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Interrelationships Among Monorchiid Trematodes with Special Emphasis on Some Northwestern Atlantic Genera

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INTERRELATIONSHIPS AMONG MONORCHIID TREMATODES WITH SPECIAL EMPHASIS ON SOME NORTHWESTERN ATLANTIC GENERA

by

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ABSTRACT

The Monorchiidae Odhner, 1911 is a cosmopolitan family of flukes (Trematoda: Digenea) comprising species that parasitize the digestive tract of estuarine and marine fishes as adults. Compared with other oceans, recent morphological or molecular taxonomic work conducted on monorchiid species from the northwestern Atlantic Ocean has been sparse (Manter, 1931; Overstreet, 1969; Andres et al., 2018; Wee et al., 2018, 2019, 2020). Therefore, the present work investigated the interrelationships of some monorchiids from the northwestern Atlantic Ocean with emphasis on several genera and investigated if *Lasiotocus minutus* (Manter, 1931) Thomas, 1959 constitutes a complex of cryptic species. New morphological and molecular data are provided for 3 species; new molecular data are provided for 5 species; 6 new monorchiid species are described and illustrated. Phylogenetic analysis of the 28S rDNA fragment revealed *Genolopa* Linton, 1910 represents a natural lineage, supporting that presence of spines in the genital atrium and a bipartite, anteriorly spined terminal organ are key diagnostic features for the genus, and provided further evidence that *Lasiotocus* Looss in Odhner, 1911 is polyphyletic. Phylogenetic analysis of the 28S rDNA fragment and morphological analysis of *L. minutus* did not support a complex of cryptic species because all isolates of the 28S rDNA region were identical across locations and definitive hosts. However, more data are needed to come to a well-supported conclusion, such as molecular data from additional DNA regions (ITS2 rDNA, mtDNA) and data from more geographic locations and intermediate hosts.

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First and foremost, I would like to thank my thesis director, Dr. Robin M. Overstreet, for his unwavering support, knowledge, instruction, and confidence in me while completing my degree. I will always be grateful for his open-door policy whenever I had questions and for his impressive attention to detail. Thank you for accepting me as a student in your lab, introducing me to the wonderful world of parasitology, and encouraging my interests in natural history, even outside of my thesis work. I also would like to thank my committee members (Dr. Kevin Dillon, Dr. Stephen "Ash" Bullard, and Dr. Zachary Darnell) for their input on my thesis work and their questions that expanded my thought process and the way I approached problems. I would like to thank Dr. Kevin Dillon, specifically, for acting as my major advisor. Additionally, I would like to thank Dr. Ash Bullard for his continual encouragement and motivation to work hard and publish. These conversations usually occurred when I was in a rut and inspired me to keep working and moving forward; I will always be thankful for that.

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This thesis is not intended as a scientific record, as per Article 8.2 of the International Code of Zoological Nomenclature (ICZN), for the taxonomic names and nomenclatural acts contained within the thesis as per Article 8.3 of the ICZN. This thesis is not a contribution to the primary scientific literature; nor should it be cited as such.

DEDICATION

No words can adequately describe my thanks to my boyfriend, family, and friends who supported me while obtaining this degree. Thank you for your patience, support, and belief in me. There are three people I would specifically like to thank and to whom I dedicate this work. The first two people are my parents. Thank you for always supporting and encouraging me to go after what I want from life and for being such a strong safety net on which I can always rely.

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LIST OF ABBREVIATIONS

AFA	alcohol-formalin-acetic acid
BI	Bayesian inference analysis
bp	base pair
DFA	Discriminant function analysis
ICZN	International Code of Zoological Nomenclature
mtDNA	mitochondrial DNA
PCA	Principle components analysis
PCR	polymerase chain reaction
rDNA	ribosomal DNA
USM	The University of Southern Mississippi
USNM	Smithsonian Institution National Museum of Natural History

CHAPTER I – GENERAL INTRODUCTION TO THE MONORCHIIDAE

1.1 Life History

The phylum Platyhelminthes refers to an enormous group of flatworms that comprises clades of free-living forms and clades of parasitic forms. Most of the parasitic forms are in 3 groups: Trematoda, Cestoda, and Monogenea. Trematoda contains 2 subclasses: Aspidogastrea and Digenea. Most digeneans reach sexual maturity in vertebrates but a few do precociously in invertebrates. The digenean life cycle generally consists of larval stages that undergo asexual reproduction in an intermediate host and adults that undergo sexual reproduction in the definitive host (Figure 1.1) (Ginetsinskaya, 1968; Yamaguti, 1975; Bullard and Overstreet, 2008).

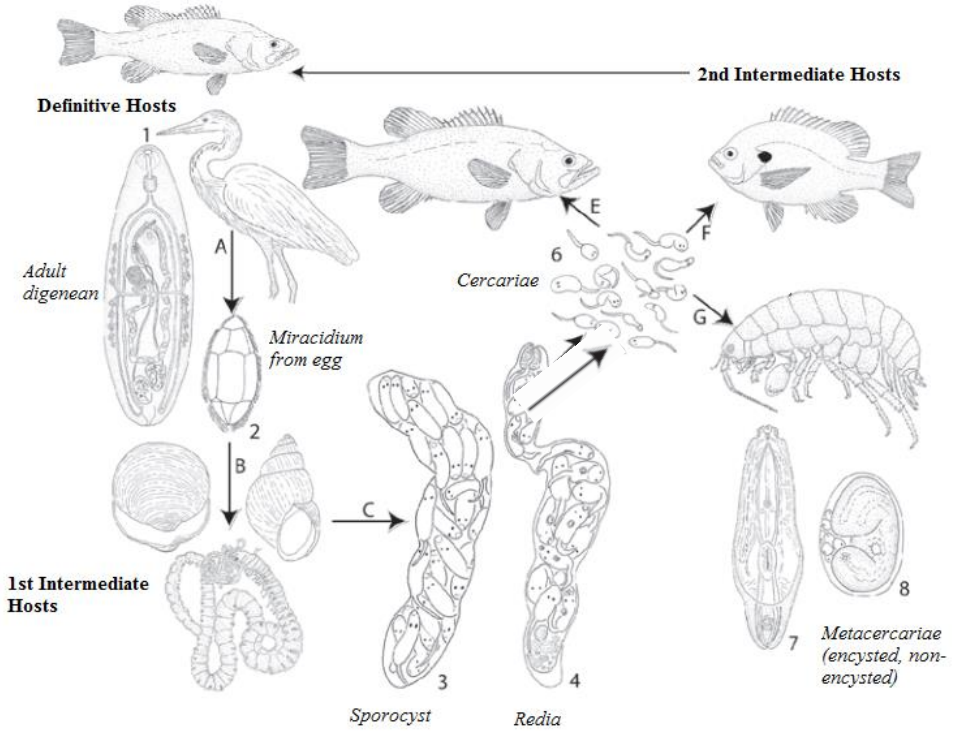

Figure 1.1 *General 3 host life cycle of hypothetical digeneans (modified from Bullard and Overstreet [2008]).*

Italicized terms refer to digenean life history stages. Bold terms refer to digenean hosts for the various life history stages.

Great diversity and complexity exist among digenean life cycles, which vary by having 2 to 5 obligatory hosts depending on the species. However, most digeneans have a 3-host life cycle (Figure 1.1). The first host is *usually* a mollusc; the second host can be a variety of taxa, such as a mollusc, arthropod, annelid, or fish (or even a hard substrate in the environment), depending on the digenean group. A vertebrate serves as the definitive host, in which the parasite undergoes sexual reproduction. Exceptionally, some species of digeneans mature precociously in the second intermediate host. Global digenean species diversity is tremendous, with approximately 18,000 species already described from various hosts and habitats (Yamaguti, 1971; Bray et al., 2016; Choudhury et al., 2016; Cribb et al., 2016). The complex life histories are likely a result of alternation of generations, which is the separation of asexual and sexual reproduction that occurs within linkage of different hosts.

Members of the Monorchiidae are the focus of this thesis. The known monorchiid life cycles follow a 3-host life cycle (Figure 1.2): a bivalve first intermediate host, a bivalve second intermediate host, and a marine perciform, cyprinodontiform, mugiliform, antheriniform, albuliform, or aulopiform fish definitive host, with few exceptions. The monorchiid life cycle typically differs from that in other digenean families because members use a bivalve as the second intermediate host, and the second intermediate host is often the same species as the first intermediate host. The molluscan second intermediate host is commonly a gastropod for non-monorchiid digenean groups. Life cycles (partial and full) are known for only 14 of the approximately 250+ accepted species of monorchiids. Known monorchiid life cycles are listed below in Table 1.1.

