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Investigation of DNA Variability and Phylogenetic Relationships of Perlesta (Plecoptera: Perlidae) in Mississippi

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INVESTIGATION OF DNA VARIABILITY AND PHYLOGENETIC RELATIONSHIPS OF PERLESTA (PLECOPTERA: PERLIDAE) IN MISSISSIPPI

by

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ABSTRACT

The genus *Perlesta* Banks, 1906 (Plecoptera: Perlidae) consists of 35 species, 33 native to the United States and Canada and two native to China. For over a century these small, brown stonefly adults and freckled yellow nymphs have gone by the name of the type species of the genus, *Perlesta placida*, but taxonomic work in the genus since 1989 has resulted in the recognition of additional species. These species were mostly recognized and described using morphological characteristics, but two areas that are lacking include (1) linking nymphs to adults and (2) phylogenetic analysis of all species occurring in Mississippi using DNA data. Three species of *Perlesta* have been reported for Mississippi (*P. lagoi*, *P. placida*, and *P. shubuta*), but *P. placida* has no DNA sequences in public DNA repositories. In this project, DNA was gathered from nymphs, females, and males of *Perlesta* collected in Mississippi to assess their utility for DNA barcoding (linking members of a species by consistent, diagnostic DNA sequences) and to infer a phylogeny using nuclear and mitochondrial DNA. This study revealed a broader degree of molecular variation in individuals from Mississippi relative to species from other states, which suggests greater infraspecific DNA variation in these species or possibly the presence of cryptic species.

ACKNOWLEDGMENTS

This project would not have been possible without the help and guidance of many wonderful people. My thesis advisor, Dr. Mac H. Alford, has mentored and guided me throughout the entirety of this project. He has answered countless questions asked at all times of the day and night. His knowledge and passion for biology have helped inspire and foster my love for plants and insects. Dr. Bill P. Stark let me borrow personal samples and resources and met with me numerous times to discuss the tricks and trades of studying stoneflies. Dr. Donald A. Yee has served on my committee and has continued to invest in and shape me as a researcher. He has done so ever since I had the opportunity to work on mosquito-related projects as an undergraduate in his research lab. I would also like to thank Dr. Audrey Harrison for giving me advice, helping me improve my sampling techniques, and loaning me samples. Dr. Boris Kondratieff also sent me samples.

I also want to thank my wife, Sara, for the overwhelming support throughout the years. I sincerely appreciate the people who helped me sample including Joshua Wilkinson, Brett Valentine, and Will McFarland. I'd like to give a special thanks to my dad, Dr. Brett Valentine, for going sampling with me multiple times and helping me build emergence traps for this project.

I am very grateful to the kind people who let me sample on their private property, which included Mr. Tom and Mrs. Polly Rosenblatt (Ft. Adams), Mr. David and Mrs. Edie Varnado (McComb), Mr. Shannon Willis (Carson), Skyler de Regt (Bentonia), and my grandfather, Dr. James Valentine (Vicksburg).

Lastly, I would like to thank my mom, Mrs. Alesia Valentine, Dr. Tommy Rauch, Dr. Brett Golson, Mrs. Karen Golson, Dr. David C. Beckett, Mr. Donaven McLaurin, and Miss Melissa Gutierrez for their contributions and investments into my life.

DEDICATION

This thesis is dedicated to my late grandfather, Dr. Tom Rhea Phillips, Jr., who played a crucial role in cultivating my love for entomology and botany.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

COI	Mitochondrial Cytochrome c Oxidase I, sometimes abbreviated *COX1* or *CO1*
DNA	Deoxyribonucleic acid
GTR+I+G	General Time Reversible (nucleotide substitution model) with Gamma distribution and invariant sites
MCL	Maximum Composite Likelihood
ML	Maximum Likelihood
MPT	Most parsimonious tree
PCR	Polymerase chain reaction
UV	Ultraviolet (light)

CHAPTER I – INTRODUCTION

1.1 The Stoneflies

Stoneflies (Plecoptera) are a group of aquatic, hemimetabolous insects with approximately 3,800 described species across 16 families (DeWalt et al., 2015; Fochetti and Tierno de Figueroa, 2008; South et al., 2019, 2021). Stoneflies are found on every continent except Antarctica, but the North American and European species are the most studied (Fochetti and Tierno de Figueroa, 2008). As hemimetabolous insects stoneflies display incomplete metamorphosis with three life stages: egg, nymph, and adult (DeWalt et al., 2015; Merritt and Cummins, 1984; Stewart and Stark, 2002). The egg and nymph stages in most species are exclusively aquatic, whereas the adults are almost entirely terrestrial. Most stoneflies reproduce from spring to summer, but the families Capniidae and Taeniopterygidae emerge and reproduce in the winter (DeWalt et al., 2015).

Stoneflies have univoltine life cycles, spending most of their time as nymphs, and dying within one to two days of emerging (Stewart and Stark, 2002). Stoneflies can be found in all types of water, but most are restricted to fast moving, lotic streams, creeks, and rivers. Stoneflies are used as bio-indicators for healthy water quality and are the least resistant aquatic insect order to pollution due to their habitat requirements and sensitivity to disturbances in their environments (Barbour et al., 1999; Lecerf et al., 2006; Strayer, 2006). In recent decades, insect populations have been in decline due to habitat loss, pesticides, and climate change (Barbour et al., 1999; Master et al., 2000). However, aquatic insects face specific challenges presented by water pollution, enhanced erosion,

and watercourse alteration (Master et al., 2000; Pautasso and Fontaneto, 2008; Williams, 2011). Stoneflies in the family Perlidae are the most at-risk for decline, showing the greatest number of extinct species since the 1950s (DeWalt, 2005). Although no largescale case studies have been conducted in the past 15 years, continued human-induced and environmental pressures, in addition to limited environmental protection policies, have likely continued the imperilment of stoneflies (DeWalt, 2005; Hallmann et al., 2017; Sánchez-Bayo and Wyckhuys, 2019).

Stoneflies are valuable to stream ecosystems, where nymphs are important for nutrient cycling by feeding extensively on detritus in earlier larval stages. Mature nymphs are predominately predaceous and provide a food source for vertebrates (Stark et al., 1998). In many species, nymphs have not been associated with their adult counterparts, and our limited understanding of these larval forms creates inconsistencies in our knowledge about stoneflies and hinders efforts to transfer biomonitoring data into conservation assessments (Barbour et al., 1999; Sweeney et al., 2011; Robinson et al., 2016; Grubbs and DeWalt, 2018). Furthermore, linking nymphs to adults may aid adult sampling by providing additional information to researchers about what adult species may be collected at sites during certain times of the year (Robinson et al., 2016). Successfully linking nymphs to adults may also reveal undescribed species (perhaps unknown as adults).

1.2 The Golden Stones, *Perlesta*

Thirty-three species of *Perlesta* (Banks, 1906) (Plecoptera: Perlidae) have been described from North America with two species described from China (Murányi and Li,

2016; South et al., 2019; Stark, 1989). For over 100 years in the United States and Canada, these small, brown stoneflies with yellow wing margins and variable head coloration were recognized as a single species, the type species of the genus, *Perlesta placida* (Hagen, 1861; Stark, 1989). After careful studies by Stark (1989) and others (Poulton and Stewart, 1991; Kirchner and Kondratieff, 1997; Stark and Rhodes, 1997; DeWalt et al., 1998; Kondratieff and Baumann, 1999; Kondratieff and Kirchner, 2002, 2003), several additional species were described, but because of their similarities and presumably close relationships, they have often been referred to as the *Perlesta placida* complex. These new species were diagnosed based on male paraprocts, genital structures, female subgenital plates, and the chorion surface and stalks of eggs (Stark, 1989; Stewart and Stark, 2002). These species were described and delimited using morphological characteristics, but two gaps in our knowledge of *Perlesta* can possibly be addressed through use of DNA sequence data: (1) linking nymphs to adult males and females through DNA barcoding and (2) elucidating phylogenetic relationships. Only 11 of the 33 proposed Nearctic *Perlesta* species have nymphs associated with adults (DeWalt, 2002; Kirchner and Kondratieff, 1997; Poulton and Stewart, 1991; Stark, 1989; Stark and Rhodes, 1997; Stewart and Stark, 2002), and one species from Mississippi, *Perlesta placida*, has never been included in a phylogenetic analysis (e.g., South et al., 2019). Currently, 18 of the 33 *Perlesta* species are represented in GenBank, and 17 were included in phylogenetic analysis with the exception of *P. shubuta* (South et al., 2019).

Three described species of stoneflies are known from Mississippi: *Perlesta lagoi*, *P. placida*, and *P. shubuta* (Stark, 1989; Stewart and Stark, 2002). Currently, *P. placida*

has no DNA sequences available in DNA repositories (e.g., GenBank). The goals of this project were (1) to use nuclear and mitochondrial DNA data to infer a molecular phylogeny of *Perlesta*, building on the data of South et al. (2019), and (2) to use DNA data to link nymphs to adult specimens ("DNA barcoding"), which will guide a search for unique morphological characteristics for recognizing the species of the nymphs. Filling in the molecular gaps with DNA barcoding will enrich the GenBank database, aid in better understanding of *Perlesta*, and facilitate the identification of unknown stoneflies for future studies and conservation effort (Sweeney et al., 2011; Li et al., 2017; Grubbs and DeWalt, 2018).

