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Creating a Georgia Southern Spider Collection: Can DNA Barcoding Help?

Guy B. Hobbs

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CREATING A GEORGIA SOUTHERN SPIDER COLLECTION: CAN DNA BARCODING HELP?

by

GUY HOBBS

(Under the Direction of Lorenza Beati)

ABSTRACT

 With over 280 spider (Araneae) species recorded within the State of Georgia, USA, the need for a well-documented natural history collection with a usable voucher system is critical to continually assess spider diversity and their future ecological impact in this region. Spider identification can be daunting for the inexperienced taxonomist; it is time consuming and sometimes requires destructive procedures. Previous works have successfully used an alternative method, DNA barcoding, to correctly identify spider species while preserving their morphology. This study set forth to create the core of a well-documented spider collection within Georgia Southern University's Institute for Coastal Plain Science and use DNA barcoding as a diagnostic tool. We collected 334 spiders from varying locations within the Coastal Plain and optimized a DNA extraction protocol from spider legs. Cytochrome C Oxidase Subunit I (COI), the gene commonly used in DNA barcoding, and an additional nuclear gene Histone 3 type A (H3A) were amplified and sequenced from a total of 132 and 150 spiders, respectively. The COI dataset identified 16 families, 33 genera, and 36 different species, while H3A identified 15 families, 23 genera, and 15 species. In addition, based on these gene sequences, we generated phylogenetic inferences of the COI and, when possible, of a concatenated dataset.

INDEX WORDS: Araneae, DNA barcoding, Cytochrome c oxidase subunit 1, Histone 3 type A

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MASTER OF SCIENCE

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CHAPTER 1

INTRODUCTION

The Coastal Plain of Georgia is an ecologically heterogeneous region. Many habitats from the marshes of the Altamaha River, to the sand hills and loblolly forests of the interior possess a plethora of spider species (Chamberlain and Ivey 1944). Anthropogenic influences, such as alterations of landscapes, introduced invasive species, and climate change, have already shown to have an impact on the biodiversity of Coastal Plain organisms (Guillebeau 2008; Hsiang et al. 2017; Mitchel et al. 2014; Pimentel 2005; Turner 1988), making this region a potentially important biomonitoring area.

Spiders (Arachnida: Araneae) play a vital role in terrestrial food webs by regulating population sizes and biodiversity of their prey and predators. Therefore, it is important to be able to monitor their diversity and density both spatially and temporally. However, with almost 40,000 species described worldwide and 3,000 in North America alone (World Spider… c2021), spiders are taxonomically challenging as the identification of many species requires extensive systematic expertise. Spiders of Georgia have been documented as early as the beginning of the 1800s with John Abbots' illustrations (unpublished illustrations, 1772-1804). Later, Chamberlain and Ivie (1944) published a seminal monograph on the spiders of Georgia that includes 282 species and 567 illustrations in part borrowed from Abbot's work. Recent publications have explored other regions of the Southern United States. In central and northern Florida, Corey and Taylor (1988) sampled and identified spiders in pine ponds, sand pine scrub and flatwood habitats recording a total of 57, 42 and 48 species respectively. Another study in southwest Georgia sampled three isolated pinewood wetlands describing 27 taxa from nine families of spiders (Tietjen et al. 2017). Within the wetlands, the families Tetragnathidae and Pisauridae were most abundant with *Dolomedes triton* and *Tetragnatha laboriosa* as the top two most common species. The most relevant publication in recent times is a study of ground-layer spiders of the Georgia Piedmont Floodplain (Draney 1997). This survey of natural floodplain forests, agricultural lands (tillage and non-tillage) and grasslands bordering these habitats in Clarke County, Georgia identified 145 species belonging to 26 families. All these studies involved endemic species exclusively occurring in Georgia and more widespread taxa found in other areas of the US.

While taxonomic keys (Bradley 2012; Gertsch 1979; Ubick et al. 2005), identification guides, and online resources (How to identify… c2021; Morphology and Physiology… c2015) are available for spider identification, the microscopic process of diagnosing spider species can be challenging and time consuming, particularly for less experienced taxonomists. Intraspecific polymorphism, sexual dimorphism, absence of keys for spiderlings, the need for dissections, all contribute to make spider identification complicated. With the advent of molecular methods, alternative procedures that, supposedly, do not require professional morphological taxonomic skills have been developed. Hebert et al. (2003a; 2003b) proposed a new genetic approach, called "barcoding of life." It involves the creation of a taxonomic depository of a highly conserved metazoan gene sequence (Cytochrome C Oxidase subunit I [COI]) from morphologically well identified specimens. The sequence depository is then used for sequence comparisons and taxonomic identification. This method has been used for many organisms, especially arthropods (Bucklin et al. 2011; Lavinia et al. 2017; Smith 2005) with spiders included (Barret and Hebert 2005; Blagoev et al. 2013; Blagoev et al. 2016). These three publications, which generated over 48,000 North American COI sequences deposited in GenBank and BOLD (Ratnasingham 2007), provided convincing evidence that the method can be used to correctly identify spiders molecularly.