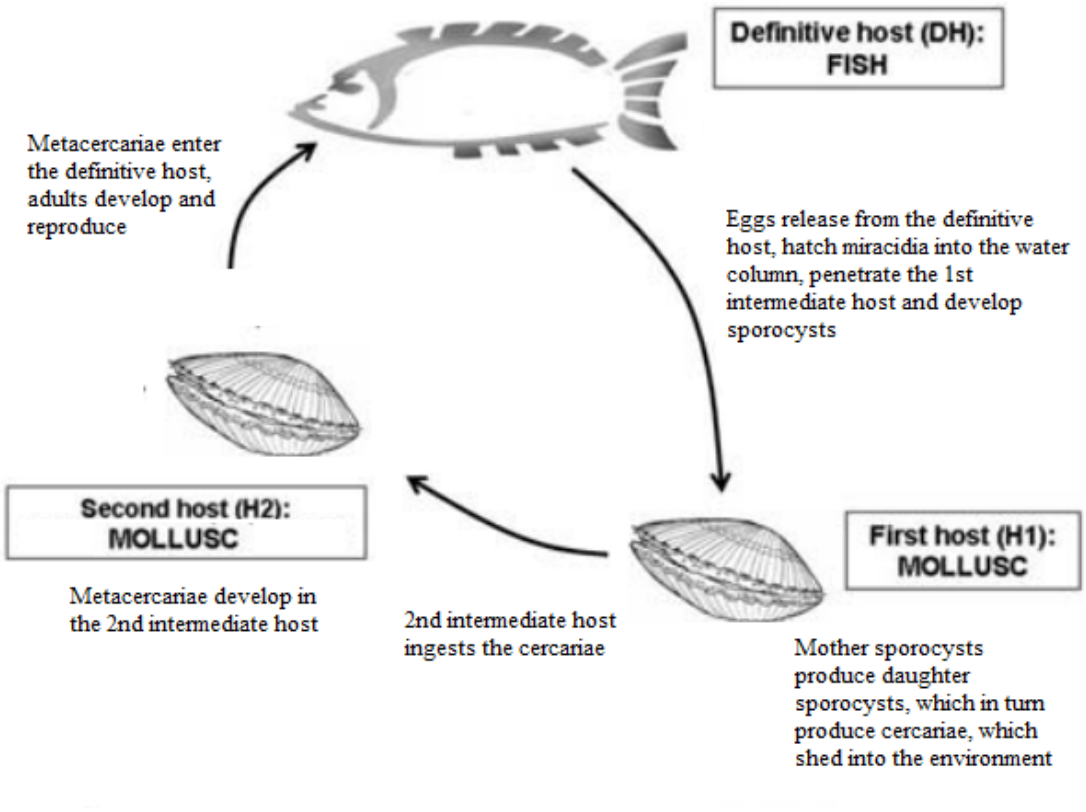

Figure 1.2 *General monorchiid 3-host life cycle (modified from Bartoli and Boudouresque [2007]).*

Table 1.1 *Known monorchiid life cycles, including monorchiid species, host species, geographic location, and reference of the reported study.*

Monorchiid species	1st Intermediate Host	2nd Intermediate Host	Definitive Host	Geographic Location	Reference
	Mollusc	**Mollusc**	**Fish**		
Proctotrema bartolii	Mactridae	Mactridae	Atherinidae, Eleginopsidae	Argentina	Gilardoni et al. (2013)
Lasiotocus elongatus	Veneridae	Veneridae	Atherinopsidae	USA (NC)	Stunkard (1981b)
Lasiotocus minutus	Veneridae	Veneridae	Atherinopsidae, Fundulidae	USA (MA, ME)	Stunkard (1981a)
Lasiotocus cf. *minutus**	Cyrenoididae	N/A	Fundulidae, Poeciliidae	USA (MS)	unpublished thesis by Smedley (2000)

Table 1.1
(continued)

Cercaria caribbea XXXVI of Cable (1956)	Veneridae	Unknown	Unknown	Puerto Rico	Cable (1956)
Lasiotocus sp. of Smedley (2000) *	Dreissenidae	Gobiidae (fish)	Gobiidae, others unknown	USA (MS, LA)	unpublished thesis by Smedley (2000)
Unknown	Veneridae	Veneridae	unknown	Argentina	Cremonte et al. (2001)
Monorcheides cumingiae	Semelidae	Semelidae, Tellinidae	Eels, flounders	USA (MA)	Martin (1940)
Telolecithus pugetensis	Veneridae	Tellinidae, Littorinidae	Embiotocidae	USA (OR, WA, CA)	De Martini and Pratt (1964)
Paratimonia gobii	Semelidae	Semelidae	Gobiidae	Europe	Maillard (1975)
Monorchis parvus	Cardiidae	Cardiidae	Sparidae	Portugal	Bartoli et al. (2000)
Monorchis monorchis *	Unknown	Antedonidae (echinoderm)	Blenniidae	France	Prévot (1967)
Postmonorchis donacis	Donacidae - possibly	Donacidae	Embiotocidae, Sciaenidae	USA (CA)	Young (1953)
Postmonorcheides maclovini	Lasaeidae	Lasaeidae	Eleginopsidae	Argentina	Bagnato et al. (2016)

* Indicates a life cycle that is an exception to the generalized monorchiid life cycle

Although most of the life cycle patterns thus far documented among monorchiids have consisted of a 3-host life cycle, there are some known exceptions. Two truncated life cycles for species in *Lasiotocus* were reported in an unpublished thesis (Smedley, 2000). Smedley (2000) demonstrated that specimens identified as *L.* cf. *minutus* in coastal Mississippi have a life cycle in which the tailless cercaria remains in the sporocyst in the first intermediate host, which in turn is eaten by the definitive host, thus skipping the need for a true second intermediate host. Smedley (2000) also demonstrated that a second, undescribed species belonging in *Lasiotocus* has a life cycle in which the cercaria

from the first intermediate host directly infects a fish second intermediate host by penetrating and encysting in the host's flesh. The metacercaria is progenetic in the second intermediate host, where it develops and matures. Further, Prévot (1967) demonstrated that the metacercaria of *Monorchis monorchis* (Stossich, 1890) Monticelli, 1893 occurs in at least an echinoderm second intermediate host. As is the case with most trematodes, complete or partial monorchiid life cycle data are available for only a small fraction of species. Therefore, it is difficult to determine if these perceived exceptions to the generalized monorchiid life cycle are truly that. They do represent unusual digenean life cycles.

1.2 Phylogenetic Affinities

The phylogenetic relationships among the Digenea have been investigated using cercarial morphology and molecular data. La Rue (1957) and Cable (1974) investigated the phylogenetic relationships among the Digenea using cercarial morphology. Olson et al. (2003) were the first to investigate the phylogenetic relationships among many families of the Digenea using molecular data (Figure 1.3) and have been followed by a more recent paper by Pérez-Ponce De León and Hernández-Mena (2019) who included additional taxa in their dataset.

La Rue (1957) and Cable (1974) classified the Monorchiidae within the Plagiorchioidea using cercarial morphology, and both Olson et al. (2003) and Pérez-Ponce De León and Hernández-Mena (2019) classified the Monorchiidae within the

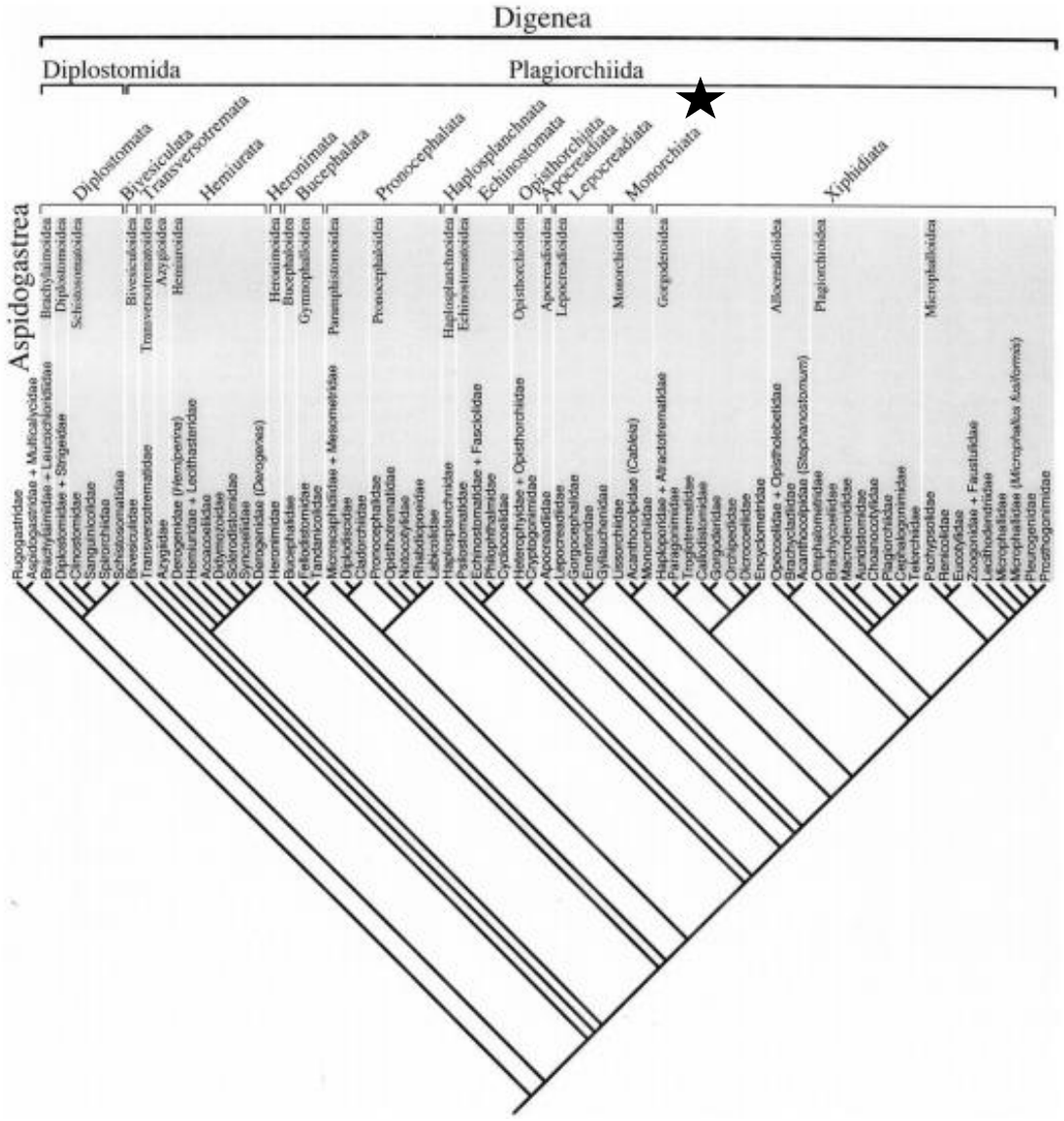

Figure 1.3 *Digenean phylogeny based on 18S and partial 28S rDNA gene sequence data.*

Modified from Olson et al. (2003).