CHAPTER II – MATERIALS AND METHODS

2.1 Sampling Procedures

Adult *Perlesta* were collected during the months of May, June, and July of 2019, 2020, and 2021 using a beat sheet during the day, ultra-violet (UV) light trapping at night, and emergence traps (Cadmus et al., 2016; DeWalt et al., 2015). UV light trapping was the most dependable method of catching adults during emergence. The UV light trap was set up on a 10′ × 10′ white tarp at sunset in open areas next to bridges or moving water. An aspirator was used to collect males, females, and mating pairs that landed on the trap or tarp. Upon capture, the posterior ends of the males were squeezed and held in 95% ethanol for approximately two minutes to preserve extracted genitalia for identification (Stark, 1989). I collected approximately 600 *Perlesta* adults.

Nymphs were collected from March to May of 2019, 2020, and 2021 in rivers, creeks, and streams by disturbing detritus and sweeping with a D-net. All nymphs and adults were preserved in 95% ethanol (EtOH) and stored out of direct sunlight at room temperature (Stark, 1989). After 48–72 hrs the ethanol was drained and refilled with 95% ethanol to prevent DNA degradation. Approximately 50 nymphs were collected. All voucher specimens will be sent to the Mississippi Entomological Museum (MEM) located in the Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology at Mississippi State University, and additional specimens may be sent to the INHS or other entomological collections.

Loans of adult *Perlesta* specimens were borrowed from Dr. Bill P. Stark (Mississippi College), Dr. Boris Kondratieff (Colorado State University), and Dr. Audrey

Harrison (U.S. Army, Engineer Research and Development Center). These private and public loan specimens were used in assessing morphological variation and for comparing key characteristics of *Perlesta*.

2.2 Sampling Localities

Sampling locations were based on locations where *Perlesta* and other stonefly species had previously been collected in the state (Bankhead, 2017; Stark, 1989). New locations were chosen for meeting water conditions adequate for *Perlesta*: small, lotic streams with fast-moving, cold water over sandy/gravel substrate (Merritt and Cummins, 1984; Snellen and Stewart, 1979). Rivers, creeks, and streams in and surrounding national forests and state parks were used as key sampling sites due to their distribution across the state, less disturbance and pollution, and availability of tent camping near sampling sites. The samples from Snellen and Stewart (1979), although originally identified as *P. placida*, were later found to be *P. decipiens* (Stark, 1989). *Perlesta* stoneflies were collected from 12 counties in Mississippi (Figure 2.1) and two counties (Sequatchie and Hamilton, not mapped) in Tennessee (Fig. 2.1).

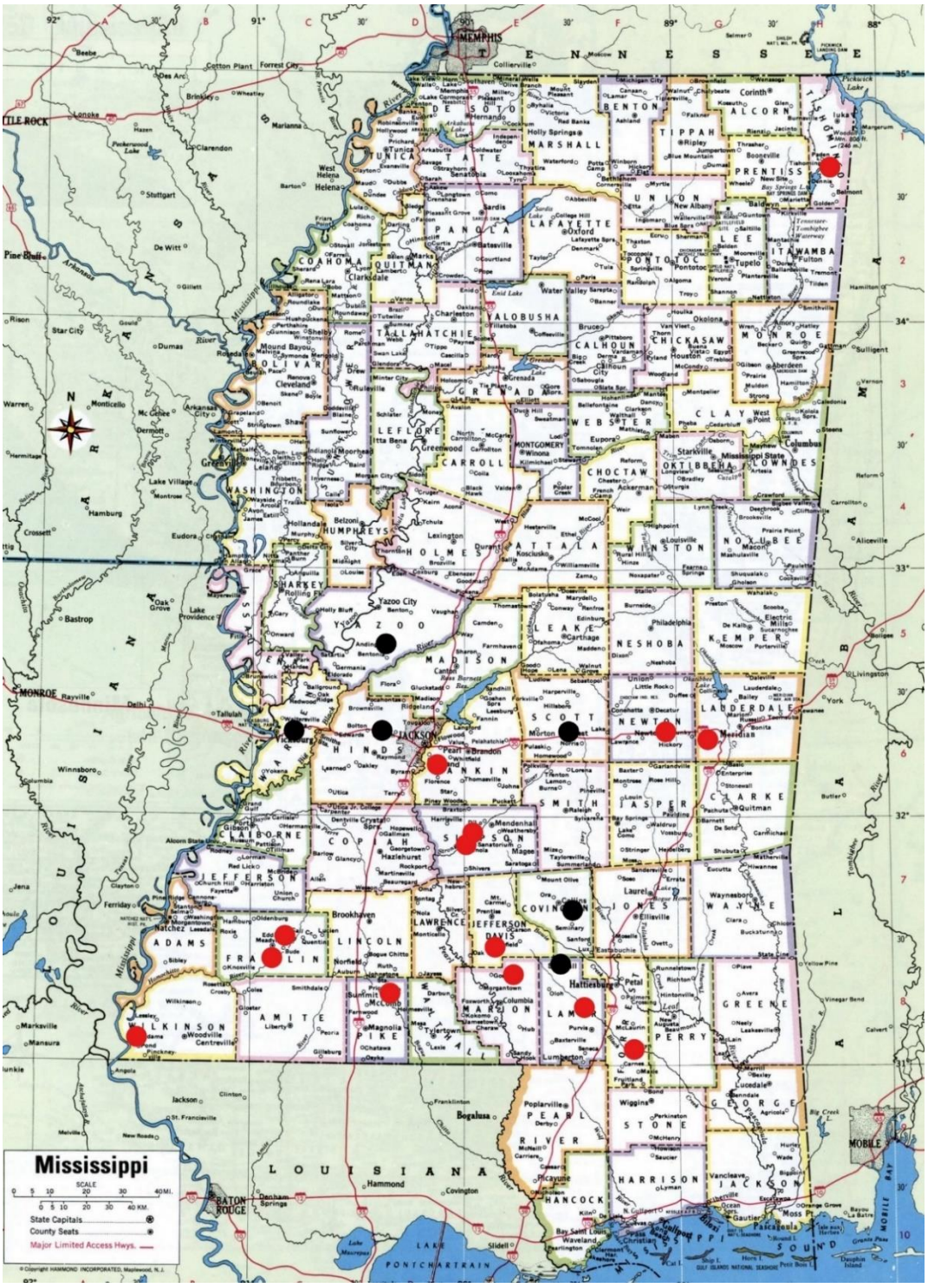


Figure 2.1. Collection sites (2018–2021) for *Perlesta* spp.

Red dots represent successful *Perlesta* sampling sites. Black dots represent unsuccessful sampling sites.

2.3 Morphology

Collected adult stoneflies were identified to currently recognized species using keys in Stark (1989, 2002, 2004). The primary morphological features used in identifying male *P. placida* and *P. shubuta* were their paraprocts and aedeagi. *Perlesta placida* has a long, slender aedeagus with a small ventral caecum (Figure 2.2A). The dorsal aedeagal patch of *P. placida* covers over half of the surface. The paraprocts are slender and long with a reduced apical spine (Figure 3C). *Perlesta shubuta* is characterized by having a shorter aedeagus with a more prominent ventral caecum (Figure 2.2B). The paraprocts of *P. shubuta* are not as long or slender as in *P. placida*, and *P. shubuta* has a more pronounced apical spine (Figure 2.3D). The aedeagal patch is wide basally, and broad at the base of the caecum (Stark, 1989). *Perlesta lagoi*, although not shown here, is characterized by short paraprocts with a small apical spine and a slender dorsal patch. (Stark, 1989).

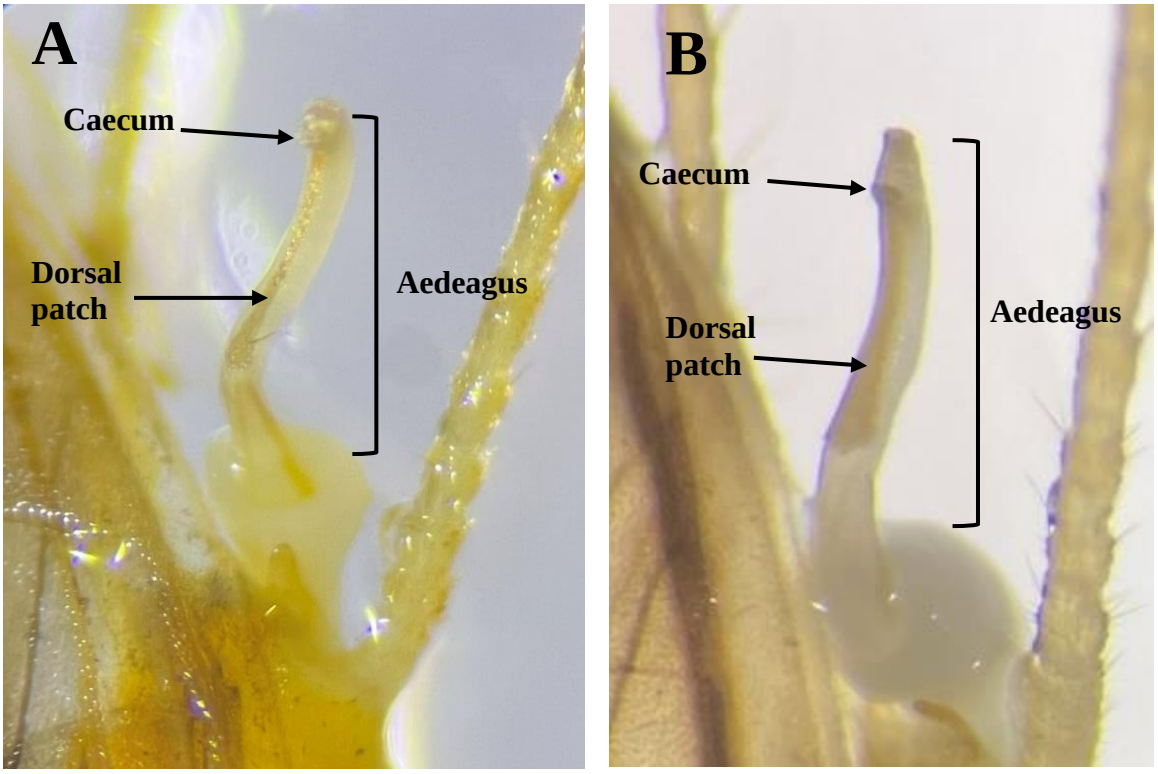


Figure 2.2 Male *Perlesta* aedeagi.