Our study had several main goals: (1) assemble a core collection of spiders from the Georgia Coastal Plain, (2) optimize a method that allows the extraction of DNA while preserving diagnostic morphological features, (3) identify our samples by comparing COI sequences to GenBank accessions, and (4) add H3A nuclear gene sequences (Histone 3 type A) to the mitochondrial dataset for comparison of results and for phylogenetic analyses.

CHAPTER 2 MATERIALS AND METHODS

Sampling

Spiders were collected from May 2017 to May 2020 from multiple habitats within the Coastal Plain of Georgia (Fig. 2.1 and Appendix 1) in an opportunistic way aiming at increasing diversity rather than monitoring densities. Older samples collected from March 2010 to June 2011 were added to our collection (Appendix 1). A variety of techniques were involved in our work: active hand catching both diurnal and nocturnal (visual detection in webs, on vegetation, under logs and stones), the usage of sweep nets, drag cloths, and pitfall traps with a 90% ethanol soapy solution.

The locations chosen for sampling were as follows: Georgia Southern University campus, two mixed forest habitats (Bo Ginn National Fish Hatchery near Magnolia Springs State Park and a private property south of Statesboro); one pond habitat (Bird Pond) south of Statesboro, where spiders were collected by the edge of the body of water and inside the surrounding mixed forest; one sandhill habitat, an east facing sandhill slope with cultivated slash pines and a small pecan grove transitioning into a riparian zone; and one saltwater marshland habitat located near St. Simons island. In addition, we received donated collected samples from multiple environments (homes, outbuildings, pools and other man-made public places) which we grouped under a "miscellaneous" section. Specimens are now housed within the Institute for Coastal Plain Science at Georgia Southern University. Each specimen is preserved in 90% ethanol, and each vial contains a label with a unique identifier (ex. ARA1, ARA2, etc.) and collection data (locality, geographical coordinates, date of collection, name of collector).

Preliminary identifications and imaging

Collected spiders were identified at least at the family level through traditional taxonomic methods. This included using identification manuals and keys available for spiders of this region (Bradley 2012; Gertsch 1979; Ubick et al. 2005). Identifications at the species or genus level were performed only for common and easy to recognize spiders that did not require dissection for a definitive diagnosis. By using a stacking BK Plus Lab System camera (Visionary Digital),

we generated voucher images of the frontal, dorsal and ventral sides of 173 of the collected spiders.

DNA extraction

In order to test our primer sets (Table 2.1) and optimize polymerase chain reaction (PCR) conditions, we first extracted DNA from whole bodies of spiders (after images were taken) by using the Qiagen DNAeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Once PCR conditions were fully tested and optimized, and because one of our main goals was to preserve spiders and deposit them in a newly created collection, we developed an alternative method for extracting DNA that would preserve diagnostic voucher spider bodies. Three extraction kits, Qiagen DNAeasy Blood and Tissue kit, QIAamp DNAmicro (Qiagen, Germantown, MD), and ChargeSwitch (Invitrogen, Carlsbad, CA), were compared for their ability to extract DNA from 2 spider legs at a time. Twenty spiders from different families and of different body sizes were selected for the test. Six legs were removed from each specimen and, of those, 2 legs were assigned to each of the kits. In addition, for the QIAamp DNAmicro kit, two additional legs were removed to test the difference between DNA elution in H2O or in AE (Qiagen, Germantown, MD) buffer.