Plagiorchiida using molecular tools. The Monorchiidae belongs within the Monorchiata, established by Olson et al. (2003) (starred in Figure 1.3 and Figure 1.4), which comprises the Monorchiidae, Lissorchiidae, and Deropristidae (Olson et al., 2003; Searle et al.,

2014; Sokolov et al., 2020). A recent paper by Sokolov et al. (2020) provided the first molecular data for a deropristid species, *Skrjabinopsolus nudidorsalis* Sokolov, Voropaeva, and Atopkin, 2020, showing the Deropristidae belongs in the Monorchiata and is sister to the Lissorchiidae and Monorchiidae (Figure 1.4).

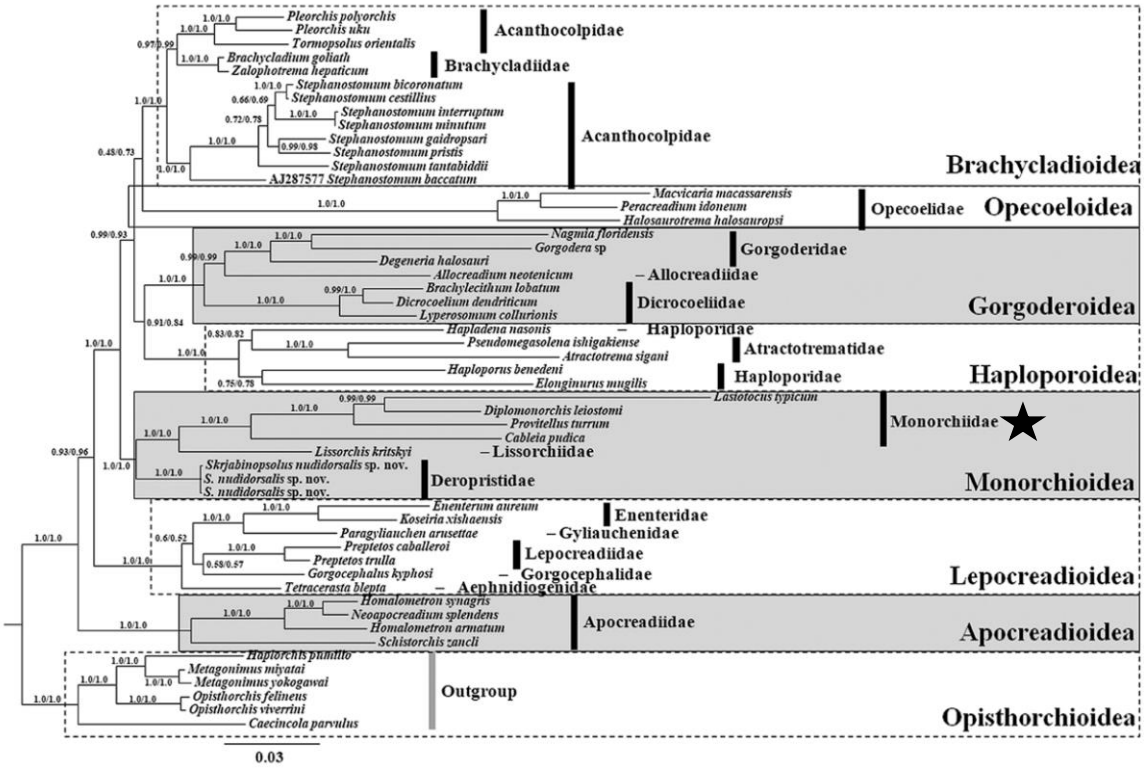

Figure 1.4 *Phylogenetic position of members of the Deropristidae based on 18S and partial 28S rDNA fragments.*

Using Bayesian inference and maximum likelihood analyses from Sokolov et al. (2020), Figure 1.

Historically, deropristids were believed to be members of various families such as Acanthocolpidae and Lepocreadiidae, and many lissorchiids were believed to be members of the Monorchiidae (Ivanov and Murygin, 1936; Skrjabin, 1958; Yamaguti, 1971; Bray, 2005; Madhavi, 2008). However, recent evidence (over the past few decades)

from life history and molecular investigations focusing on these groups has resulted in the current classification that divides them into 3 distinct families in the Monorchiata. There are several lines of evidence to support the separation of these 3 families other than phylogenetic data. The Deropristidae consists of species found in freshwater, possibly marine, habitats, and deropristids with known life cycles have oculate cercariae and use oligochaetes as a second intermediate host (Peters, 1961; Skrjabin, 1974). Like the adult lissorchiids and monorchiids, adult deropristids have a spinous tegument and complex terminal genitalia (consisting of a cirrus sac with an internal seminal vesicle and spinous cirrus and a spinous metraterm). Adult deropristids have a median genital pore and an unequally bipartite internal seminal vesicle (Bray, 2005). The Lissorchiidae consists of species found in freshwater habitats only, and species with known life cycles use gastropods as the first intermediate host, in which rediae produce non-oculate cercariae (Onyejekwe, 1972; Besprozvannykh et al., 2012). Adult members of the Lissorchiidae also have distinct morphological differences compared with deropristids and monorchiids such as having a lateral or sublateral genital pore. The Monorchiidae consists of species predominantly found in estuarine and marine habitats, and species with known life cycles use bivalves as the first intermediate host, in which sporocysts produce cercariae that are oculate or non-oculate (Yamaguti, 1975; Stunkard, 1981a,b; Smedley, 2000; Gilardoni et al., 2013). Adult monorchiids have a terminal organ, which is a distinct sac-like structure at the terminal end of the uterus (discussed in detail in the next section), instead of a "simpler" metraterm and have a median genital pore.

The Monorchiata is a sister taxon to the Xiphidiata (see Figure 1.3). Digeneans within Xiphidiata are morphologically distinguished from the Monorchiata by having a

cercaria with a penetrating stylet, which allows the xiphidatans to penetrate their arthropod second intermediate hosts (Olson et al., 2003). Digeneans within the Monorchiata do not have a penetrating stylet (Cable and Hunninen, 1942; Peters, 1961). They have cercariae with penetration glands, which allow the monorchiatans to penetrate the soft tissues of their molluscan second intermediate hosts (Yamaguti, 1975; Smedley, 2000; Cremonte et al., 2001; Gilardoni et al., 2013).

The Monorchiidae consists of approximately 40 genera (Madhavi, 2008), but there is a paucity of molecular data available for members of this family. Only 16 genera, shown below in Table 1.2, have species with representative sequence data available. *Lasiotocus* Looss in Odhner 1911 is the most speciose genus within the Monorchiidae, consisting of 49 accepted species worldwide (provided by the *World Register of Marine Species* [WoRMS]), and yet it has only 3 species with representative sequence data. None of those is the type-species, *Lasiotocus mulli* (Stossich, 1883) Looss in Odhner, 1911.

Table 1.2 *Monorchiid genera with representative sequences available.*

Monorchiid species	Host Family	Location	Gene Region	GenBank #	Reference
Allobacciger annulatus	Pomacanthidae	Australia	18S, ITS2, 28S, cox1	MK993436 MK955779 MK955782 MK975248	Wee et al., 2020
Allobacciger brevicirrus	Nemipteridae	Australia	18S, ITS2, 28S, cox1	MK993435 MK955778 MK955781 MK975246	Wee et al., 2020
Allobacciger polynesiensis	Pomacanthidae	Moorea	18S, ITS2, 28S, cox1	MK993434 MK955777 MK955780 MK975243	Wee et al., 2020
Cableia pudica	Monacanthidae	Hawaii	28S	AY222251	Olson et al., 2003
*Diplomonorchis leiostomi**	Sciaenidae	USA - GoM	28S	AY222252	Olson et al., 2003

Table 1.2
(continued).