(**A**) Dorsal view of *Perlesta placida* (660) aedeagus collected in Simpson County, MS. (**B**) Dorsal view of *Perlesta shubuta* (504) aedeagus collected in Wilkinson County, MS.

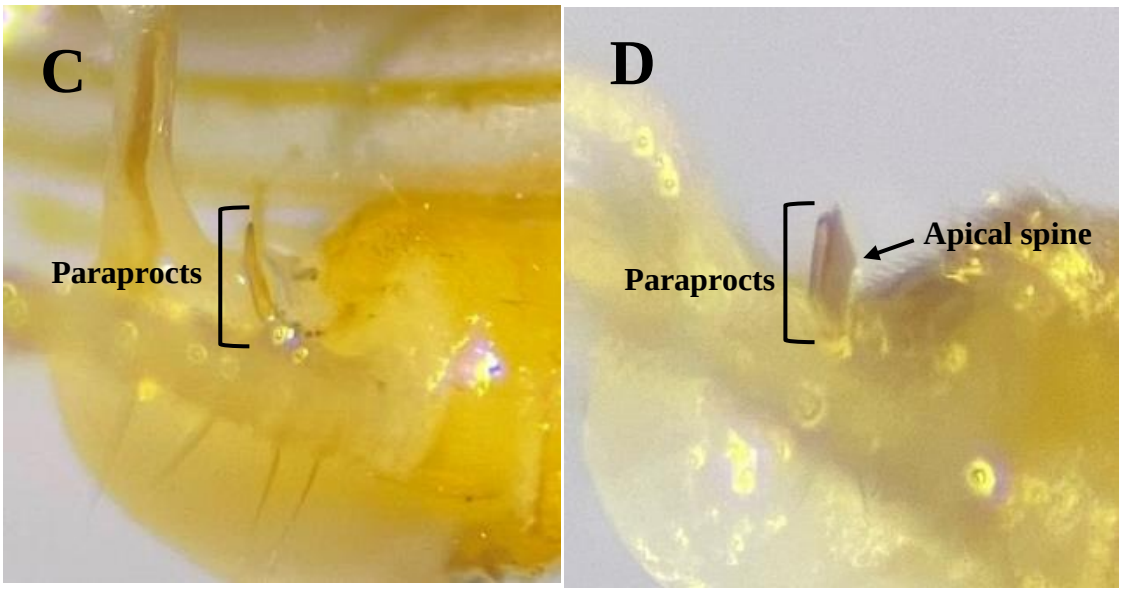


Figure 2.3 *Perlesta* paraprocts.

(**C**) Lateral view of *Perlesta placida* (659) paraprocts collected in Simpson County, MS. (D) Lateral view of *Perlesta shubuta* (657) paraprocts collected in Tishomingo County, MS.

Female *Perlesta* are highly variable, making identification most accurate in conjunction with eggs and identified males collected at the same locality. However, females found in Mississippi can be distinguished predominately by their subgenital plates. The subgenital plates of *Perlesta placida* have small, rounded lobes (Figure 2.4E), while the plates of *Perlesta shubuta* have truncate lobes, creating a small v-notch (Figure 2.4F; Stark, 1989, 2004). *Perlesta lagoi* has relatively large subgenital lobes, with rounded edges and a v-notch (Stark, 1989).

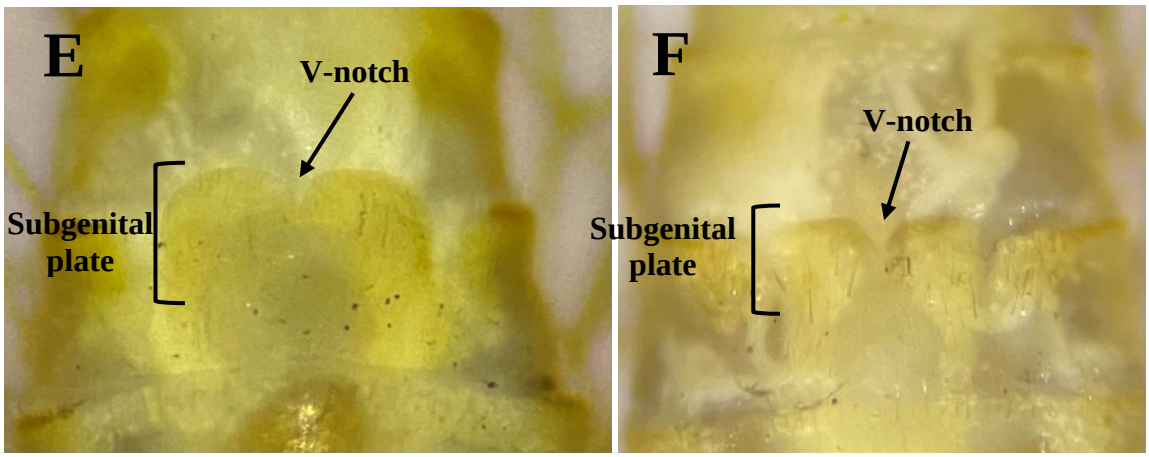


Figure 2.4 *Perlesta* female subgenital plates.

(**E**) Female *Perlesta placida* subgenital plate. Dorsal view of *Perlesta placida* (488) subgenital plate collected in Rankin County, MS. (**F**) *Perlesta shubuta* (649) subgenital plate collected in Forrest County, MS.

Perlesta eggs are characterized by size, chorion surface, and egg stalks. *Perlesta placida* has oval eggs with a short, sessile collar, and a chorion surface with minute pits. *Perlesta shubuta* eggs are oval with a short, almost sessile, button-like collar with a smooth chorion surface (Stark, 1989, 2004).

Variations in color patterns as well as setal patterns on the head, cerci, and legs of nymphs are used to distinguish between species of *Perlesta* (Stark and Harrison, 2019), but *P. shubuta* is currently the only Mississippi species that has a nymph linked to an adult. *Perlesta shubuta* nymphs were identified using two keys (Stark, 1989, 2002; Morse et al., 2017). The primary features used for identifying *P. shubuta* nymphs were the presence of a dark, transverse ocellar band and large setal "spots" on the head (Figure 2.5G) compared to smaller setal dots in other associated nymphs (Stark, 1989, 2002; Morse et al., 2017).

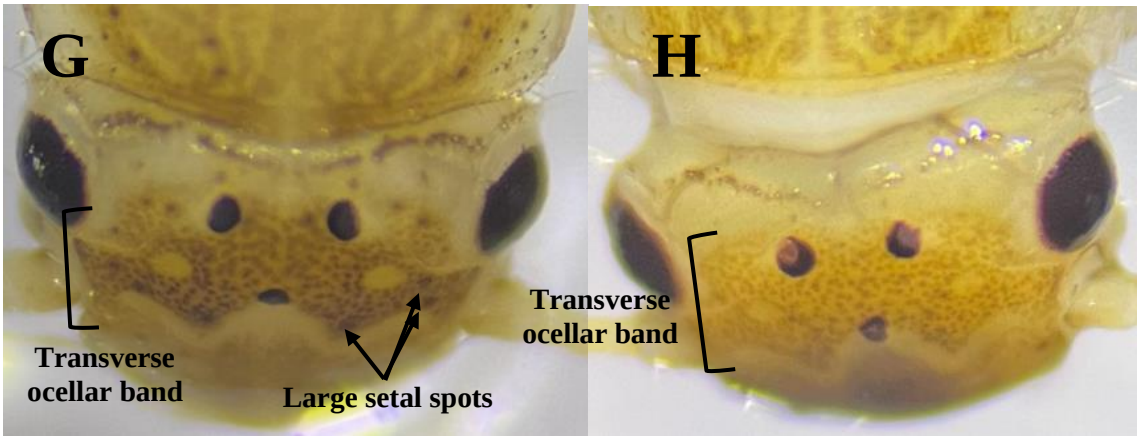


Figure 2.5 *Perlesta* nymphs.

(**G**) *Perlesta shubuta* head with dark, transverse ocellar band, and large, dark setal "spots." (**H**) Presumably *Perlesa placida*, with lighter transverse ocellar band, and lack of larger setal spots.

2.4 DNA

The genomic DNA of *Perlesta* was extracted from a single leg of individuals utilizing a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's procedures. DNA regions were amplified using the protocol of Samarakoon et al. (2013) for a fragment of the nuclear gene encoding for the 16S ribosomal subunit and for the mitochondrial gene encoding for the cytochrome c oxidase I (COI) subunit using polymerase chain reaction (PCR). The PCR protocol for the 16S and COI regions was completed using a Thermo Electron Corporation PCR Sprint Thermal Cycler SPRT001 and a BioRad MJ Mini Thermal Cycler. The DNA amplification program was as follows: 94°C for 3 min, then 35 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 45 s, and a final step of 72°C for 5 min. The PCR products were separated by electrophoresis and observed on an agarose gel stained with ethidium bromide. DNA fragments were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and sent to Eurofins in Louisville, KY, for sequencing.

