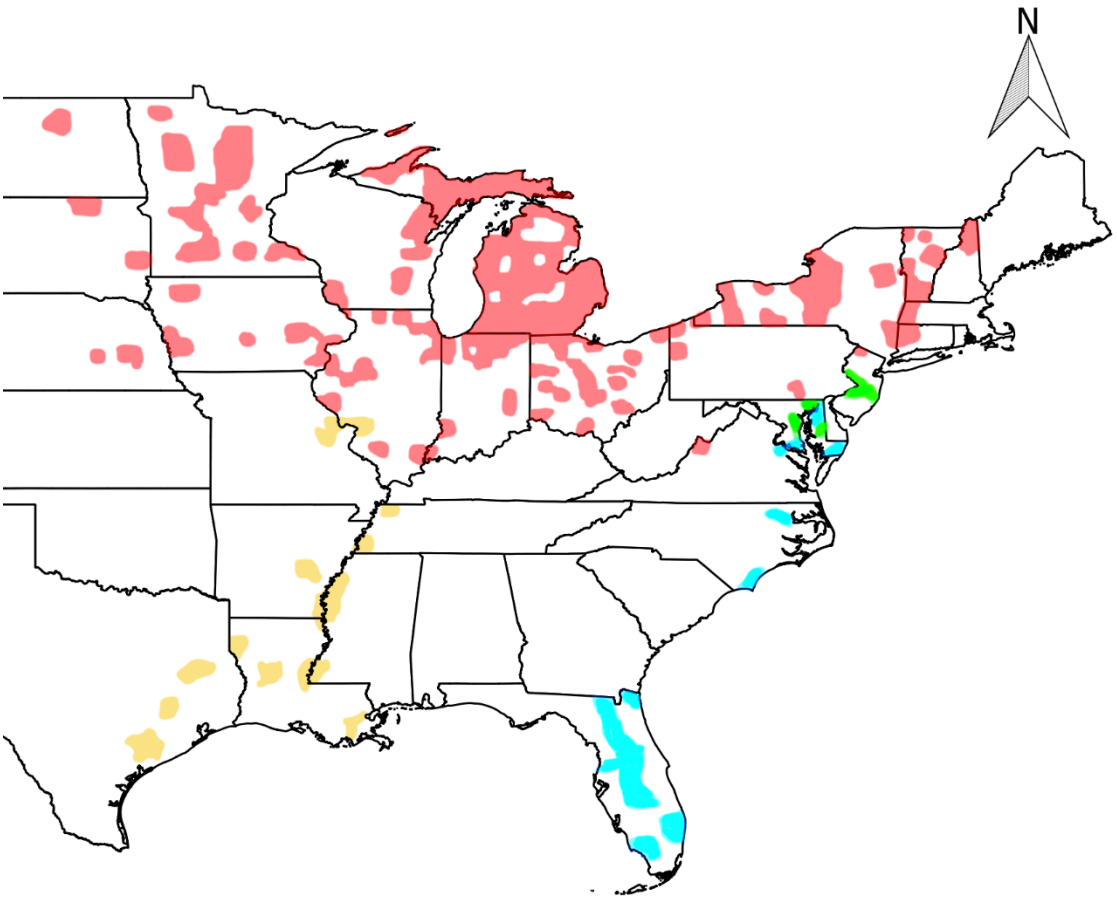

Figure 9. Distributions of Southeastern US *Oxyloma* Prior to This Work.

Areas in map colored red represent *Oxyloma retusa*, straw yellow *Oxyloma salleana*, blue *Oxyloma effusa*, and green *Oxyloma subeffusa*. Map largely based on Hubricht (1985).