Helicometroides longicollis *	Haemulidae	Australia	28S, ITS2	KJ658287 KJ658288	Searle et al., 2014
Hurleytrematoides sp. A	Chaetodontidae	Australia, Palau	ITS2	JN969580	McNamara et al., 2014
Hurleytrematoides zebrasomae	Chaetodontidae	Australia, Palau	ITS2	JN969575	McNamara et al., 2014
Hurleytrematoides sasali	Chaetodontidae	Australia, Palau	ITS2	JN969570	McNamara et al., 2014
Hurleytrematoides prevoti	Chaetodontidae	Australia	ITS2	JN969568	McNamara et al., 2014
Hurleytrematoides pasteuri	Chaetodontidae	Australia	ITS2	JN969567	McNamara et al., 2014
Hurleytrematoides morandi	Chaetodontidae	Australia, Palau, Moorea	ITS2	JN969557, JN969559	McNamara et al., 2014
Hurleytrematoides loi	Carangidae	Australia	28S	MK501989	Wee et al., 2019
Hurleytrematoides loi	Chaetodontidae	Australia, Palau	ITS2	JN969549	McNamara et al., 2014
Hurleytrematoides kulbickii	Chaetodontidae	Moorea	ITS2	JN969544	McNamara et al., 2014
Hurleytrematoides justinei	Tetraodontidae	Australia	ITS2	JN969543	McNamara et al., 2014
Hurleytrematoides galzini	Carangidae	Australia	28S	MK501988	Wee et al., 2019
Hurleytrematoides galzini	Chaetodontidae	Australia	ITS2	JN969541	McNamara et al., 2014
Hurleytrematoides fijiensis	Chaetodontidae	Australia	ITS2	JN969538	McNamara et al., 2014
Hurleytrematoides faliexae	Chaetodontidae	Australia, Moorea, Palau	ITS2	JN969536, JN969537	McNamara et al., 2014
Hurleytrematoides deblocki	Chaetodontidae	Australia, Palau, Moorea	ITS2	JN969529, JN969533	McNamara et al., 2014
Hurleytrematoides coronatum	Chaetodontidae	Australia, Palau, Moorea	ITS2	JN969518, JN969522	McNamara et al., 2014
Hurleytrematoides combesi	Chaetodontidae	Australia	ITS2	JN 969516	McNamara et al., 2014
Hurleytrematoides chaetodoni	Chaetodontidae	Western Atlantic Ocean	ITS2, 28S	MH244116	Andres et al., 2018
Hurleytrematoides boucheti	Chaetodontidae	Palau	ITS2	JN969514	McNamara et al., 2014
Hurleytrematoides bartolii	Chaetodontidae	Australia	ITS2	JN969512	McNamara et al., 2014
Lasiotocus arrhichostoma	Haemulidae	Australia	28S	KJ658289	Searle et al., 2014

Table 1.2
(continued).

Lasiotocus lizae	Mugilidae	Vietnam	28S	LN831724	Atopkin et al., 2017
Lasiotocus typicum	Carangidae	North Sea, UK	28S	AY222254	Olson et al., 2003
*Madhavi fellaminutus**	Mullidae	Australia	28S	MG920219	Wee et al., 2018
Monorcheides centropygis	Pomacanthidae	Moorea	ITS2	JN969511	McNamara et al., 2014
*Monorchis monorchis**	Sparidae	Corsica	28S	AF184257	Tkach et al., 2001
Monorchis parvus	Sparidae	France	18S, ITS1	Y18936	Bartoli et al., 2000
Monorchis sp. JBPI	Blenniidae	France	18S, ITS1	AJ277375	Jousson et al., 2000
Monorchis lewisi	Sparidae	Australia	28S	MF503309	Cribb et al., 2018
Opisthomonorcheides delicatus					unpublished
Opisthomonorcheides pampi	Carangidae	Bali	ITS2	KX839158	Bray et al., 2017
Opisthomonorcheides ovacutus	Carangidae	Bali	ITS2	KX839157	Bray et al., 2017
Ovipusillus geminus	Carangidae	Australia	28S	MK501987	Wee et al., 2019
Ovipusillus mayu	Carangidae	Australia	28S	MF503310	Cribb et al., 2018
Parachrisomon delicatus	Mullidae	Australia	28S	MG920218	Wee et al., 2018
*Postmonorcheides maclovini**	Eleginopsidae	Argentina	18S, ITS1, 5.8S	KC920684	Bagnato et al., 2016
Postmonorchis sp. SA-2013					Unpublished – Carella, 2013
Proctotrema addisoni	Haemulidae	Australia	28S	KJ658291	Searle et al., 2014
Proctotrema CG-2015	Gaimardiidae	Argentina	ITS1, 5.8S	KP765716	Bagnato et al., 2015
Provitellus chaometra	Carangidae	Australia	28S	MK501984	Wee et al., 2019
Provitellus infrequens	Carangidae	Australia	28S	MK501985	Wee et al., 2019
Provitellus infibrova	Carangidae	Australia	28S	MK501986	Wee et al., 2019
*Provitellus turrum**	Carangidae	Hawaii	28S	AY222253	Olson et al., 2003

***** Indicating the type-species of the genus.

1.3 Morphology

There are several morphological features used to distinguish the Monorchiidae from other digenean families. A structure known as the terminal organ serves as the most important synapomorphy for the family (Yamaguti, 1934; Madhavi, 2008). Adult monorchiids are distinguished by the presence of a terminal organ, a structure unique among digeneans. The terminal organ is essentially a sac-like outcropping of the terminal portion of the uterus. Odhner (1911) erected the family and simply referred to the terminal organ as the vagina. Subsequent authors referred to the terminal organ as the vagina, metraterm, vaginal sac, metraterm sac, or metraterm pouch (Nicoll, 1915; Manter, 1931). Yamaguti (1934) was the first to use the term "terminal organ" in reference to this structure, but the term was not used consistently until the 1950s (Srivastava, 1938; Manter, 1940; Hopkins, 1941; Dollfus, 1948; Thomas, 1959; Manter and Pritchard, 1961; Bartoli and Prévot, 1966). Monorchiids also have the combination of a spiny tegument, spinous portions in the terminal genitalia, and vitellaria restricted to a small area in the body. Most species have just 1 testis (others have 2, 8, or more), and some species have filamentous eggs (Yamaguti, 1971; Madhavi, 2008). The most important characteristics used to differentiate the many genera that comprise the Monorchiidae are the distribution of vitellarium and aspects of the terminal genitalia. The latter include the shape and size of the terminal organ, the junction of the uterus with the terminal organ, the shape of the seminal vesicle, and the presence of and the patterns of spines on the terminal organ, cirrus, and genital atrium (Nahhas and Powell, 1965; Madhavi, 2008).

1.4 Project Goals

The goals of this thesis are to investigate several aspects of taxonomic and phylogenetic interrelationships in the Monorchiidae, focusing on some species found in the northwestern Atlantic Ocean in the genera *Genolopa* Linton, 1910, *Lasiotocus* Looss in Odhner, 1911, *Diplomonorchis* Hopkins, 1941, and *Postmonorchis* Hopkins, 1941, using a combination of morphological and molecular techniques. The monorchiid diversity in the northwestern Atlantic Ocean has not been explored recently and molecular sequence data has only rarely been mined from species in the region; only 2 identified species are presently available in a public database. The molecular data that will be obtained during this project will be used to elucidate evolutionary history relationships among monorchiids and between monorchiids and their definitive hosts. Results from this project will also address monorchiid diversity through morphological analyses and serve to reveal complexes of cryptic species. Additionally, data from this project in the northwestern Atlantic Ocean will be used in conjunction with monorchiid data reported from the Indo-Pacific Ocean to better understand global diversity and interrelationships within the Monorchiidae.

CHAPTER II - PHYLOGENETIC AFFINITY OF *GENOLOPA* LINTON, 1910 (DIGENEA: MONORCHIIDAE) WITH DESCRIPTIONS OF TWO NEW SPECIES

2.1 Introduction

Taxonomy of monorchiids is based on morphological features present in adult stages like with most digenean families, and classification of the family has most recently been summarized by Madhavi (2008). The status of *Genolopa* Linton, 1910, originally erected for *Genolopa ampullacea* Linton, 1910 that parasitizes grunts (Perciformes: Haemulidae) in the Dry Tortugas near southern Florida, has been controversial among taxonomists for nearly a century. Early confusion and controversy regarding the genus stemmed primarily from the failure by Linton to report genital atrium spination in his descriptions. Various taxonomists interpreted Linton's species in opposing ways and advocated conflicting classifications of the species into other monorchiid genera (Manter, 1931, 1942; Hopkins, 1941; Thomas, 1959; Manter and Pritchard, 1961; Yamaguti, 1971). Manter (1942) noted that spines present in the genital atrium in Linton's specimens represent an informative generic feature. Manter (1942) also described the cirrus and terminal organ spines from the type specimens and additional specimens of *G. ampullacea* he collected from the Dry Tortugas.

Currently, the presence of spines in the genital atrium, along with the presence of a bipartite, anteriorly spined terminal organ are used as the primary features differentiating *Genolopa* from other similar monorchiid genera. For example, in the diagnoses for *Lasiotocus* Looss in Odhner, 1911, *Proctotrema* Odhner, 1911, and *Parachrisomon* Madhavi, 2008 all species lack spines in the genital atrium. Furthermore, diagnoses for 3 other monorchiid genera (*Proctotrematoides* Yamaguti, 1938,

Paraproctorema Yamaguti, 1934, and *Monorchicestrahelmins* Yamaguti, 1971) differ little from that of *Genolopa*. However, species in *Proctotrematoides* uniquely possess spines in a distinctive muscular, "flask-shaped" diverticulum attached to the genital atrium (Machida, 2005). Species in *Paraproctorema* have spines in the genital atrium similar to the arrangement in species of *Genolopa*, but the terminal organ is unipartite and fully spined rather than bipartite and partially spined, and a conspicuous bulb-like sphincter occurs where the uterus meets the terminal organ in species of *Paraproctotrema*. Similarly, species in *Monorchicestrahelmins* all have spines in the genital atrium but have a unipartite, spined terminal organ without a bulb-like sphincter where the uterus meets the terminal organ (Madhavi, 2008).

Investigation of the accepted classification of the Monorchiidae using modern molecular techniques is highly desirable. To date, only 3 of the aforementioned genera are represented by species with publicly available sequence data (Olson et al., 2003; Searle et al., 2014; Atopkin et al., 2017; Wee et al., 2018). Only 2 genera have representative species sequenced from western Atlantic monorchiids (Olson et al., 2003; Andres et al., 2018). Currently, no species of *Genolopa* is represented among the publicly available molecular data.