The cytochrome c oxidase subunit I (COI) gene was chosen for this study as it is one of the more conservative protein-coding genes and has a large reference database (Folmer et al., 1994). However, the inconsistent primer binding sites across the COI gene, which were designed to work for all invertebrates, prompted the use of additional primers that were specifically designed here for use in insects, using the reference sequence of *Drosophila yakuba* used in Folmer et al. (1994). The mitochondrial 16S rRNA gene was chosen due to studies showing less amplification bias than COI and consistent variation in sequence abundance specifically in stoneflies (Elbrecht et al., 2016).

Table 2.1 Primers and Sequences

Primer Name	Primer Sequence
COI	
LCOI-1490	5′-GGTCAACAAATCATAAAGATATTGG-3′
HCO-2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′
LCO-Insect-1490	5’-TWTCWACMAATCATAAARATATTGG-3’
HCO-Insect-2198	5’-TAMACTTCWGGRTGACCAAARAAYCA-3’
16S	
Terry-16S-A	5′-CGCCTGTTTATCAAAAACAT-3′
Terry-16S-B	5′-CTCCGGTTTGAACTCAGATCA-3′

2.5 Linking Males, Females, and Nymphs

If consistent variation exists, analyzing the DNA of nymphs found at sampling sites with identified adult species would supplement the morphological data. Ideally, DNA data can be used to match nymphs to adult males and females and assign them to the appropriate species. With corresponding congeners, morphological features (or variation) that correspond to the species can be assessed. In this case, the “DNA barcodes” could provide the basis for nymph identification. The different nymph species could then be compared to each other to discern what—if any—the differentiating morphological characteristics are.

2.6 Phylogenetics

DNA data were collected primarily of the species in Mississippi, and these data were combined with data available in GenBank, primarily from the study of South et al. (2019) who sequenced the COI region from 17 *Perlesta* species for phylogenetic reconstruction. The newly collected data were cleaned using Sequencher 5.0 (Gene Codes Corp., Ann Arbor, Michigan), aligned using ClustalX (Larkin et al., 2007), and exported for phylogenetic analysis using WinClada (Nixon, 2002) and MEGA-X (Kumar

et al., 2018). Sequences were trimmed to a uniform 471 nucleotides for the 16S region and 612 nucleotides for the COI region to minimize effects from missing data. Parsimony and maximum likelihood (ML) were used to infer phylogenetic relationships. For parsimony analyses, 10 sequential ratchet runs (Nixon, 1990) of 200 iterations were performed, followed by a heuristic search on the recovered trees, saving a total of 5000 trees doing 500 replications, with each replication saving 5 trees. Then, all unsupported nodes were hard collapsed, and a strict consensus was calculated for all trees. Lastly, 5000 jackknife replications were run, each consisting of 10 search replications holding 2 trees. For maximum likelihood analyses, the General Time Reversible nucleotide substitution model and a gamma distribution with invariable sites (GTR+I+G) was used with 1,000 bootstrap replications as used in South et al. (2019) and suggested by MEGA (Kumar et al., 2018).

2.7 Species Concept

Perlesta species have historically been delimited by trait-based species concepts. Species have been distinguished using combinations of morphological characteristics including coloration, egg morphology, and male and female genital structures (Stark, 1989), corresponding to a Morphological Species Concept or Phylogenetic Species Concept (PSC) *sensu* Nixon and Wheeler (1990; Nixon and Davis, 1992). A recent study by South et al. (2019) examined the COI region to support the description of a new species, where a phylogenetic analysis of the DNA data showed strong support for monophyletic groups that corresponded to the species inferred by morphological data. Monophyly of species is not required by the PSC (unlike the Monophyletic Species

Concept *sensu* Mishler and Brandon [1987] or the Genealogical Species Concept *sensu* Baum and Shaw [1995]), but monophyly does provide additional evidence of close relationship and the passing of enough time for divergence, formation of autapomorphies, and extinction of intermediates. For example, in the South et al. (2019) study, all the sampled species form monophyletic groups in phylogenetic analysis of mitochondrial COI data, but this does not necessarily have to be true, as species arise from pre-existing species which may persist. For this study, I utilized the Unified Species Concept of de Queiroz (2005), where species concept and species delimitation are considered two different issues. In his definition, the different kinds of "concepts" in the past typically represent primacy of different kinds of data (reproductive compatibility or barriers, diagnostic morphological features, monophyly, ecological niche, etc.), which he argues should all be kinds of evidence in support of species recognition/differentiation in the process of species delimitation. When enough different kinds of data are accumulated, which is left to the particular researcher and organisms studied, different species may be recognized.

For this study, DNA data were the primary data collected for assessing species boundaries, but morphological characteristics were also used to support species identification. However, the process of delimiting species was iterative. The same DNA sequences, or similar DNA sequences that form a clade or paraphyletic grade, were initially interpreted to have come from the same species, but when the DNA sequences were different (differences that led to placement in different clades in phylogenetic

analysis), then the specimens were revisited to compare morphological features to the taxon with which it was first associated.

CHAPTER III – RESULTS

3.1 Sampling Outcomes

Although a goal of this project was to collect all three Mississippi *Perlesta* species, *Perlesta lagoi* was not collected despite multiple sampling attempts across multiple sampling seasons at sites where it was collected in the past, including the location where the holotype was collected (Stark, 1989). One specimen (*Perlesta shubuta* 504) was collected in Wilkinson County, MS, and shared similar features to *P. lagoi*, but was ultimately identified as *P. shubuta* due to its dorsal patch and caecum. *Perlesta lagoi* has not been collected in Mississippi in the last few decades (Stark, pers. comm.). Although *Perlesta* species were collected in 12 counties, only sampling in three counties (Franklin, Marion, and Simpson) resulted in the collection of all the stages/sexes of stoneflies at the same locality. Surprisingly, multiple days of using a D-net to sample streams, creeks, and other suitable habitats for *Perlesta* nymphs were unsuccessful, only to collect an abundance of adults in the same locations at night with a UV light trap. The majority of specimens used in this project were collected from May to June of 2021. Although the emergence of *Perlesta* adults was approximately three weeks later than usual in 2021, this may have been due to a cooler spring and persistent rain into the summer. Morphological identification was corroborated independently with Dr. Bill P. Stark (Mississippi College).

3.2 Phylogenetic Analysis of COI DNA Sequences

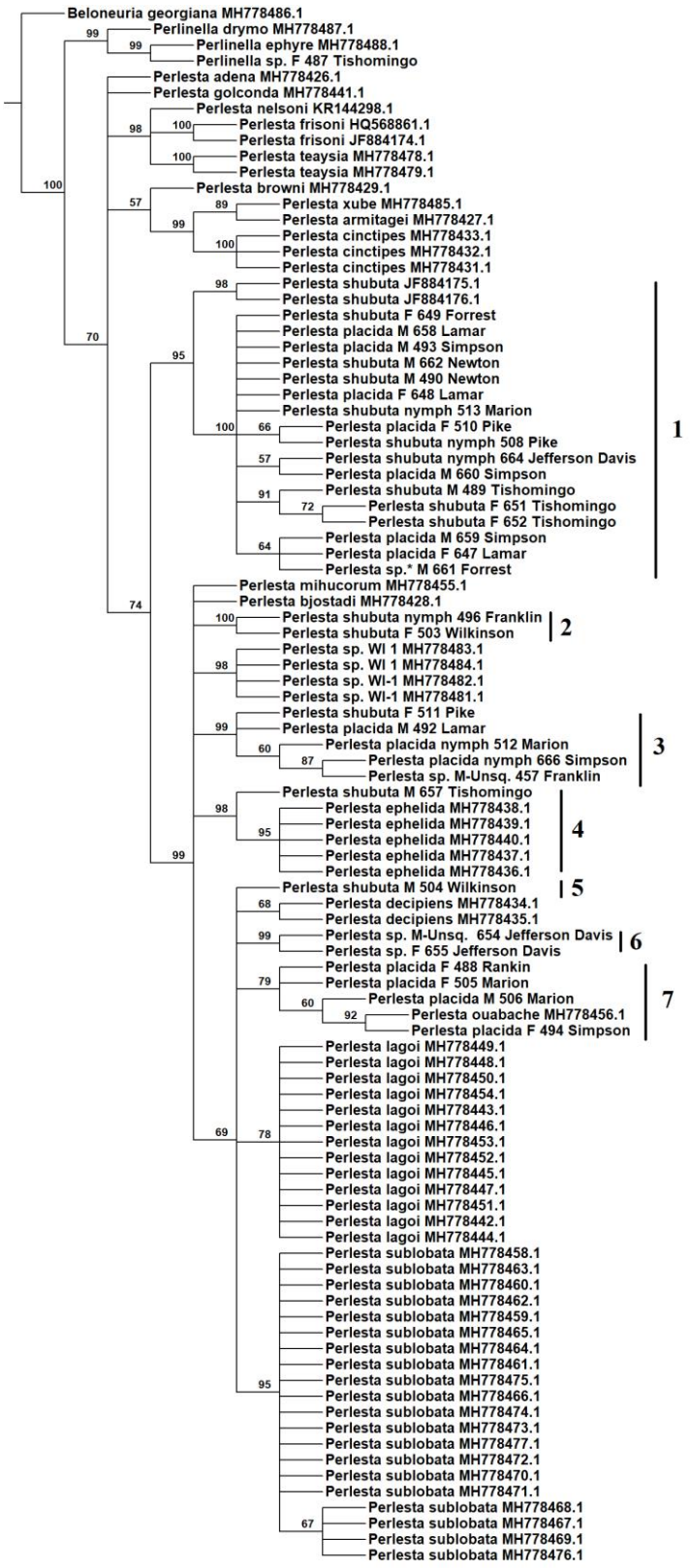
DNA sequences of mitochondrial COI were obtained for 32 *Perlesta* individuals, including 14 males, 12 females, and 6 nymphs. The outgroups used in these analyses were *Beloneuria georgiana, Perlinella drymo,* and *Perlinella ephyre*. At least one individual was sequenced for each sampling locality. The parsimony analysis resulted in 23,644 most parsimonious trees (MPTs) of length 1212, a consistency index (CI) of 0.33, and a retention index (RI) of 0.79 (Farris, 1989). A strict consensus tree with all unsupported nodes collapsed was then calculated. Jackknife values (Farris, 1996) were mapped onto the strict consensus tree. Sequences from GenBank are referenced with their GenBank number. Sequences from this study are annotated as male (M), female (F), or nymph (N) and the name of the county where the individual was collected (Fig. 3.1).