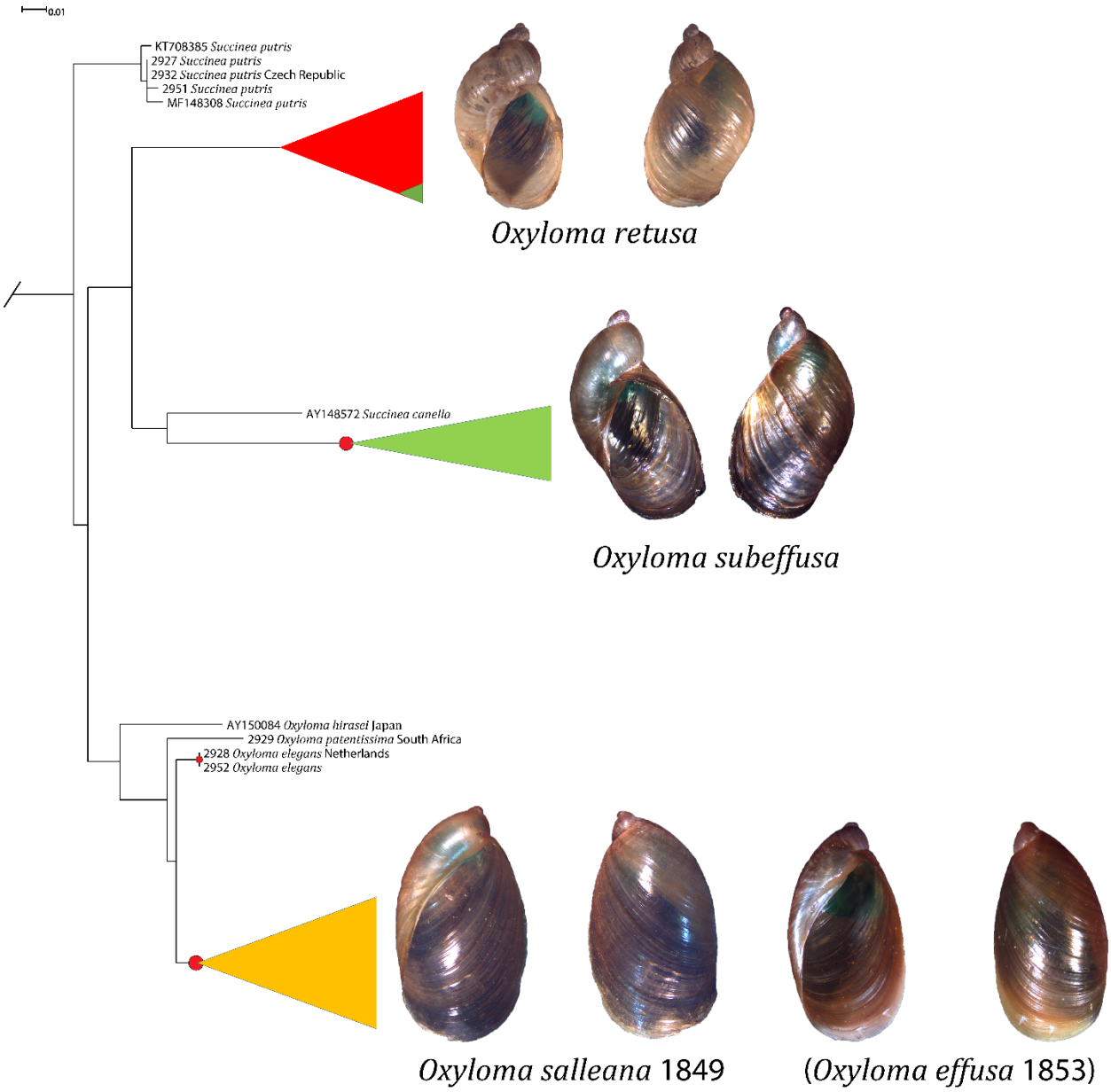

Figure 10. Collapsed Maximum Likelihood Tree of COI-LSU Concatenated Sequences with Proposed Species-level Clade Assignments.

Red dots at nodes indicate ≥95% support by ultrafast bootstrap approximation. Break on the tree represented by a dash indicates continuation of the tree.