DNA amplification

Amplification of the mitochondrial COI (approx. 600 bp) (Hebert et al. 2003; Folmer et al. 1994) and the nuclear H3A (approx. 320 bp) gene sequences (Colgan et al. 1998) were performed by using primers described in the literature (Table 2.1). The amplification of COI from spider DNA often requires the combination of different primer sets (Astrin et al. 2016; Blagoev et al. 2013; Blagoev et al. 2016; Hebert et al. 2003b). Thermocycler conditions for amplifying spider DNA were optimized for each combination of COI primers and for the nuclear H3A gene primers by running annealing temperature gradients. Once PCR conditions were established, we used primer sets F1-R1 and F2-R2 (Table 2.1) for comparing amplicons from DNA extracted from legs with the four methods. Identical amounts of extracted DNA were amplified from each of the four extracts obtained from each spider by using the DreamTaq HotStart DNA Polymerase kit (Thermo Scientific, Waltham, MA). The PCR mixture included: 2.5µl of 10x DreamTaq Buffer, 0.5µl of a 10mM DNTPs mix, 1.25µl of each primer

(10pmole/µl), 2.5µl of DNA, 0.26 µl DreamTaq HotStart DNA Polymerase (1.25 U), and 16.74µl of molecular grade H2O. For each set of primers, four 1.5% agarose gels with 21 lanes each (20 for spiders and 1 for the molecular weight standard) were prepared. The four sets of amplicons were distributed in the same order in the gels and gel patterns were compared for specificity (one band versus, more than one band or an indistinct smear) and for intensity.

Sequence assembly and preliminary identification through BLAST

Successful DNA amplicons were purified by using ExoSAP-IT (Thermo Scientific, Waltham, MA) and shipped for sequencing of both complementary strands to Eurofins Genomics (Louisville, KY). The complementary strands were assembled by using the online platform, Benchling (Benchling… c2021). Consensus sequences were saved in the standard FASTA format. Each sequence was compared to homologous GenBank entries by using BLAST (Altschul et al. 1990). Query coverage and percent identity were recorded for the closest sequences retrieved with BLAST.

DNA distances and phylogenetic analyses

Sequences were aligned with Mesquite 3.61 [\(Maddison and Maddison 201](https://www.sciencedirect.com/science/article/pii/S1877959X1630053X?via%3Dihub#bib0140)9). Codon organization was considered when aligning the COI and H3A data sets. For the COI data matrix, Kimura-2 distance (K2P) (Kimura 1980) were calculated and used to generate a barcode histogram in order to verify whether our data provided a visible barcoding gap between intra and interspecific distances. When both sequences were available for the same spider, a concatenated COI and H3A aligned matrix was created. The COI and the concatenated data sets were analyzed by Bayesian analysis (BA) with a general time-reversible nucleotide substitution model and posterior probability values were calculated with MrBayes 3.2.4 [\(Huelsenbeck and Ronquist](https://www.sciencedirect.com/science/article/pii/S1877959X1630053X?via%3Dihub#bib0110) [2001;](https://www.sciencedirect.com/science/article/pii/S1877959X1630053X?via%3Dihub#bib0110) Ronquist et al. 2012b). Two analyses, with four chains each, were run simultaneously for BA analyses (1,000,000 generations). Trees were sampled every 100 iterations with a 25% burning fraction. The 50% majority-rule consensus tree of the remaining trees was inferred, and posterior probabilities were recorded for each branch. Filistatidae were used as outgroups in our analyses because they were found to be a basal lineage in the Araneaomorpha (Garrison N et al. 2016). For the concatenated data set, we used Linyphiidae as outgroups because we did not obtain H3A sequences from Filistatidae samples.

Table 2.1. PCR primers used in this study.

Gene	Primer	Sequence $(5'-3')$	References
COI	chelicerate forward1 (F1)	TACTCTACTAATCATAAAGACATTGG	Hebert et al. (2003)
	chelicerate reverse1 (R1)	CCTCCTCCTGAAGGGTCAAAAAATGA	Hebert et al. (2003)
	chelicerate reverse2 (R2)	GGATGGCCAAAAAATCAAAATAAATG	Hebert et al. (2003)
	$LCO1490$ (F2)	GGTCAACAAATCATCATAAAGATATTGG	Folmer et al. (1994)
H3histone	H ₃ AF	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. (1998)
	H3AR	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (1998)

Figure 2.1. Collection sites. Site (a) is Magnolia Springs State Park, (b) a privately owned sandhill property, (c) Georgia Southern University campus, (d) Bird Pond, (e) Saltwater marsh near St. Simons Island.

CHAPTER 3 RESULTS AND DISCUSSION

Sampling

A total of 334 spiders were collected: 93 from Bird Pond, 87 from the sandhill habitat, 15 from saltwater marshes, 24 from the GSU campus, 81 from mixed forest habitats, and 34 assigned to the "miscellaneous" section (Fig. 3.1).