In the strict consensus COI tree, *Perlesta* from Mississippi appeared in seven clades (Fig. 3.1). *Perlesta placida* and *P. shubuta* were not confined to exclusive clades of individual species. The first clade included the most samples from this study with seven *P. placida* adults, six *P. shubuta* adults, three *P. shubuta* nymphs, and one adult male that could not be keyed out to species due to ambiguous morphological features. This individual shared morphological features with both *P. placida* and *P. golconda.* Namely, this individual's head patterns and dorsal caecum/ridge were similar to *P. golconda*, although Mississippi is currently outside its range, although South et al. (2019) reported it from Louisiana. This clade received strong support (100% jackknife support), and the nearest sister species identified and uploaded to GenBank was *P. shubuta* with

strong support (95% jackknife support) (identification presumably by stonefly expert Dr. R. E. DeWalt (Illinois Natural History Survey). A nested clade within the first clade shows relatively strong support for *P. shubuta* collected in Tishomingo County (91% jackknife support). The remaining nested clades show weak support (<70% jackknife support).

The second clade shows strong support (100% jackknife support) for a *P. shubuta* nymph and a female *P. shubuta*. The third clade is composed exclusively of individuals from this study with one *P. shubuta* adult, one *P. placida* adult, two *P. placida* nymphs, and one unsqueezed male adult. This clade shows strong support (99% jackknife support) and a nested clade with moderate support (87% jackknife support) between a *P. placida* nymph and an unsqueezed/unidentified *Perlesta* adult male. The fourth clade contains one adult male, *P. shubuta*, with strong support sister to a clade of *P. ephelida* (98% jackknife support).

The sixth clade includes an unsqueezed/unidentified *Perlesta* male and unidentified female from Jefferson Davis County, which formed a strongly supported (99% jackknife support) nested clade, while a *P. shubuta* (fifth clade) from Tishomingo County fell outside any nested clades. This male's (504) aedeagus armature and caecum looked like *Perlesta lagoi* but was ultimately keyed out to *Perlesta shubuta* after soaking in potassium hydroxide and based on paraprocts and aedeagal features by Dr. Stark. The seventh clade had moderate support (79% jackknife support) with four *P. placida* adults. One *P. ouabache* individual showed strong support (92% jackknife support) with a *Perlesta placida* female as a sister taxon.


Beloneuria georgiana MH778486.1
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Perlesta golconda MH778441.1
Perlesta nelsoni KR144298.1
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Perlesta cinctipes MH778431.1
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Perlesta shubuta nymph 513 Marion
Perlesta placida F 510 Pike
Perlesta shubuta nymph 508 Pike
Perlesta shubuta nymph 664 Jefferson Davis
Perlesta placida M 660 Simpson
Perlesta shubuta M 489 Tishomingo
Perlesta shubuta F 651 Tishomingo
Perlesta shubuta F 652 Tishomingo
Perlesta placida M 659 Simpson
Perlesta placida F 647 Lamar
Perlesta sp.* M 661 Forrest
Perlesta mihucorum MH778455.1
Perlesta bjostadi MH778428.1
Perlesta shubuta nymph 496 Franklin
Perlesta shubuta F 503 Wilkinson
Perlesta sp. WI 1 MH778483.1
Perlesta sp. WI 1 MH778484.1
Perlesta sp. WI-1 MH778482.1
Perlesta sp. WI-1 MH778481.1
Perlesta shubuta F 511 Pike
Perlesta placida M 492 Lamar
Perlesta placida nymph 512 Marion
Perlesta placida nymph 666 Simpson
Perlesta sp. M-Unsq. 457 Franklin
Perlesta shubuta M 657 Tishomingo
Perlesta ephelida MH778438.1
Perlesta ephelida MH778439.1
Perlesta ephelida MH778440.1
Perlesta ephelida MH778437.1
Perlesta ephelida MH778436.1
Perlesta shubuta M 504 Wilkinson
Perlesta decipiens MH778434.1
Perlesta decipiens MH778435.1
Perlesta sp. M-Unsq. 654 Jefferson Davis
Perlesta sp. F 655 Jefferson Davis
Perlesta placida F 488 Rankin
Perlesta placida F 505 Marion
Perlesta placida M 506 Marion
Perlesta ouabache MH778456.1
Perlesta placida F 494 Simpson
Perlesta lagoi MH778449.1
Perlesta lagoi MH778448.1
Perlesta lagoi MH778450.1
Perlesta lagoi MH778454.1
Perlesta lagoi MH778443.1
Perlesta lagoi MH778446.1
Perlesta lagoi MH778453.1
Perlesta lagoi MH778452.1
Perlesta lagoi MH778445.1
Perlesta lagoi MH778447.1
Perlesta lagoi MH778451.1
Perlesta lagoi MH778442.1
Perlesta lagoi MH778444.1
Perlesta sublobata MH778458.1
Perlesta sublobata MH778463.1
Perlesta sublobata MH778460.1
Perlesta sublobata MH778462.1
Perlesta sublobata MH778459.1
Perlesta sublobata MH778465.1
Perlesta sublobata MH778464.1
Perlesta sublobata MH778461.1
Perlesta sublobata MH778475.1
Perlesta sublobata MH778466.1
Perlesta sublobata MH778474.1
Perlesta sublobata MH778473.1
Perlesta sublobata MH778477.1
Perlesta sublobata MH778472.1
Perlesta sublobata MH778470.1
Perlesta sublobata MH778471.1
Perlesta sublobata MH778468.1
Perlesta sublobata MH778467.1
Perlesta sublobata MH778469.1
Perlesta sublobata MH778476.1
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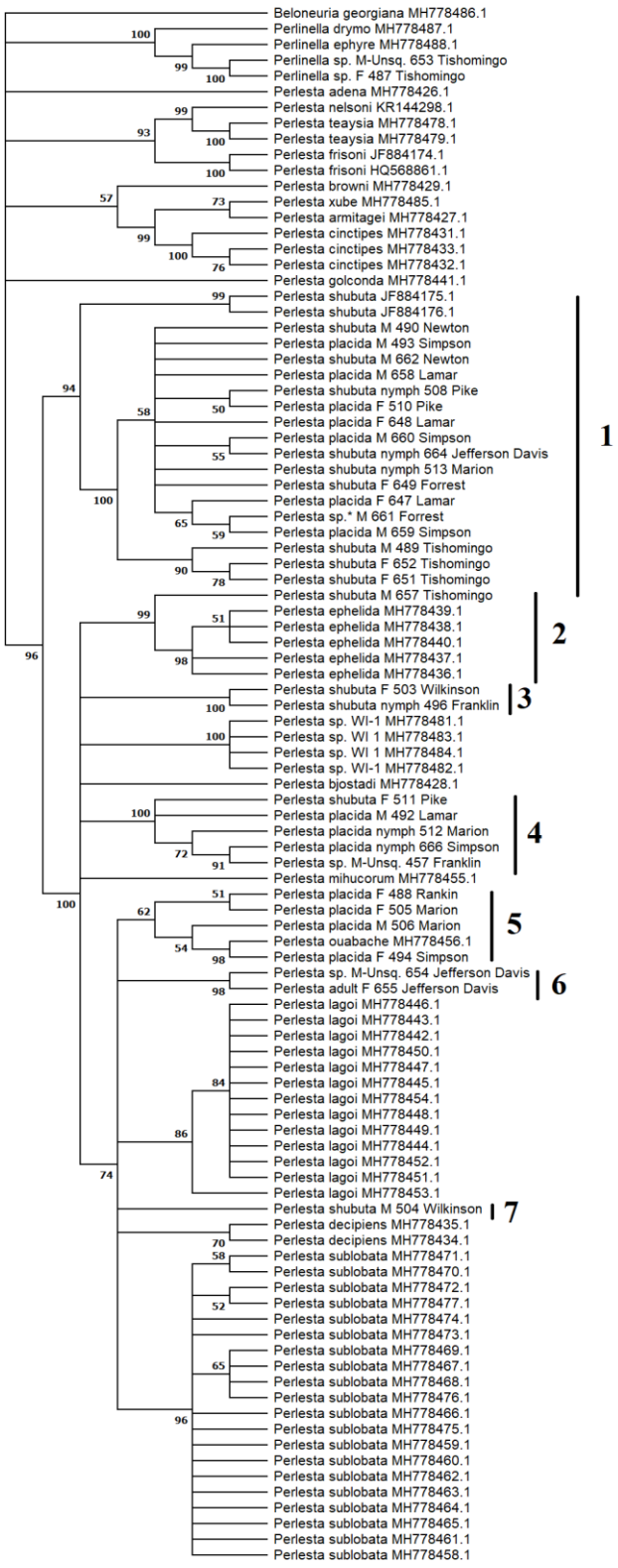
Figure 3.1 COI Strict Consensus Tree

Strict consensus tree with all unsupported branches collapsed of 23,644 most parsimonious trees obtained in a phylogenetic analysis of mitochondrial COI data. L=1212, CI=0.33, RI=0.79, jackknife values above the branches. Sequences from GenBank are referenced with their GenBank number. Sequences from this study are annotated as male (M), female (F), or nymph (N), a lab DNA extraction number, and the name of the county where the individual was collected. Unidentified males without aedeagus exuded are labeled (M-Unsq). Outgroup taxon: *Beloneuria georgiana*, *Perlinella drymo*, and *Perlinella ephyre.* Clades where Mississippi *Perlesta* appear are labeled with numbered lines.