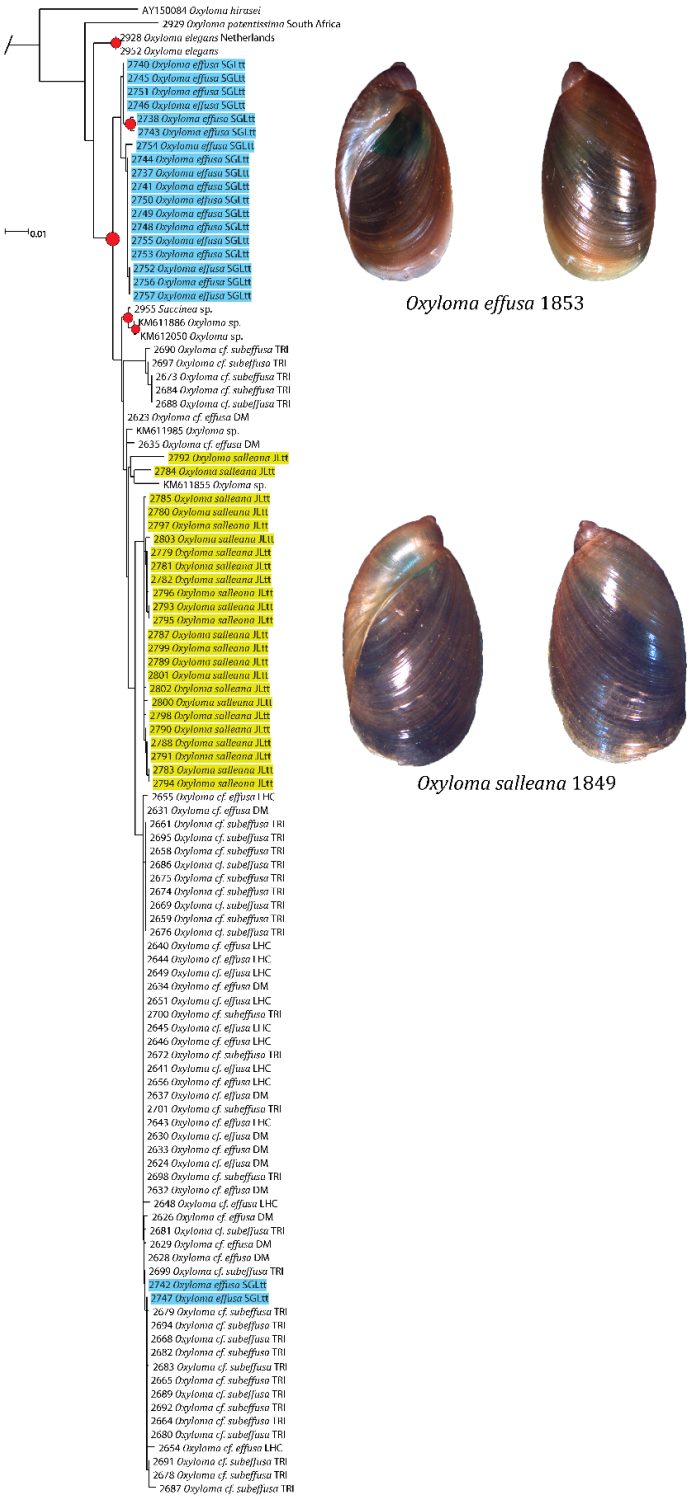

Figure 11. Expanded *Oxyloma salleana* Clade of Maximum Likelihood Tree of COI-LSU Concatenated Sequences.

Red dots at nodes indicate ≥95% support by ultrafast bootstrap approximation.