Preliminary identifications and imaging

A total of 173 spiders were imaged. Identification attempts were performed on these samples and resulted in them being all identified at the family level. The most common spiders, such as *Leucauge* sp., *Micrathena* sp., *Neoscona* sp.*, Peucetia viridans* and *Trichonephila clavipes* were identified to species level. The voucher images will be made available to the public through an ad-hoc web page (ongoing work). Once the non-destructive method for extracting DNA was fully optimized, because spiders could be preserved, the unnecessary imaging of the remaining 161 spiders was ended.

DNA extraction

Comparison of the four extraction methods showed that for both sets of primers, the QIAamp DNAmicro kit with elution in AE buffer gave the best results with 70-75% of the samples being amplified (one sharp band of the correct size and of high intensity), versus 55- 60% for the DNAeasy Blood and Tissue kit, 45-65% for the Invitrogen kit, and 65-70% for the DNAmicro kit followed by elution in H₂O (Table 3.2). After eliminating some of the most common spiders from our sample, in order to avoid too many redundant results, we extracted DNA from a total of 204 spiders (61%). One study (Blagoev et al. 2016) has managed to generate COI sequences from 27,269/30,679 (89%) spider DNA samples obtained from a single spider leg. Their method was more successful but based on fully automated procedures.

DNA amplification

By testing different annealing temperatures through temperature gradients, the best PCR conditions for our primer sets were determined to be as listed in Table 2.1. A total of 132 amplicons were obtained with the COI primers sets (64%), 150 for H3A (73%), and 70 for both (34%). H3A is a very short nuclear gene (~ 320 bp) that might be easier to amplify than a longer mitochondrial gene $($ \sim 600 bp). Also, to reduce expenses, we used only the F1-R1 and F2-R2 combination of COI primers, while in some cases the F1-R2 or the F2-R1 combinations might have been more suitable. Once purified and sequenced, the amplicons yielded a total of 95 and 94 usable COI and H3A sequences, respectively.

Preliminary identification through BLAST and K2P distance calculations

We decided to use BLAST to compare our sequences to accessions in GenBank rather than use the BOLD platform for simplicity of use and because, while the BOLD sequences are also found in GenBank, not all GenBank accessions will be retrieved from BOLD. We are aware, however, that the algorithm used to identify species is slightly different in the two systems.

Of the 95 COI sequences, 79 were identified at the species level with BLAST similarity values > 98% ($K2P > 97.93$), 6 at the genus level only and 10 at the family level only. $K2P$ distances between genera varied from 5.69 to 22.46% (in Theridiidae) indicating that distances between genera were sometimes as important as distances between families (12.00 to 29.39%). The barcoding gap histogram based on K2P distances showed that the intraspecific peak is clearly separated from the intraspecific peak (Fig. 3.2). The barcoding gap was not as definite in a previously published study (Barrett and Hebert 2005) which, however, included a larger variety of species. If the taxonomic breath of our sample had been more important, we might also have found taxa with higher intraspecific divergence values. Of the 94 H3A sequences, 30 were identified at the species level with BLAST similarity values > 98% (K2P > 98.20), 30 at the genus level only, and 34 at the family level only. COI was better at identifying specimens at the species level because there are more and more diverse COI sequences available for comparison in GenBank (over 48,000 vs. 1,075). Also, most available GenBank H3A sequences were generated from European or South American spider species (Arnedo et al. 2004; Piacentini and Ramirez 2019; Wheeler et al. 2017) that do not occur in North America, explaining why most identifications were at the supra-specific levels. Although H3A proved to contribute little to our

identification effort, at least for the overlapping samples, that were primarily identified though COI barcoding and microscopic confirmation, we can now provide the scientific community with a new set of H3A sequences.

The COI dataset include specimens from 16 families, 33 genera, and 36 different species. Considering the relatively small sample, this represents a diverse and heterogeneous group of taxa. The H3A dataset identified 15 families, 23 genera, and 15 species. Interestingly, COI and H3A primers did not equally amplify different spider families. For instance, COI primers appeared to be better at amplifying Lycosidae, Pisauridae, and Salticidae DNA (Fig. 3.3) than H3A primers which detected more easily Tetragnathidae (Fig. 3.4).