A phylogeny was also inferred using the Maximum Likelihood (ML) method (Fig. 3.2) using MEGA X (Kumar et al., 2018). The General Time Reversible model was selected as used by South et al. (2019) (Nei and Kumar, 2000). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4896)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 29.17% sites). This analysis involved 99 nucleotide sequences and total of 612 positions in the final dataset.

The Maximum Likelihood tree yielded similar results to the consensus tree from parsimony, and each colorfully outlined clade includes the same sequences. The first clade (Fig. 3.2) has strong support (94% bootstrap support) and good support for the nested monophyletic clade of *P. shubuta* from Tishomingo County, Mississippi (90% bootstrap support). The second, third, and fourth clades showed almost identical support to the same numbered clades in the consensus tree obtained by parsimony (Fig. 3.1). The fifth clade showed moderately strong support for *P. ouabache* and *P. placida* at the terminal branches, but the overall clade had weak support (62% bootstrap support) compared to the parsimony tree (79% bootstrap support). The sixth clade had strong support (98% bootstrap support) for sister taxa at the terminal branches for an unsqueezed *Perlesta* male and unidentified female from Jefferson Davis County, Mississippi. The seventh clade, *Perlesta shubuta* (504), was left in an unresolved position as seen in the consensus tree obtained by parsimony (Fig. 3.1).

The Maximum Likelihood tree produced in this study affirms the relationships obtained in South et al. (2019). All the supported clades found in his ML tree were the same in this one, with similar bootstrap support. The *P. shubuta* and *P. placida* sequences from this study mostly made clades (albeit clades with representatives of both species) around the *Perlesta* species used in his study. The two exceptions were (1) in the second clade (Fig. 3.2) where a *P. shubuta* sequence showed strong support (99% bootstrap support) as sister to *P. ephelida*, and (2) in the fifth clade, where a *P. placida* was sister to *P. oubache* (98% bootstrap support).


Beloneuria georgiana MH778486.1
Perlinella drymo MH778487.1
Perlinella ephyre MH778488.1
Perlinella sp. M-Unsq. 653 Tishomingo
Perlinella sp. F 487 Tishomingo
Perlesta adena MH778426.1
Perlesta nelsoni KR144298.1
Perlesta teaysia MH778478.1
Perlesta teaysia MH778479.1
Perlesta frisoni JF884174.1
Perlesta frisoni HQ568861.1
Perlesta browni MH778429.1
Perlesta xube MH778485.1
Perlesta armitagei MH778427.1
Perlesta cinctipes MH778431.1
Perlesta cinctipes MH778433.1
Perlesta cinctipes MH778432.1
Perlesta golconda MH778441.1
Perlesta shubuta JF884175.1
Perlesta shubuta JF884176.1
Perlesta shubuta M 490 Newton
Perlesta placida M 493 Simpson
Perlesta shubuta M 662 Newton
Perlesta placida M 658 Lamar
Perlesta shubuta nymph 508 Pike
Perlesta placida F 510 Pike
Perlesta placida F 648 Lamar
Perlesta placida M 660 Simpson
Perlesta shubuta nymph 664 Jefferson Davis
Perlesta shubuta nymph 513 Marion
Perlesta shubuta F 649 Forrest
Perlesta placida F 647 Lamar
Perlesta sp.* M 661 Forrest
Perlesta placida M 659 Simpson
Perlesta shubuta M 489 Tishomingo
Perlesta shubuta F 652 Tishomingo
Perlesta shubuta F 651 Tishomingo
Perlesta shubuta M 657 Tishomingo
Perlesta ephelida MH778439.1
Perlesta ephelida MH778438.1
Perlesta ephelida MH778440.1
Perlesta ephelida MH778437.1
Perlesta ephelida MH778436.1
Perlesta shubuta F 503 Wilkinson
Perlesta shubuta nymph 496 Franklin
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Perlesta sp. WI 1 MH778483.1
Perlesta sp. WI 1 MH778484.1
Perlesta sp. WI-1 MH778482.1
Perlesta bjostadi MH778428.1
Perlesta shubuta F 511 Pike
Perlesta placida M 492 Lamar
Perlesta placida nymph 512 Marion
Perlesta placida nymph 666 Simpson
Perlesta sp. M-Unsq. 457 Franklin
Perlesta mihucorum MH778455.1
Perlesta placida F 488 Rankin
Perlesta placida F 505 Marion
Perlesta placida M 506 Marion
Perlesta ouabache MH778456.1
Perlesta placida F 494 Simpson
Perlesta sp. M-Unsq. 654 Jefferson Davis
Perlesta adult F 655 Jefferson Davis
Perlesta lagoi MH778446.1
Perlesta lagoi MH778443.1
Perlesta lagoi MH778442.1
Perlesta lagoi MH778450.1
Perlesta lagoi MH778447.1
Perlesta lagoi MH778445.1
Perlesta lagoi MH778454.1
Perlesta lagoi MH778448.1
Perlesta lagoi MH778449.1
Perlesta lagoi MH778444.1
Perlesta lagoi MH778452.1
Perlesta lagoi MH778451.1
Perlesta lagoi MH778453.1
Perlesta shubuta M 504 Wilkinson
Perlesta decipiens MH778435.1
Perlesta decipiens MH778434.1
Perlesta sublobata MH778471.1
Perlesta sublobata MH778470.1
Perlesta sublobata MH778472.1
Perlesta sublobata MH778477.1
Perlesta sublobata MH778474.1
Perlesta sublobata MH778473.1
Perlesta sublobata MH778469.1
Perlesta sublobata MH778467.1
Perlesta sublobata MH778468.1
Perlesta sublobata MH778476.1
Perlesta sublobata MH778466.1
Perlesta sublobata MH778475.1
Perlesta sublobata MH778459.1
Perlesta sublobata MH778460.1
Perlesta sublobata MH778462.1
Perlesta sublobata MH778463.1
Perlesta sublobata MH778464.1
Perlesta sublobata MH778465.1
Perlesta sublobata MH778461.1
Perlesta sublobata MH778458.1
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Figure 3.2 COI Maximum Likelihood Tree

Maximum Likelihood phylogenetic reconstruction of 99 *Perlesta* COI sequences using the GTR+I+G nucleotide substitution model. 32 sequences are from this study, while 67 are from GenBank submissions indicated by their accession numbers. Sequences from this study are annotated as male (M), female (F), or nymph (N) and the name of the county where the individual was collected. Unidentified males without aedeagus exuded are labeled (M-Unsq). Outgroup taxa: *Beloneuria georgiana*, *Perlinella drymo*, and *Perlinella ephyre*. Bootstrap scores from 1,000 replicates are shown at nodes.

3.3 Phylogenetic Analysis of 16S DNA Data

DNA of the 16S region, aligned and trimmed to 471 bp, was amplified for 41 *Perlesta* individuals, including 20 males, 15 females, and 6 nymphs. The outgroups used in these analyses were *Perlinella drymo, Perlinella sp.* 653, and *Perlinella sp.* 487. At least one individual was sequenced for each sampling locality. One *Perlesta decipiens* sequence was used from GenBank. The phylogenetic analysis using parsimony resulted in 10,394 most parsimonious trees with length 91, a CI of 0.81, and an RI of 0.96. A strict consensus tree with all unsupported nodes collapsed was calculated from these MPTs, and values from a jackknife analysis were mapped onto the tree above the branches. Sequences from GenBank are referenced with their GenBank number. Sequences from this study are annotated as male (M), female (F), or nymph (N), a lab DNA extraction number, and the name of the county where the individual was collected. Unidentified males without aedeagus exuded are labeled (M-Unsq) (Fig. 3.3).

In the strict consensus tree based on a parsimony analysis of 16S, *Perlesta* from Mississippi formed two major clades (Fig. 3.3). Again, as seen in the COI results, *P. placida* and *P. shubuta* were not confined to exclusive clades of individual species. The

first clade showed weak support (51% jackknife support) with seven *P. placida* adults, six *P. shubuta* adults, three *P. shubuta* nymphs, and one adult male that could not be keyed out to species due to ambiguous morphological features (see first paragraph of COI Analysis). The second clade showed strong support (96% jackknife support) with four *P. placida* adults, three *P. shubuta* adults, one *P. decipiens* adult from GenBank, and three unidentified/unsqueezed adults. The three unidentified adults formed a nested clade with weak support (56% jackknife support). The third clade is nested within the second clade and has moderate support (78% jackknife support) with one *P. placida* adult, one *P. placida* nymph, one *P. shubuta* nymph, and eight unidentified adults.