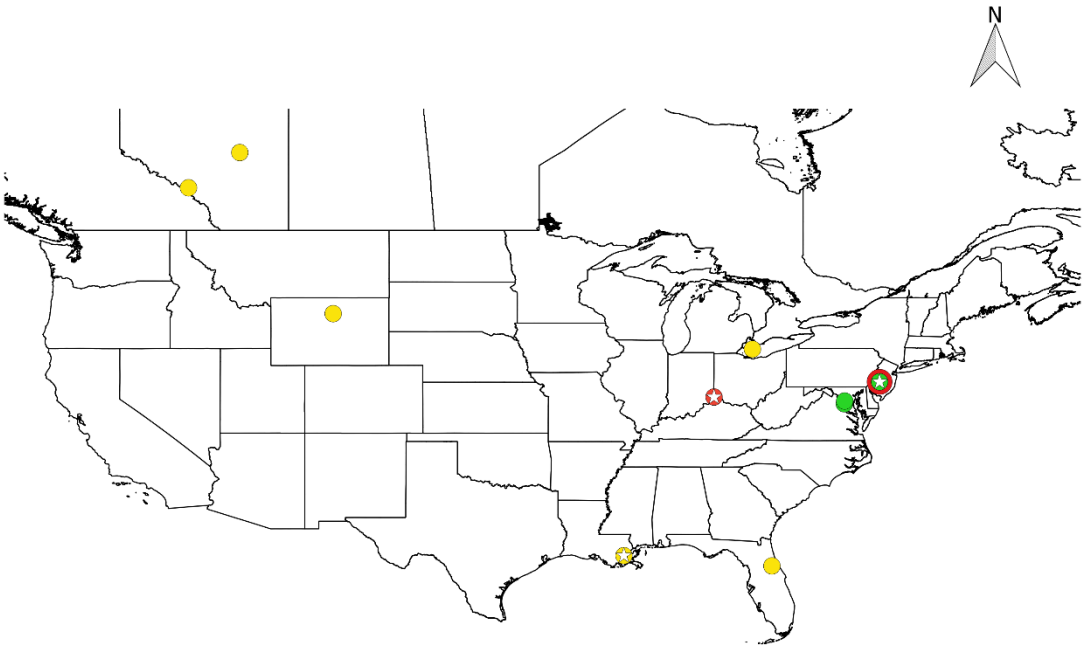

Figure 12. Map of Type Localities of Southeastern US *Oxyloma*.

Points colored with red represent presence of *Oxyloma retusa*, yellow *Oxyloma salleana*, and green *Oxyloma subeffusa*. The type localities are represented by a white star.