This study utilizes novel molecular sequence data from 3 species of *Genolopa*, including the type-species (*G. ampullacea*), to estimate the phylogenetic position of the genus among other monorchiids. Reliability of 2 generic-level features currently used to differentiate species of *Genolopa* from other monorchiid genera (presence of a spiny genital atrium, and bipartite, anteriorly spined terminal organ) is scrutinized here using molecular analysis. Two new species of *Genolopa* are described from fishes from the

Florida Keys, and novel molecular sequence data for *Postmonorchis orthopristis* Hopkins, 1941 and 3 species of *Lasiotocus* are provided.

2.2 Material and Methods

2.2.1 Specimen Collection and Morphological Analysis

Various hosts (listed in the taxonomic summaries sections with specific localities) were sampled using baited hook and line and cast nesting from areas in Florida (April 2017, March 2018, August 2018, September 2018), North Carolina (August 2018), and New Jersey (August 2018). Worms were collected from fish held on ice for no more than 12 hr after capture following the methods described by Cribb and Bray (2010). One modification to the methods of Cribb and Bray (2010) was post-fixing some worms in 10% formalin specifically for morphological analysis after they had originally been preserved in 70%–80% ethanol. Preserved worms were hydrated using distilled water, stained using VanCleave's hematoxylin or Mayer's hematoxylin, de-stained following methods of Curran et al. (2007), and dehydrated in a graded ethanol series, before being cleared in clove oil or methyl salicylate. Cleared specimens were mounted on microscope slides in Canada balsam or Damar gum. Morphological data were collected using an Olympus BX53 compound microscope in conjunction with iSolutions Lite (Version 8.2) © software (IMT, Inc., Vancouver, British Columbia, Canada). Measurements were provided as ranges in micrometers (µm) and, where appropriate, followed by the measurement taken directly from a holotype in parentheses. Specimens were illustrated using a drawing tube and then digitized using Adobe® Photoshop® CS6 (Adobe Inc., San Jose, California). The type series for *G. ampullacea* was borrowed from the Smithsonian

National Museum of Natural History (USNM), Washington DC, for comparison with the present material.

Herein the regions of the bipartite terminal organ were defined relative to the body axis of the worm. The “anterior region” of the terminal organ was the part that opened into the genital atrium, often spined, and the “posterior region” of the terminal organ was the blind portion that was opposite the anterior region, often not spined, vesicular, as defined by Madhavi (2008) in reference to bipartite terminal organs. “Proximal/distal” terminology was not used in reference to regions of the terminal organ because those terms have been defined in contradicting ways previously when applied to the monorchiid terminal organ (Overstreet, 1971; Madhavi, 2008). Additionally, the terms dextral and sinistral were observer-independent, as if viewed from the body/specimen, not the view of the illustration. The terms median and submedian were defined relative to the longitudinal axis or median plane that bisected a bilateral animal into 2 mirrored halves.

2.2.2 Molecular Sequencing

Molecular vouchers consisted of hologenophores and paragenophores (Pleijel et al., 2008). Paragenophores were cleared in nuclease-free water, wet-mounted, and photographed before extraction for further potential morphological analysis (Andres et al., 2018). Genomic DNA was extracted from molecular vouchers using a QIAgen DNAeasy blood and tissue kit (Qiagen Inc., Valencia, California) following the manufacturer’s instructions modified to extend the initial tissue lysing stage to 18 hr.

The complete second internal transcribed spacer unit (ITS2) and the partial 28S rDNA regions (including domains D1-D3) were targeted and amplified from the

extracted DNA by polymerase chain reaction (PCR) using a MJ mini cycler (Bio-Rad, Hercules, California). The ITS2 rDNA region was amplified using the forward primer ITSf and the reverse primer 300R (Tkach and Snyder, 2007). Internal sequencing primers for the ITS2 rDNA region included digl2r (Tkach and Snyder, 2007) and d58r (Curran et al., 2006). The partial 28S rDNA region was amplified using the forward primer digl2 (Tkach and Snyder, 2007) or LSU5 (Littlewood, 1994) and the reverse primer 1500R (Tkach and Snyder, 2007) targeting the 5′ end of the 28S rDNA region. Internal sequencing primers for the partial 28S rDNA region included 300F, ECD2, and 900F (Tkach and Snyder, 2007).

The PCR reactions were conducted in a total volume of 25 µL that contained 10.5 µL extracted DNA, 12.5 µL Taq buffer (DreamTaq Master Mix 2X, Thermo Fisher Scientific, Waltham, Mississippi), and 1 µL of each forward and reverse primer at 10 mM/µL concentration. The PCR cycling profile was as follows: 3 min denaturation at 94 C; 40 cycles of 30 sec denaturation at 95 C, 45 sec annealing at 52 C, 2 min extension at 72 C, and 3 min extension hold at 72 C. Samples were then held at 4 C after completion of the reaction protocol. The PCR products then underwent gel electrophoresis; subsequent bands were cut from the gel and extracted using a QIAquick Gel Extraction Kit following the manufacturer's instructions (Qiagen Inc., Valencia, California). Sanger sequencing reactions were conducted by Eurofins Genomics LLC (Louisville, Kentucky) and GENEWIZ (South Plainfield, New Jersey). Sequencing of the ITS2 rDNA region was successful for some of my species only. Consequently, the present phylogenetic analysis is based on sequence data from the partial 28S rDNA region. The 5′ end of the partial 28S rDNA region was determined by annotation in the ITS2 Database using the

'Metazoa' model (Keller et al., 2009; Ankenbrand et al., 2015). Successfully generated sequence regions were provided to GenBank and accession numbers were provided below in Table 2.1, taxonomic summaries, and the supplemental molecular data section. Although the ITS2 sequences were not used for phylogenetic analysis in this study, they were made publicly available for use in future works.

2.2.3 Phylogenetic Analysis

Contiguous sequences were assembled using Sequencher™ version 5.0 (GeneCodes Corp., Ann Arbor, Michigan). New sequences derived from 2 new species of *Genolopa* (found in taxonomic summaries below) and 5 other newly generated monorchiid sequences were combined with available partial 28S rDNA sequences of some monorchiids and related species in GenBank (listed in Table 2.1). Sequences were aligned and masked with the GUIDANCE2 web-server (http://guidance.tau.ac.il) (Landan and Graur, 2008; Sela et al., 2015) using the MAFFT alignment algorithm, 100 bootstrap repeats, 1,000 cycles of iterative refinement, and the *localpair* algorithm. Alignment (column) positions with confidence scores <0.4 were excluded from subsequent Bayesian inference (BI) analysis (Andres et al., 2018). The alignment was then trimmed on both ends to the shortest sequence, excluding *Lasiotocus lizae* Liu, 2002 because the partial 28S rDNA sequence was much shorter than for the other species in the alignment, and edited by eye in BioEdit (version 7.2.5) (Hall, 1999). Phylogenetic analysis was conducted using BI with MrBayes 3.2.7 software (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). The best nucleotide substitution model was estimated with jModeltest version 2.1.10 (Darriba et al., 2012) and both the Akaike information criterion (AIC) and Bayesian information criterion (BIC) predicted the GTR

+ I + Γ model as the best estimator. Therefore, the BI analysis was conducted using the closest approximation to this model. The BI analysis was performed using the following model parameters: "nst = 6," "rates = invgamma," "ngen = 1,000,000," "samplefreq = 500," "printfreq = 500," and "diagnfreq = 5,000." The values of the samples of the substitution model parameters were summarized using "sump." Tree and branch lengths were summarized using "sumt." The first 25% of trees were discarded using the following settings: "relburnin = yes," "burninfrac = 0.25." Nodal support was estimated by posterior probabilities. All other settings were left as default values. Two species in the Lepocreadiidae and 1 species in the Lissorchiidae were included in the alignment, with *Bianium arabicum* Sey, 1996 (a lepocreadiid) serving as the outgroup for the analysis (Wee et al., 2018, 2019). FigTree version 1.4.3 (Rambaut and Drummon, 2012) was used to visualize the phylogeny and Adobe® Photoshop® CS6 (Adobe Inc., San Jose, California) was used for subsequent editing.

Table 2.1 *Partial 28S rDNA sequence data used in the phylogenetic analysis.*

Species	**Host Species**	**GenBank Accession Number**	**Reference**
Monorchiidae Odhner, 1911			
Cableia pudica	*Cantherines pardalis*	AY222251	Olson et al., 2003
Diplomonorchis leiostomi *	*Leiostomus xanthurus*	AY222252	Olson et al., 2003
Genolopa ampullacea *	*Haemulon flavolineatum*	**MN984474**	Panyi et al., 2020
Helicometroides longicollis *	*Diagramma labiosum*	KJ658287	Searle et al., 2014
Hurleytrematoides chaetodoni *	*Chaetodon striatus*	MH244116	Andres et al., 2018
Hurleytrematoides galzini	*Gnathanodon speciosus*	MK501988	Wee et al., 2019
Hurleytrematoides loi	*Gnathanodon speciosus*	MK501989	Wee et al., 2019
Lasiotocus arrhichostoma	*Diagramma labiosum*	KJ658289	Searle et al., 2014
Lasiotocus glebulentus	*Mugil curema*	**MN984476**	Panyi et al., 2020
Lasiotocus lizae	*Liza longimanus*	LN831723	Atopkin et al., 2017
Lasiotocus sp.	*Menidia menidia*	**MN984477**	Panyi et al., 2020
Lasiotocus trachinoti	*Trachinotus carolinus*	**MN984478**	Panyi et al., 2020
Lasiotocus typicum	*Trachurus trachurus*	AY222254	Olson et al., 2003
Madhavia fellaminuta	*Upeneus tragula*	MG920219	Wee et al., 2018
Monorchis lewisi	*Acanthopagrus australis*	MF503309	Cribb et al., 2018
Monorchis monorchis *	*Diplodus vulgaris*	AF184257	Tkach et al., 2001
Ovipusillus mayu *	*Gnathanodon speciosus*	MF503310	Cribb et al., 2018

Table 2.1 (continued).