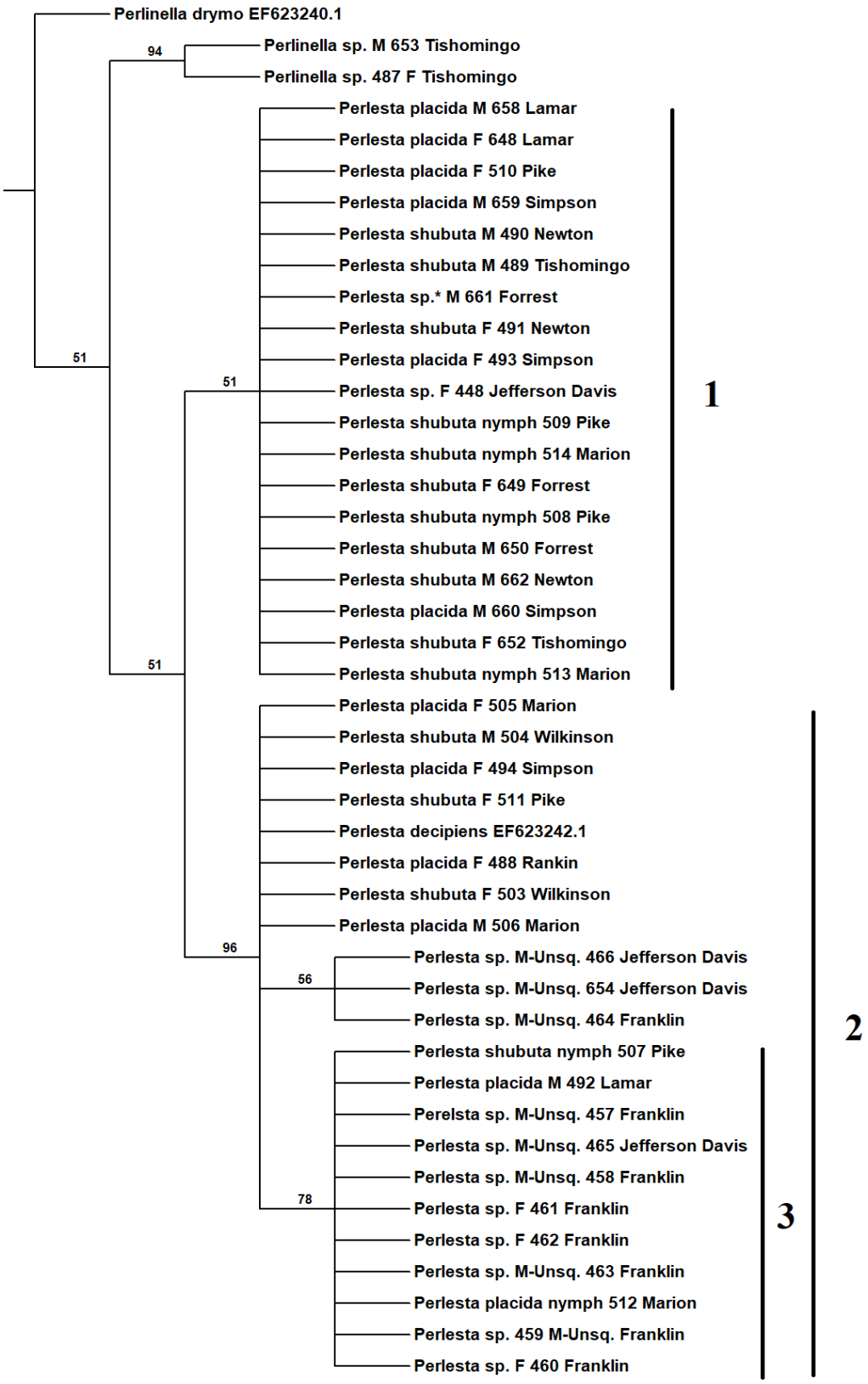
Perlinella drymo EF623240.1
94
Perlinella sp. M 653 Tishomingo
Perlinella sp. 487 F Tishomingo
Perlesta placida M 658 Lamar
Perlesta placida F 648 Lamar
Perlesta placida F 510 Pike
Perlesta placida M 659 Simpson
Perlesta shubuta M 490 Newton
Perlesta shubuta M 489 Tishomingo
Perlesta sp.* M 661 Forrest
Perlesta shubuta F 491 Newton
51
Perlesta placida F 493 Simpson
51
Perlesta sp. F 448 Jefferson Davis
1
Perlesta shubuta nymph 509 Pike
Perlesta shubuta nymph 514 Marion
Perlesta shubuta F 649 Forrest
Perlesta shubuta nymph 508 Pike
Perlesta shubuta M 650 Forrest
Perlesta shubuta M 662 Newton
Perlesta placida M 660 Simpson
Perlesta shubuta F 652 Tishomingo
51
Perlesta shubuta nymph 513 Marion
Perlesta placida F 505 Marion
Perlesta shubuta M 504 Wilkinson
Perlesta placida F 494 Simpson
Perlesta shubuta F 511 Pike
Perlesta decipiens EF623242.1
Perlesta placida F 488 Rankin
Perlesta shubuta F 503 Wilkinson
Perlesta placida M 506 Marion
96
Perlesta sp. M-Unsq. 466 Jefferson Davis
56
Perlesta sp. M-Unsq. 654 Jefferson Davis
Perlesta sp. M-Unsq. 464 Franklin
2
Perlesta shubuta nymph 507 Pike
Perlesta placida M 492 Lamar
Perelsta sp. M-Unsq. 457 Franklin
Perlesta sp. M-Unsq. 465 Jefferson Davis
Perlesta sp. M-Unsq. 458 Franklin
78
Perlesta sp. F 461 Franklin
3
Perlesta sp. F 462 Franklin
Perlesta sp. M-Unsq. 463 Franklin
Perlesta placida nymph 512 Marion
Perlesta sp. 459 M-Unsq. Franklin
Perlesta sp. F 460 Franklin

Figure 3.3 16S Strict Consensus Tree

Strict consensus tree with all unsupported branches collapsed of 10,394 most parsimonious trees obtained in a phylogenetic analysis of nuclear 16S data. L=91, CI=0.81, RI=0.96, jackknife values above the branches. Sequences from GenBank are referenced with their GenBank number. Sequences from this study are annotated as male (M), female (F), or nymph (N), a lab DNA extraction number, and the name of the county where the individual was collected. Unidentified males without aedeagus exuded are labeled (M-Unsq). Outgroup taxa: *Perlinella drymo. Perlinella sp.* 653, *Perlinella sp.* 487. Clades where Mississippi samples appear are marked with numbered lines.

A phylogeny was also inferred by using the Maximum Likelihood method (Fig. 3.4). The analysis and construction of trees used the same approach as above in the COI ML analysis. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2860)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 40.76% sites). This analysis involved 44 nucleotide sequences. There were a total of 471 base-pair positions in the final dataset. These analyses were conducted in MEGA X (Kumar et al., 2018).

Mississippi *Perlesta* species appeared in two major clades in the 16S Maximum Likelihood Tree (Fig. 3.4). Again, *P. placida* and *P. shubuta* were not confined to exclusive clades of individual species. The first clade showed moderately weak support (77% bootstrap support) with seven *P. placida* adults, six *P. shubuta* adults, three *P. shubuta* nymphs, and one unidentified adult male (*Perlesta* sp. 662). The second clade showed weak support (64% bootstrap support) with four *P. placida* adults, three *P. shubuta* adults, one *P. decipiens* adult from GenBank, and three unidentified/unsqueezed adults. The three unidentified adults formed a nested clade with weak support (55%

bootstrap support). The third clade is nested within the second clade and had moderate support (83% bootstrap support) with one *P. placida* adult, one *P. placida* nymph, one *P. shubuta* nymph, and eight unidentified adults.