Phylogenetic tree comparisons

Phylogenetic trees were obtained after duplicate sequences had been removed from the aligned matrix. Bayesian analyses provide better distance evaluation based on more complex evolutionary models than K2P. The COI trees associated with barcoding in the literature are usually based on K2P distances of amino acid sequences and often show strong monophyly for each considered species, but little support for basal lineage topology (Barrett and Hebert 2005). By using DNA sequences, we achieved better overall resolution in some of the basal groups (families Desidae and Corinnidae) (Fig. 3.5). Nevertheless, the remaining families originated from a large polytomy. Within, the polytomy, all families were monophyletic (posterior probability values > 0.75) with the exception of Theridiidae. *Archaearanea tepidariorium* does not cluster with the other members of the family. The large divergence levels within a nonmonophyletic Theridiidae has been observed in other more complex phylogenetic analyses (Liu et al 2016) where *Archaeaeanea* was found to belong to a different subfamily (Theridiinae) than *Latrodectus* and *Steatoda* (Latrodectinae), the other two genera represented in our sample.

While H3A has been used successfully, in conjunction with other genes, for phylogenetic analyses of spiders (Wheeler et al. 2017) these studies used morphologically identified specimens. Given the results we obtained, it does not appear that, until more H3A sequences from the United States are made available to the scientific community, this gene is a viable barcoding tool. Nevertheless, when the two genes were concatenated (Figure 3.6), the structure of the phylogeny was very well resolved even basally. With the exception of the Theridiidae, all families, genera, and species were monophyletic (with posterior probabilities > 0.75).

PCR step	Cycles	F1R1 & F2R2	Cycles	H ₃ A
Initial Denaturation		95° C for 5 min		95° C for 5 min
Denaturation		95 \degree C for 20 sec		95° C for 20 sec
Annealing	10	44° C +0.4 $^{\circ}$ /cycle for 45 sec		60° C -1 [°] C/cycle for 30 sec
Elongation		65° C for 40 sec		72° C for 30 sec
Denaturation		94° C for 20 sec		95° C for 20 sec
Annealing	20	48° C for 45 sec	28	53° C for 30 sec
Elongation		65° C for 40 sec		72° C for 30 sec

Table 3.1. Optimized polymerase chain reaction programs for COI and H3A.

Table 3.2 Comparison of four methods of DNA extraction from spider legs. QDBT= Qiagen DNAeasy Blood & Tissue Kit, ICS= Invitrogen ChargeSwitch Kit, QQDMW= Qiagen QIAamp DNAmicro Kit eluted in H2O and QQDMAE = Qiagen QIAamp DNAmicro Kit eluted in AE buffer

Figure 3.1. Number of spiders collected in each type of environment. Donated samples were assigned to the miscellaneous section.

Figure 3.2. Counts of pairwise comparisons per KP2 divergence values.

Figure 3.3. Number of identifications per spider family (COI gene)

Figure 3.4. Number of identifications per spider family (H3A gene)

Figure 3.5. Phylogenetic analysis of Cytochrome C Oxidase subunit 1 (COI) sequences. Separate families are delimited by differently colored boxes. Supported lineages from Bayesian analysis (> 0.75 posterior probability) are marked on branches with a blue oval.

Figure 3.6. Phylogenetic analysis of concatenated COI and H3A gene sequences. Separate families are delimited in differently shaded boxes. Supported lineages from Bayesian analysis (> 0.75 posterior probability) are marked on nodes with a blue oval.

CHAPTER 4

CONCLUSIONS

In conclusion, this study provided us with a taxonomically diverse and well-documented spider sample that will constitute the initial core of the future GSU spider collection. DNA extraction methodologies preserving simultaneously the spider bodies, and more importantly, their diagnostic features were optimized as an essential part of a workflow that should allow this collection to rapidly grow.

COI barcoding proved to be useful because many of the species we collected had already been molecularly characterized and their sequences had already been deposited in GenBank. This was not the case for the H3A sequences. Nevertheless, one should not forget that morphological verifications are always advisable, because taxonomic identifications preceding GenBank sequence submissions are sometimes wrong. Of the samples that were only identified by COI barcoding at the genus and family level, morphological identification will also be necessary. They might correspond either to a known species for which a COI sequence has yet to be generated, or to a new species. Given the rate at which new spider species are validated (World Spider… c2021) it would not be surprising to discover unknown taxa for the U.S. and Georgia in particular.

Phylogenetically, the combination of COI and H3A sequences in a single analysis proved to be more informative than the use of COI only. When corroborated by COI identifications, our H3A dataset will constitute a small but interesting addition to the relatively limited set of sequences available in GenBank.

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APPENDICES

APPENDIX 1: FIELD COLLECTIONS

APPENDIX 2: BLAST SEARCH RESULTS FOR COI AND H3A