Parachrisomon delicatus	*Upeneus tragula*	MG920218	Wee et al., 2018
Postmonorchis orthopristis *	*Haemulon flavolineatum*	**MN984475**	Panyi et al., 2020
Proctotrema addisoni	*Diagramma labiosum*	KJ658291	Searle et al., 2014
Provitellus chaometra	*Gnathanodon speciosus*	MK501984	Wee et al., 2019
Provitellus infrequens	*Gnathanodon speciosus*	MK501985	Wee et al., 2019
Provitellus turrum *	*Pseudocaranx dentex*	AY222253	Olson et al., 2003
Lissorchiidae Magath, 1917			
Lissorchis kritskyi	*Minytrema melanops*	EF032689	Curran et al., 2006
Lepocreadiidae Odhner, 1905			
Bianium arabicum	*Lagocephalus lunaris*	MH157076	Bray et al., 2018
Lepotrema adlardi	*Abudefduf bengalensis*	MH730015	Bray et al., 2018

* Indicates type-species of the genus.

2.3 Results

2.3.1 Morphological

Monorchiidae Odhner, 1911

Genolopa Linton, 1910

2.3.1.1 *Genolopa ampullacea* Linton, 1910 (Figure 2.1)

2.3.1.1.1 Taxonomic Summary

Type host: *Haemulon macrostomum* (Günther, 1859), Spanish grunt, Haemulidae.

Type locality: Dry Tortugas, Florida.

Other hosts reported by the cited authors but specimens not confirmed as *G. ampullacea* by us: Manter (Manter, 1942): *H. album* (Cuvier, 1830), *H. carbonarium* (Poey, 1860), *H. flavolineatum* (Desmarest, 1823), *H. plumierii* (Lacepède, 1801), *H. sciurus* (Shaw, 1803), *Synodus foetens* (Linnaeus, 1766), Synodontidae; Manter (Manter, 1947): *H. aurolineatum* (Cuvier, 1830), *H. chrysargyreum* (Günter, 1859), *H. album* (Cuvier, 1830), *H. carbonarium* (Poey, 1860), *H. flavolineatum* (Desmarest, 1823), *H. macrostomum* (Günther, 1859), *H. plumierii* (Lacepède, 1801), *H. sciurus* (Shaw, 1803), *H. striatum* (Linnaeus, 1758), *Synodus foetens* (Linnaeus, 1766); Sparks (Sparks, 1957):

H. sciurus (Shaw, 1803)*;* Sogandares-Bengal (Sogandares-Bernal, 1959): *H. album* (Cuvier, 1830), *H. parra* (Desmarest, 1823), *H. plumierii* (Lacepède, 1801), *H. sciurus* (Shaw, 1803); Nahhas and Cable (Nahhas and Cable, 1964): *H. album* (Cuvier, 1830), *H. bonariense* (Cuvier, 1830), *H. flavolineatum* (Desmarest, 1823), *H. melanurum* (Linnaeus, 1758), *H. sciurus* (Shaw, 1803), *H. striatum* (Linnaeus, 1758); Rees (Rees, 1970): *H. flavolineatum* (Desmarest, 1823); Nagaty and Abdel-Aal (Nagaty and Abdel-Aal, 1972): *Cheilinus lunulatus* (Forsskål, 1775), Labridae; Fischthal (Fischthal, 1977): *H. flavolineatum* (Desmarest, 1823); Kohn et al. (Kohn, Macedo, and Fernandes, 1982): *H. sciurus* (Shaw, 1803); Centeno and Bashirullah (Centeno, 2003): *H. aurolineatum* (Cuvier, 1830), *H. bonariense* (Cuvier, 1830), *H. chrysargyreum* (Günter, 1859), *H. melanurum* (Linnaeus, 1758), *H. parra* (Desmarest, 1823), *H. steindchneri* (Jordan and Gilbert, 1882); Bashirullah and Díaz (Bashirullah and Díaz, 2015): *H. flavolineatum* (Desmarest, 1823).

Other reported localities: Bahamas (Sparks, 1957); Panama and Bimini, British West Indies (Sogandares-Bernal, 1959); Curaçao and Jamaica (Nahhas and Cable, 1964); Bermuda (Rees, 1970); Red Sea (Nagaty and Abdel-Aal, 1972); Belize (Fischthal, 1977); Rio de Janeiro State, Brazil (Kohn, Macedo, and Fernandes, 1982); Venezuela (Centeno, 2003; Bashirullah and Díaz, 2015); Puerto Rico (Dyer, Williams, and Bunkley-Williams, 1992).

Host (present study): *Haemulon flavolineatum* (Desmarest, 1823), french grunt, Haemulidae.

Locality: Islamorada, Florida (24°53′53.3112”N, 80°39′33.84”W).

Sites: intestine, pyloric ceca.

Specimens examined: USNM 1321276 (5 syntypes).

Specimens deposited: 3 vouchers: USNM 1611654, 1611655, 1611656; 2 hologenophores: USNM 1611657, 1611658.

Sequences deposited: Partial 28S rDNA, 2 identical replicates (1 submitted to GenBank: accession number MN984474).

2.3.1.1.2 Supplemental Data (Based on 5 gravid, adult specimens from *H. flavolineatum*, mounted without pressure)

Body elongate, tapering slightly at both ends, widest near mid-body, 829 to 1265 long, 202 to 253 wide. Tegument spinose; spines larger and denser anteriorly, 4 to 6 long, 1 to 3 wide at base, smaller and less dense posteriorly, 3 to 4 long, 2 to 3 wide at base. Eyespot pigment absent. Oral sucker simple, subglobular, subterminal, 70 to 82 long or 5% to 9% of body length, 66 to 82 wide. Ventral sucker circular, weakly muscularized, near anterior third of body, 50 to 57 long or 4% to 6% of body length, 50 to 57 wide. Oral sucker to ventral sucker width ratio 1:0.66 to 1:0.77. Forebody 318 to 407 long or 29% to 36% of body length. Hindbody 519 to 833 long or 57% to 65% of body length. Prepharynx about as long as pharynx. Pharynx slightly elongate to spherical, 36 to 40 long or 3% to 4% of body length, 29 to 37 wide. Esophagus 52 to 64 long or 5% to 6% of body length with cecal bifurcation closer to pharynx than ventral sucker. Ceca blind, extending well into hindbody, terminating 114 to 204 from posterior end or 9% to 18% of body length.

Testis single, subellipsoidal to slightly elongate, median to submedian, dextral, 154 to 170 long or 13% to 19% of body length, 109 to 126 wide. Post-testicular space

324 to 495 long or 35% to 39% of body length. Cirrus sac elongate, curving dextrally, dorsal to ventral sucker and ovary, opening anteriorly into genital atrium, terminating at ovarian level or mid-level of testis, 178 to 240 long or 15% to 21% of body length, 53 to 79 wide (contents consisting of internal seminal vesicle, pars prostatica, and cirrus); cirrus elongate, 73 to 80 long or 7% to 9% of body length, 19 to 34 wide when not everted, spined; spines not uniform in size, with smaller spines anteriorly and interiorly, 5 to 8 long, 2 to 3 wide at base; larger spines posteriorly and exteriorly, 8 to 12 long, 3 to 7 wide at base; seminal vesicle unipartite, elongate, in posterior region of cirrus sac, 44 to 82 long or 4% to 10% of body length, 34 to 51 wide. Genital atrium spined; spines more numerous than depicted in Figure 2.1a,b; spines forming a half ring-like structure located near where cirrus entering atrium, 30 to 38 long, 1 to 3 wide at base when cirrus not everting into genital atrium. Genital pore median, opening 10 to 21 or 1% to 2% of body length anterior to ventral sucker.

Ovary subglobular to triangular, never distinctly lobed, submedian, dextral, ventral to and slightly overlapping anterior margin of testis, 67 to 85 long or 5% to 10% of body length, 61 to 81 wide. Terminal organ slightly flask-shaped when not curving ventrally into cross sectional view or constricted, distinct, bipartite, sinistral to cirrus sac, 122 to 136 long or 10% to 15% of body length, 49 to 52 wide; posterior region unspined, muscular, blind; anterior portion separated by muscular sphincter at mid-level, opening into genital atrium, spined; spines evenly distributed, 8 to 14 long, 1 to 3 wide at base. Mehlis' gland slightly antero-sinistral to ovary (observed in only 1 specimen). Seminal receptacle not observed. Laurer's canal descending sinistrally from region of female complex to testis level, coiling, ascending in straight line to ovarian level, opening

dorsally between ovary and cirrus sac (observed in only 1 specimen). Vitellarium comprising 2 lateral groups of 7 to 9 follicles at ovarian level; follicles 27 to 37 long, 29 to 39 wide, meeting as common lateral duct, expanding as central, dorsal vitelline reservoir, connecting to female complex (usually obscured). Uterus voluminous, mostly intercecal, extending 28 to 55 or 3% to 6% of body length from posterior end to genital atrium, descending in coils from region of ootype at ovarian level, dorso-sinistral to testis, rarely overlapping testis, reaching posterior extent, ascending in coils ventrally, sinistral to testis, joining terminal organ at mid-level; post-testicular uterus occupying 271 to 454 or 83% to 92% of post-testicular space, 30% to 36% of body length. Eggs operculate, non-filamented, tanned, 15 to 20 long, 8 to 11 wide when distal.