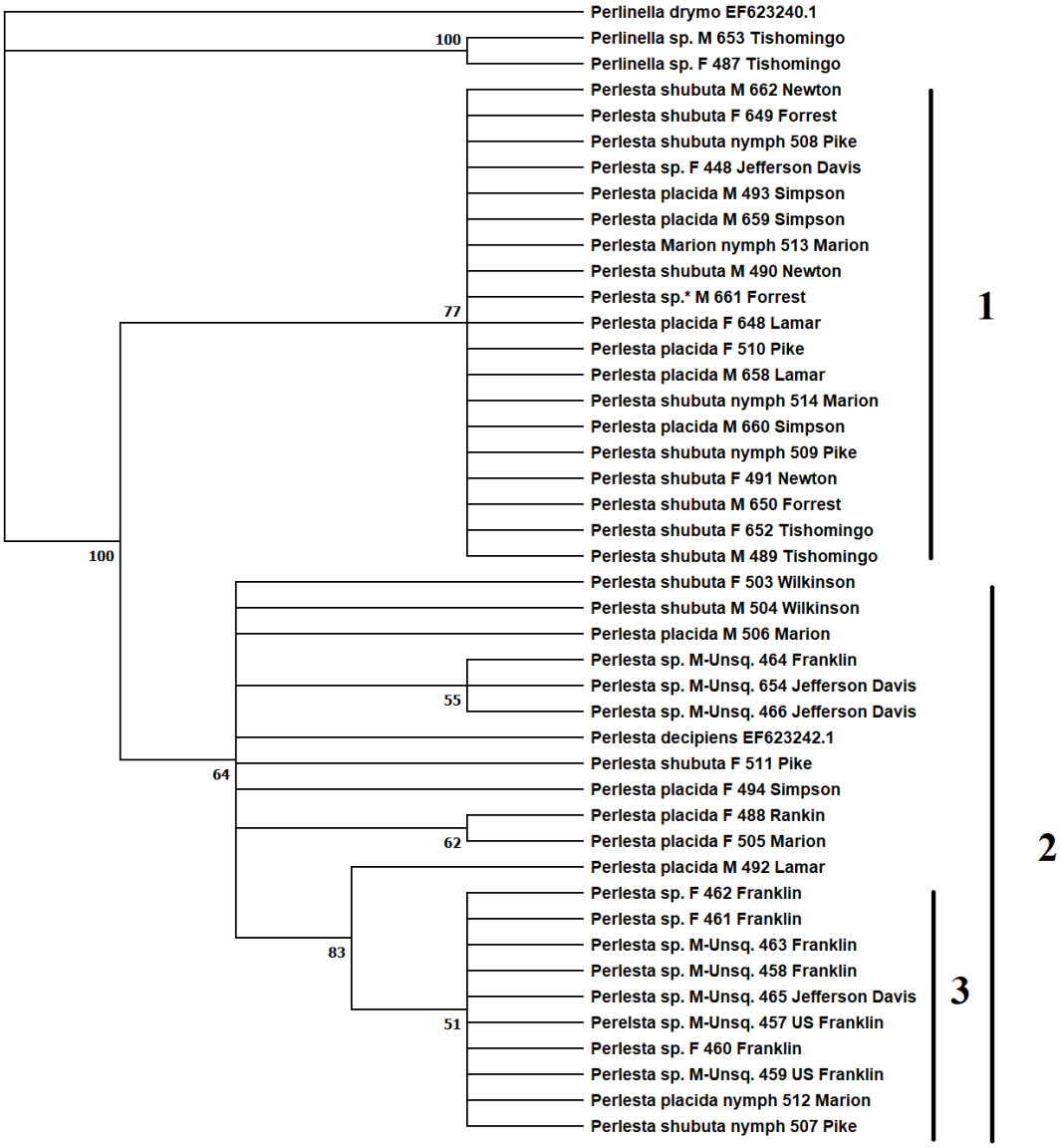
Perlinella drymo EF623240.1
Perlinella sp. M 653 Tishomingo
Perlinella sp. F 487 Tishomingo
Perlesta shubuta M 662 Newton
Perlesta shubuta F 649 Forrest
Perlesta shubuta nymph 508 Pike
Perlesta sp. F 448 Jefferson Davis
Perlesta placida M 493 Simpson
Perlesta placida M 659 Simpson
Perlesta Marion nymph 513 Marion
Perlesta shubuta M 490 Newton
Perlesta sp.* M 661 Forrest
Perlesta placida F 648 Lamar
Perlesta placida F 510 Pike
Perlesta placida M 658 Lamar
Perlesta shubuta nymph 514 Marion
Perlesta placida M 660 Simpson
Perlesta shubuta nymph 509 Pike
Perlesta shubuta F 491 Newton
Perlesta shubuta M 650 Forrest
Perlesta shubuta F 652 Tishomingo
Perlesta shubuta M 489 Tishomingo
Perlesta shubuta F 503 Wilkinson
Perlesta shubuta M 504 Wilkinson
Perlesta placida M 506 Marion
Perlesta sp. M-Unsq. 464 Franklin
Perlesta sp. M-Unsq. 654 Jefferson Davis
Perlesta sp. M-Unsq. 466 Jefferson Davis
Perlesta decipiens EF623242.1
Perlesta shubuta F 511 Pike
Perlesta placida F 494 Simpson
Perlesta placida F 488 Rankin
Perlesta placida F 505 Marion
Perlesta placida M 492 Lamar
Perlesta sp. F 462 Franklin
Perlesta sp. F 461 Franklin
Perlesta sp. M-Unsq. 463 Franklin
Perlesta sp. M-Unsq. 458 Franklin
Perlesta sp. M-Unsq. 465 Jefferson Davis
Perelsta sp. M-Unsq. 457 US Franklin
Perlesta sp. F 460 Franklin
Perlesta sp. M-Unsq. 459 US Franklin
Perlesta placida nymph 512 Marion
Perlesta shubuta nymph 507 Pike
100
100
77
64
55
62
83
51
1
2
3

Figure 3.4 16S Maximum Likelihood Tree.

Maximum Likelihood phylogenetic reconstruction of 41 *Perlesta* 16S sequences using the GTR+I+G nucleotide substitution model. 40 sequences are from this study, while 2 are from GenBank submissions indicated by their accession numbers. Outgroup taxon: *Perlinella drymo*. Bootstrap scores from 1,000 replicates are shown at nodes.

The 16S parsimony and ML trees (Figs. 3.3 and 3.4) were similar to one another. The same species appeared in each of the three clades found in both trees, with the only difference being bootstrap/jackknife support. The first clade showed stronger support in the ML tree (77% bootstrap support) than the same clade in the parsimony tree (51% jackknife support). The second clade in the parsimony analysis had strong support (96% jackknife support) while the same clade had weak support (64% bootstrap support) in the ML tree. The difference in support values could be due to the different parameters used in each support analysis.

The COI and 16S trees were congruent with one another (Figs. 3.1–3.4). The first clade across all trees for both regions contained the same species with strong bootstrap support in both the COI parsimony (95% jackknife support) and ML tree (94% bootstrap support). This clade had weak support in the 16S parsimony consensus tree (51% jackknife support) and moderate support in the ML tree (77% bootstrap support). The remaining clades are not as consistent, but still largely agree across trees and regions. However, the second clade and nested third clade in both 16S trees contain mostly the same species found in the smaller clades recovered in the COI trees. The higher number of sequences in the COI analyses probably led to more resolution in the COI trees than in the 16S trees.

CHAPTER IV – DISCUSSION

Although the data displayed variation within the genus, this variation did not align well with the morphological species. There are several possible reasons for this. Perhaps the individuals were misidentified based on morphology. This is a common issue with females and nymphs, which sometimes lack known diagnostic features, even for the three species previously reported for Mississippi. However, the key characters in males were examined closely, and identifications were verified independently without bias by Dr. Bill Stark (Mississippi College), a stonefly expert, with both of us reaching the same conclusions in all cases.

Perhaps there are incipient or cryptic species, which have been picked up by the data gathered here but which are morphologically indistinguishable with the material currently collected. This is not uncommon and has been observed in stoneflies, as well as other insects (The Heliconius Research Consortium, 2012; Grubbs and DeWalt, 2018; Young et al., 2019).

Hybridization has been observed in stoneflies, and until recently was considered rare amongst plecopterans and other aquatic insects (Ross and Ricker, 1971; Dijkstra et al., 2014; Hughes et al., 2014). However, recent studies show hybridization may be more widespread than previously thought (Grubbs and DeWalt, 2018; Young et al. 2019), and even among the individuals collected for this study, “mating pairs” often consisted of a male and female of different genera, which has also be observed by others (Zeigler, 1990; Masly, 2012). A study by Elbrecht et al. (2014) assessed the presence of cryptic species within the highly variable predacious stonefly species, *Dinocras cephalotes.* That study

looked at mitochondrial and nuclear DNA to assess the possibility of hybridization through interbreeding. *Dinocras cephalotes* haplotypes showed intraspecific COI distances above the typical barcoding gap threshold. However, nuclear DNA was also assessed to determine whether differences in the mitochondrial DNA was a result of interbreeding. Differences in nuclear DNA would likely be homogenized due to recombination if interbreeding was occurring, thus leading to differences between the mitochondrial and nuclear DNA. They found that hybridization was unlikely due to similarities between the mitochondrial and nuclear regions.

In this study, relationships may be confounded due to introgression or lineage sorting (Heinold, 2014; Boumans and Figueroa, 2016). However, the nuclear 16S and mitochondrial COI trees consistently showed similar relationships amongst species despite different modes of inheritance of the nuclear and mitochondrial DNA. The ML and parsimony trees showed similar relationships, but the ML tree helped resolve some polytomies found in the MPT (Figs. 3.1–3.4). The lack of differences between the mitochondrial COI trees and nuclear 16S trees suggests reproductive barriers may have evolved, and hybridization may not be as likely (Elbrecht et al., 2014).

Perhaps *Perlesta placida*, which many of the specimens were identified as, is a widespread species from which other species have evolved due to geographical separation following glaciation or niche specialization, which leaves it as a diverse grade (instead of clade) of individuals. This may also explain why there is low genetic divergence between *P. placida* and *P. shubuta* in all trees and why these species do not appear as monophyletic groups (Figs. 3.1–3.4). An interesting observation here was that

nymphs at many localities were not collected despite intensive collection effort, possibly pointing to a phenological or habitat difference that has not yet been recorded, which may point to the presence of incipient species or cryptic species. The *Perlesta* samples from South et al. (2019) formed monophyletic clades for multiple *Perlesta* species, which may be a result of their species being geographically isolated by the Interior Highlands (Ouachitas/Ozarks), Appalachian Mountains, or migration following glaciation (Fochetti and Figuero, 2008; Elbrecht et al., 2014). Another explanation may be that most samples from South et al., (2019) came from individuals collected from the same locality or general area, with the exception of *Perlesta sublobata*.

In conclusion, this study revealed a high degree of genetic variation in Mississippi individuals of *Perlesta*. The variation did not align with morphological identifications of the specimens, possibly indicating that the morphological variation noted between *P. placida* and *P. shubuta* may merely represent infraspecific variation instead of diagnostic differences between two species, or that the specimens need to be further assessed for cryptic species. Grubbs and DeWalt (2018) described the need for a broader comparative approach as the delineation of new species based on morphological characters continues to become more difficult due to overlapping diagnostic features found in *Perlesta*. They suggest the need for a taxonomic revision of the genus using both morphological and molecular data that looks at traditional features, as well as features that have not been previously considered for identification. Because the molecular variation did not align with the morphological species, the DNA did not provide a good tool to match nymphs to "known" *Perlesta* adults. Future studies including more males, females, and nymphs of

Perlesta species from the same localities from southeastern states in the Gulf Coastal Plain may provide insight on whether cryptic species exist. The inclusion of more mitochondrial and nuclear gene analyses may also be useful in future studies for delimiting species. Lastly, a thorough sampling of Mississippi for *Perlesta lagoi* would be helpful in better understanding the relationships between *Perlesta* species in Mississippi and aid in future biomonitoring and conservation assessments.

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