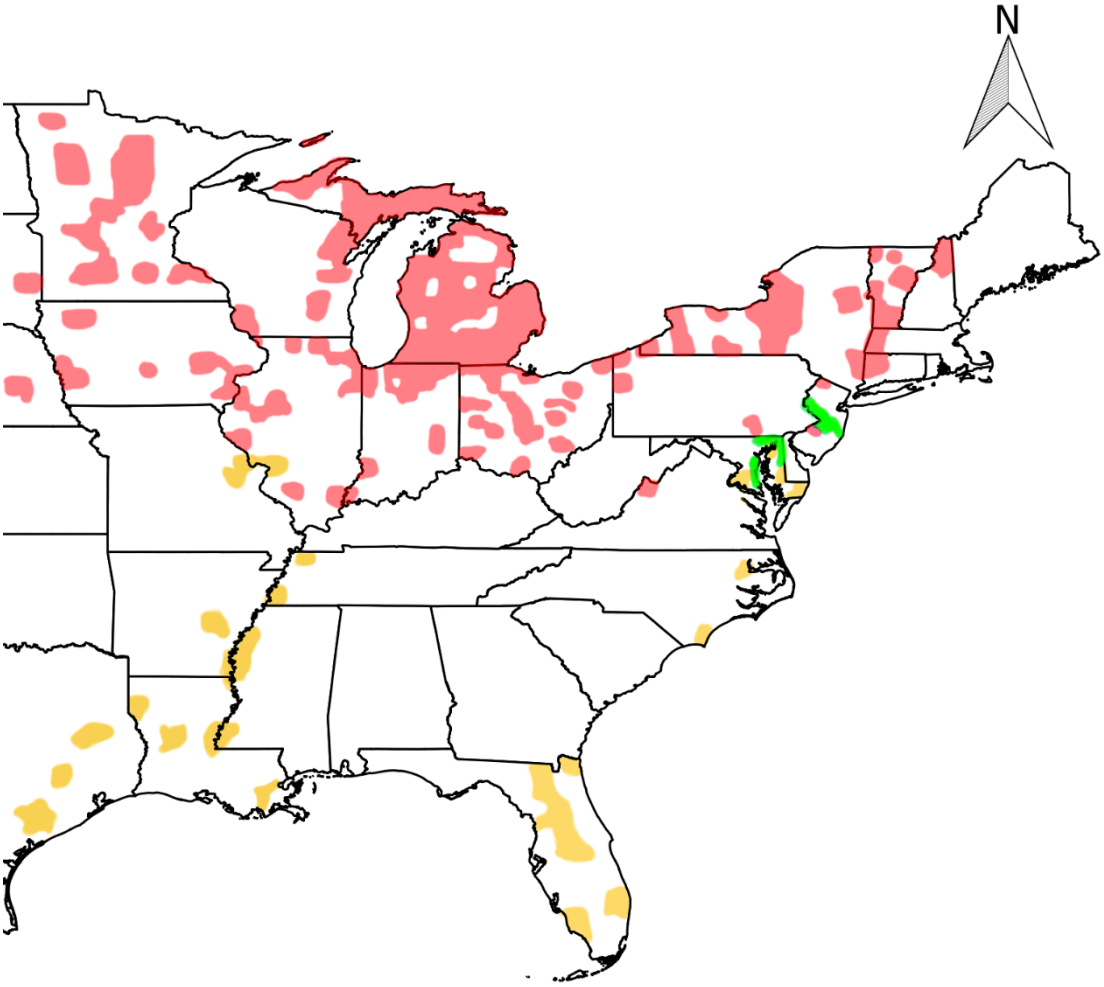

Figure 13. Updated Map of Southeastern US *Oxyloma* Geographic Distributions Based on This Study.

Areas colored in red represent *Oxyloma retusa*, straw yellow *Oxyloma salleana*, and green *Oxyloma subeffusa.*