Excretory vesicle I-shaped, extending to posterior end of cirrus sac, often obscured by voluminous egg-filled uterus; single concretion in 1 specimen; excretory pore terminal.

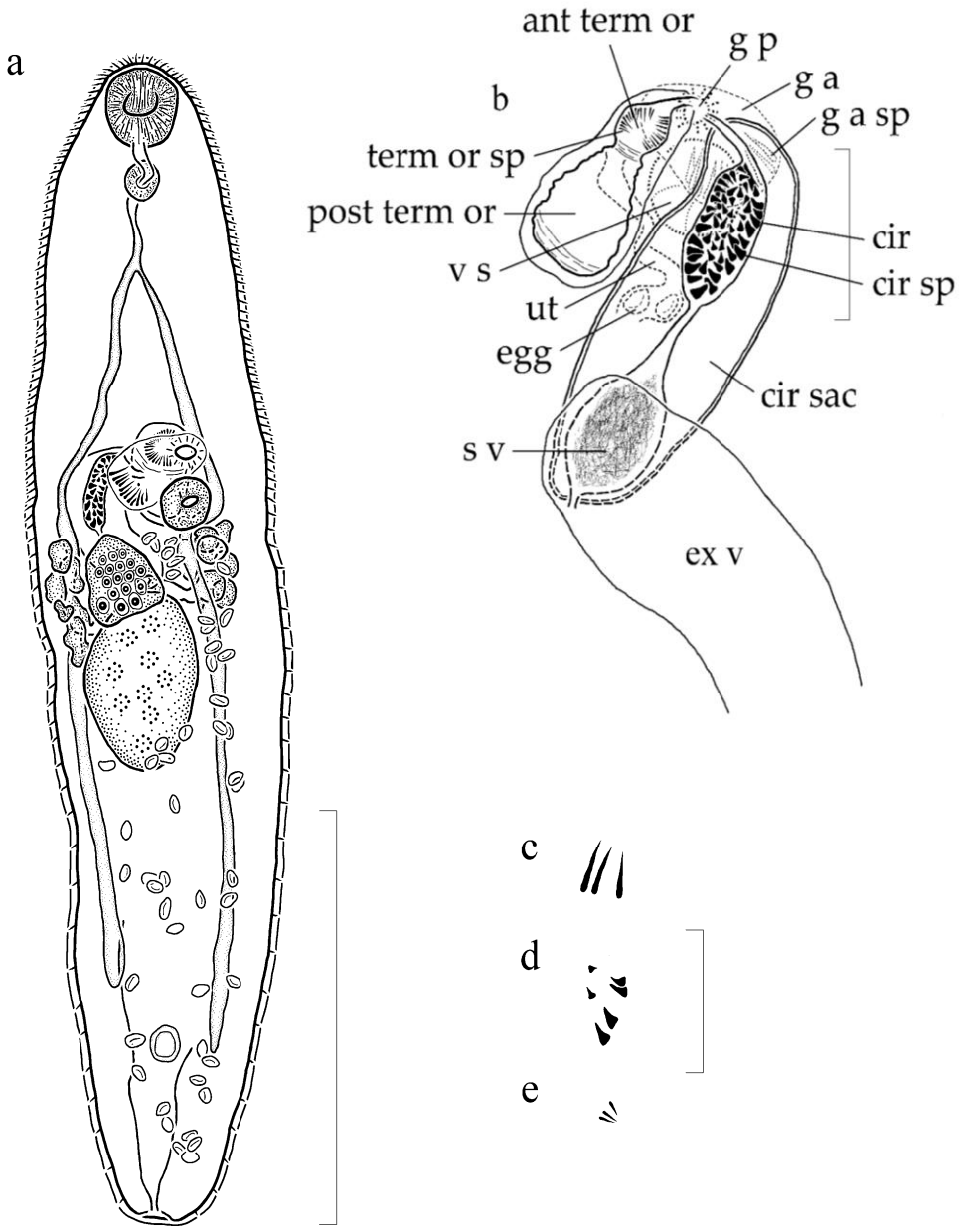

Figure 2.1 Genolopa ampullacea *Linton, 1910, from* Haemulon flavolineateum.

(a) Ventral view, whole mount, scale bar 400 µm; (b) dorsal view, terminal genitalia showing anterior region of the terminal organ (ant term or), posterior region of the terminal organ (post term or), terminal organ spines (term or sp), ventral sucker (v s), uterus (ut), eggs (egg), seminal vesicle (s v), excretory vesicle (ex v), cirrus sac (cir sac), cirrus (cir), cirrus spines (cir sp), genital atrium (g a), genital atrium spines (g a sp), and genital pore (g p), scale bar 100 µm; (c) genital atrium spines, scale bar 50 µm; (d) cirrus spines, note different sized spines, scale bar 50 µm; (e) anterior terminal organ spines, scale bar 50 µm.

2.3.1.1.3 Remarks

Measurements derived from new and previous observations from the syntypes for *G. ampullacea*, from specimens collected from the southern Atlantic Ocean, and from new supplemental data are provided in Table 2.2 for comparison. Specimens of *G. ampullacea* collected and studied for taxonomic purposes prior to the present study were fixed using various methods, very commonly using an unheated acid applied to severely compressed worms; whereas the specimens used for the present supplemental data were preserved using the preferred modern method: specimens were heat-killed with near boiling water, preserved in ethyl alcohol (then post-fixed in formalin) or formalin, and mounted without added pressure. Major differences are apparent between specimens fixed under pressure and those heat-killed without pressure (Curran et al., 2001). In flattened specimens, body width is nearly 2.5 to 3 times wider, both suckers are compressed to nearly twice their normal size, and the cirrus sac, cirrus, and terminal organs are all nearly twice as large (see Table 2.2). Comparison among these and other measurements demonstrates the importance of using fixation techniques that avoid artificial compression when conducting taxonomic comparisons. Furthermore, alcohol-formalin-acetic acid (AFA) and other acid fixation methods create slightly acidic conditions in the mounting medium that has been demonstrated to lead over time to the degradation of hard structures such as body spines and spines associated with terminal genitalia (Curran et al., 2013a). Indeed, tegumental spines appear degraded or are altogether lacking from areas in the syntypes of *G. ampullacea*, and the spines associated with the terminal genitalia are severely degraded. The present supplemental data derived from *G. ampullacea* from *H. flavolineatum* are provided for comparison with future

works. Despite the obvious effects of fixation under pressure and with an acid, certain features present in syntypes and discernable from the available descriptions of *G. ampullacea* strongly suggest that the recently collected material from *H. flavolineatum* represents *G. ampullacea*. Specifically, the percentage of post-testicular space relative to body length, the percentage of post-testicular space occupied by the uterus relative to body length, and the percentage of post-testicular space occupied by the uterus relative to the post-testicular space all agree.

Table 2.2 *Comparison of measurements of* Genolopa ampullacea.

Reference	**Linton (1910)**	**Manter (1942)**	**Manter (1942)**	**Kohn et al. (1982)**	**Present Study**	**Present Study**
Material examined	syntypes	syntypes	new material	new material	syntypes	new material
Under pressure?	yes	yes	no	yes	yes	no
Fixed with an acid?	yes	yes	unknown	yes	yes	no
Host	*H. macrostomum*	*H. macrostomum*	*H. album, H. carbonarium, H. flavolineatum, H. plumierii, H. sciurus, S. foetens*	*H. sciurus*	*H. macrostomum*	*H. flavolineatum*
Locality	Dry Tortugas, FL	Dry Tortugas, FL	Tortugas, FL	Rio de Janeiro, Brazil	Dry Tortugas, FL	Florida Keys
Genital atrium spines	-	34 to 36	34 to 36		30 to 36	30 to 38
Cirrus spines	-	12	12	5 to 10 X 7 to 12	10 to 12 X 4	5 to 8, 8 to 12 X 2 to 3, 3 to 7
Terminal organ spines	-	17	17	-	-	8 to 14 X 1 to 3
Body length	1150 to 1420	-	425 to 1275	740 to 1580	1227	829 to 1265
Body width	630	-	187 to 365	310 to 590	602	202 to 253
Oral sucker	140 *	-	50 to 96	120 to 210 X 150 to 170	131 x 174	70 to 82 X 66 to 82
Ventral sucker	120 *	-	34 to 62	49 to 82 X 56 to 94	102 x 58	50 to 57 X 50 to 57
Sucker ratio		-	3:2	1:0.37 to 1:0.45	1:0.33	1:0.66 to 1:0.77
Pharynx	40 *	-	17 to 40 X 17 to 42	37 to 56 X 34 to 70	63 x 42	36 to 40 X 29 to 37

Table 2.2
(continued).