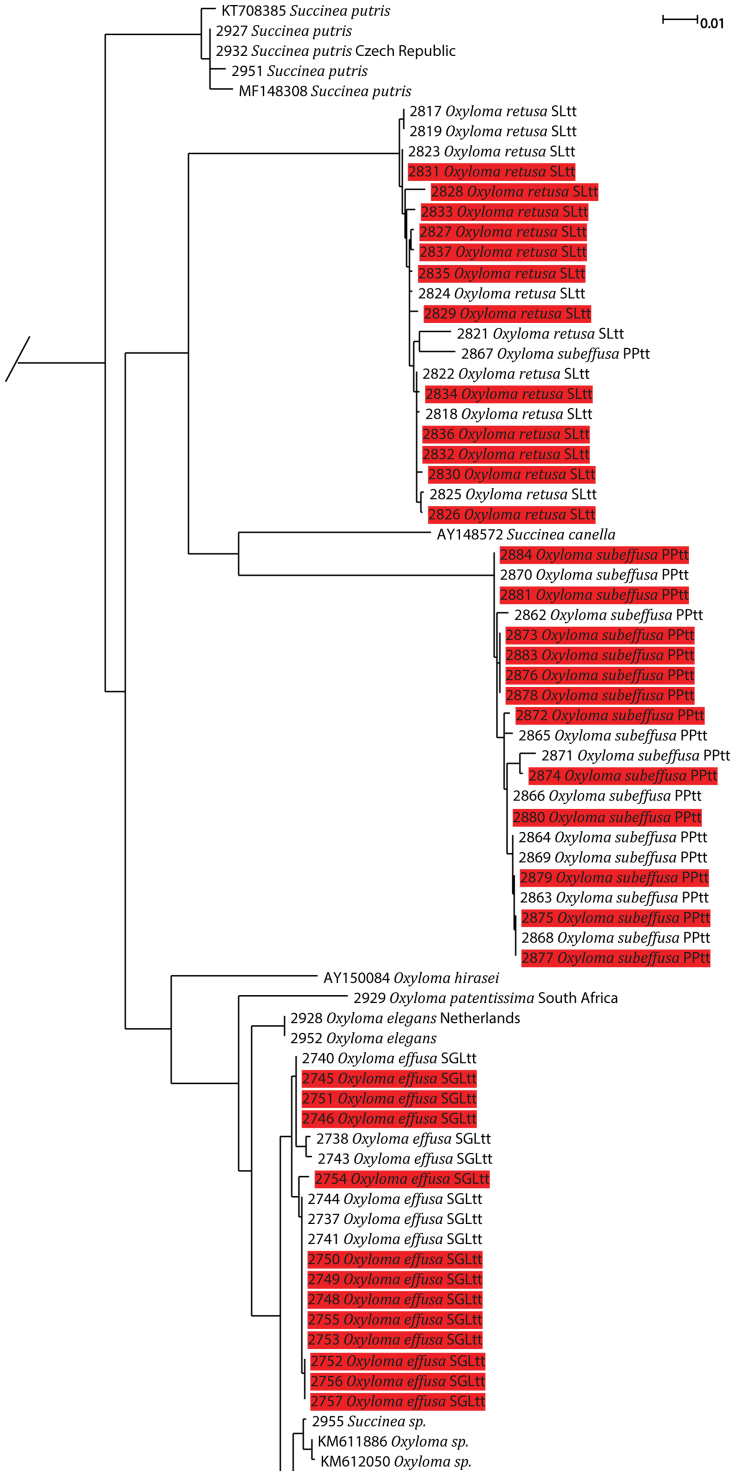

Figure 14. *Oxyloma* Specimens Used in Delimitation Analyses.

Oxyloma specimens highlighted on the tree were used in all species delimitation analyses. Tree in image is COI-LSU concatenated tree. Break in tree indicates the tree continues.

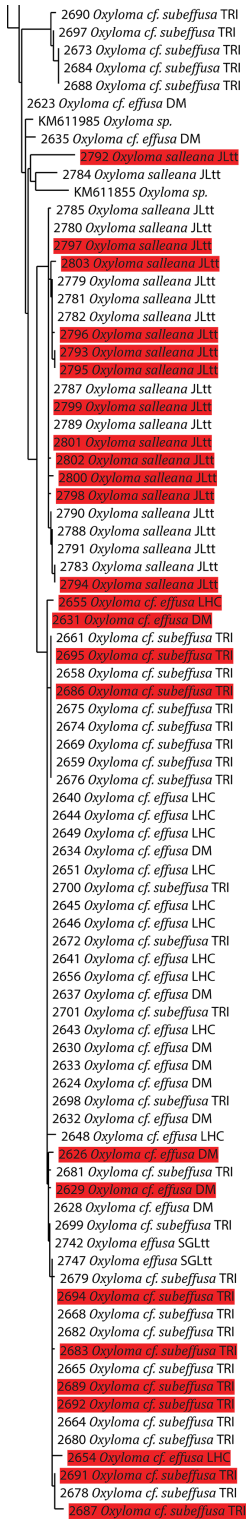

Figure 14. *Oxyloma* Specimens Used in Delimitation Analyses (Continued).

BIOGRAPHICAL SKETCH

Marco A. Martinez Cruz began working in the laboratory of Dr. Kathryn E. Perez in Spring of 2015 as a research assistant during his undergraduate studies. He became an undergraduate Research Fellow for the Howard Hughes Medical Institute during Fall of the same year, and spent a year working under the direction and mentorship of Dr. Kathryn E. Perez on projects dedicated to the understanding of taxonomy, phylogeny, and the discovery of new animal species, until obtaining the degree of Bachelor of Science in Biology, with a minor in Chemistry, at the University of Texas Rio Grande Valley in August 2016. Marco began graduate studies in Fall 2016, and was conferred the degree of Master in Biology, from the University of Texas Rio Grande Valley, in May 2018. His personal e-mail is: 94martinezmarco@gmail.com.