Cirrus sac	-	-	225 x 99	-	351 x 135	178 to 240 X 53 to 79
Cirrus	-	-	-	-	168 x 45	73 to 80 X 19 to 34
Terminal organ	-	-	150 x 85	-	242 x 106	122 to 136 X 49 to 52
Testis	-	-	-	150 to 300 X 100 to 180	186 x 155	154 to 170 X 109 to 126
Percentage of post-testicular space to body length	-	-	33%	-	37%	35 to 39%
Ovary	-	-	-	-	113 x 118	67 to 85 X 61 to 81
Eggs	17 x 10	-	18 to 22 X 9 to 11	21 to 28 X 9 to 12	14 to 16 X 7 to 10	15 to 20 X 8 to 11
Excretory vesicle	-	-	I - to ventral sucker	-	-	I – to posterior of cirrus sac

* Transverse diameter.

Measurements in micrometers (µm), dimensions shown as length by width.

Despite problems associated with differences in fixation techniques, *G. ampullacea* can be differentiated from the 12 other nominal species in the genus. Herein, *G. ampullacea* is compared with these other nominal species.

Genolopa ampullacea is similar to *Genolopa plectorhynchi* (Yamaguti, 1934) Hopkins, 1941 but is most easily distinguished from the latter by having a subglobular, rounded oral sucker instead of a funnel-shaped oral sucker, a subglobular to triangular ovary rather than a distinctly trilobed ovary, smaller eggs (15 to 20 long, 8 to 11 wide compared with 26 to 29 long, 15 to 18 wide), and the cirrus spines are not bristle-like. Additionally, the description of *G. plectorhynchi* does not mention a spiny genital atrium; however, the illustration of the terminal genitalia of *G. plectorhynchi* appears to have spines surrounding the genital atrium. These spines do, however, look more similar in shape and size to those associated with the cirrus, so it is possible that the illustration

shows a partially extruded spiny cirrus. I did not obtain type material of *G. plectorhynchi*, so we cannot confirm if true genital atrium spines exist. *Genolopa plectorhynchi* does have spines in the anterior region of the terminal organ.

Genolopa ampullacea may be differentiated from *Genolopa brevicaecum* (Manter, 1942) Manter and Pritchard, 1961 by the latter not having spines in the anterior region of the terminal organ. Additionally, the genital atrium spines shown in the illustrations of *G. brevicaecum* appear to be similar in shape and size to those associated with the cirrus compared with my material where there is a distinct difference in the size and shape of the genital atrium spines (30 to 38 long, 1 to 3 wide at base) compared with the cirrus spines (8 to 12 long, 3 to 7 wide at base). Possibly the genital atrium spines of *G. brevicaecum* are from a partially extruded spiny cirrus, but no measurement was given. I consider *G. brevicaecum* as *incertae sedis* because it violates the generic diagnosis by lacking spines in the anterior region of the terminal organ.

Genolopa ampullacea may be differentiated from *Genolopa anisotremi* (Nahhas and Cable, 1964) Yamaguti, 1971 and *Genolopa pritchardae* (Nahhas and Cable, 1964) Yamaguti, 1971 by neither *G. anisotremi* nor *G. pritchardae* having a spined genital atrium. Therefore, I consider *G. anisotremi* and *G. pritchardae* to be *incertae sedis*.

Genolopa ampullacea may be differentiated from *Genolopa microsoma* Lebedev, 1968 by the latter having a unipartite, unspined terminal organ and an unspined genital atrium both of which violate the generic diagnosis of *Genolopa*. As a result, I consider *G. microsoma* to be *incertae sedis*.

Genolopa ampullacea may be differentiated from *Genolopa cheilini* Nagaty and Abdel-Aal, 1972 by the latter having an unspined cirrus and an unspined genital atrium.

The presence of spines on the cirrus is a family level trait. Therefore, I do not believe *G. cheilini* belongs in the Monorchiidae and consider it *incertae sedis*.

Genolopa lunulata Nagaty and Abdel-Aal, 1972 is also likely not a monorchiid. The description of *G. lunulata* states the tegument is smooth, i.e., unspined, and there is no description or illustration of a terminal organ; both are key features of the familial diagnosis, so I consider *G. lunulata* to be *incertae sedis*.

Genolopa ampullacea may be differentiated from *Genolopa mintungensis* Wang, 1975 by the latter not having spines in the genital atrium and what appear to be spines in the posterior region of the terminal organ in the illustration. I was unable to obtain the original species description for *G. mintungensis*, so I am relying on supplemental data from a later publication for this comparison (Parasitology Laboratory, 1976). I consider *G. mintungensis* to be *incertae sedis* because the 2 aforementioned features violate the generic diagnosis.

Genolopa ampullacea may be differentiated from *Genolopa bychowskii* Zhukov, 1977 by the latter having an unspined tegument, an unspined genital atrium and unspined terminal genitalia. Tegumental spines and a spined cirrus are key to the familial diagnosis, so I do not believe *G. bychowskii* is a monorchiid and consider it *incertae sedis*.

Genolopa ampullacea may be differentiated from *Genolopa loborchis* Wang, 1977 by the latter having a larger body size, a distinctly lobed ovary, an irregular, unsmooth testis, and fewer vitelline follicles per vitelline group. Additionally, it is unclear if the terminal genitalia and genital atrium are spined in *G. loborchis*, so I consider it to be *incertae sedis*, possibly at the family level if the cirrus is truly unspined.

Genolopa ampullacea may be differentiated from *Genolopa mugilis* Knoff and Amato, 1992 by the smaller oral to ventral sucker width ratio (1:0.53 to 1:0.58) and smaller genital atrium spine size (7 to 13 long) in *G. mugilis* compared with the larger sucker width ratio (1:0.66 to 1:0.77) and larger genital atrium spines (30 to 38 long, 1 to 3 wide at base) in *G. ampullacea*. The genital atrium spines of *G. mugilis* are more dispersed throughout the genital atrium and appear similar to the cirrus spines in size and shape compared with the genital atrium spines of *G. ampullacea* that form a ring-like structure of long bristles near where the cirrus enters the genital atrium.

Genolopa ampullacea may be differentiated from *Genolopa magnacirrus* Thatcher, 1996 by the latter having a Y-shaped excretory vesicle, apparent spines in the posterior portion of the terminal organ, and no description or illustration of a spiny genital atrium. Therefore, I consider *G. magnacirrus* to be *incertae sedis*.

I conclude, based on the review of morphological features in presently named species in *Genolopa*, that only 3 of the nominal species should be considered as valid, *G. ampullacea, G. plectorhynchi* and *G. mugilis*. This opinion is based on the fact that these are the only species that possess spines in the genital atrium and spines in the anterior region of the bipartite terminal organ. *Genolopa cheilini, G. lunulata, G. bychowskii* and possibly *G. loborchis* are considered to be *insertae sedis* at the family level because they do not follow the morphological diagnosis for members of the Monorchiidae. *Genolopa brevicaecum, G. anisotremi, G. pritchardae, G. microsoma, G. mintungensis*, and *G. magnacirrus* violate the generic diagnosis of *Genolopa* as described above and are considered *incertae sedis*.

2.3.1.2 *Genolopa vesca* Panyi, Curran, and Overstreet, 2020 (Figure 2.2)

2.3.1.2.1 Taxonomic Summary

Type host: *Haemulon sciurus* (Shaw, 1803), blue striped grunt, Haemulidae.

Type locality: Long Key, Florida (24°47′26.93”N, 80°53′2.96”W).

Sites: intestine, pyloric ceca.

Specimens deposited: Holotype: USNM 1611648; 4 paratypes: USNM 1611649, 1611650, 1611651, 1611652; 1 hologenophore: USNM 1611653.

Sequences deposited: Partial 28S rDNA, 1 sequence (1 submitted to GenBank: accession number MN984471); ITS2 rDNA, 1 sequence (1 submitted to GenBank: accession number MN984471).

Etymology: The specific epithet is a Latin feminine adjective meaning very small in reference to the smaller tegumental spines in this species relative to the type-species.

http://zoobank.org/urn:lsid:34D4B1D8-D4BE-485B-9102-30436080EDE5

2.3.1.2.2 Description (Based on 6 gravid, adult specimens and 1 non-gravid specimen, all mounted without pressure)

Body elongate, slightly tapering at both ends, narrower anteriorly, widest near mid-body, 871 to 1223 (1223) long, 188 to 276 (211) wide. Tegument spinose; spines largest and densest anteriorly, 2 to 4 long, 1 to 3 wide at base, smaller, rounded, and less dense posteriorly, 1 to 3 long, 2 to 3 wide at base. Eyespot pigment absent. Oral sucker simple, spherical to subspherical, subterminal, 49 to 74 (74) long or 6% to 8% (6) of body length, 51 to 88 (83) wide. Ventral sucker circular, weakly muscularized, between anterior half and anterior third of body, 46 to 60 (60) long or 5% (5) of body length, 41 to 56 (56) wide. Oral sucker to ventral sucker width ratio 1:0.60 to 1:0.85 (1:0.67).

Forebody 305 to 504 (504) long or 31% to 41% (41) of body length; hindbody 477 to 652 (652) long or 53% to 60% (53) of body length. Prepharynx about half length of pharynx to about as long as pharynx, 21 to 40 (40) long or 2% to 3% (3) of body length. Pharynx spherical to slightly elongate, 34 to 43 (43) long or 3% to 4% (4) of body length, 33 to 40 (40) wide. Esophagus length variable, 35 to 101 (98) long or 3% to 9% (8) of body length. Cecal bifurcation closer to level of pharynx than level of ventral sucker, 147 to 266 (266) anterior to ventral sucker or 15% to 22% (22) of body length. Ceca blind, extending well into hindbody, terminating 81 to 157 (116) from posterior end or 8% to 16% (10) of body length.

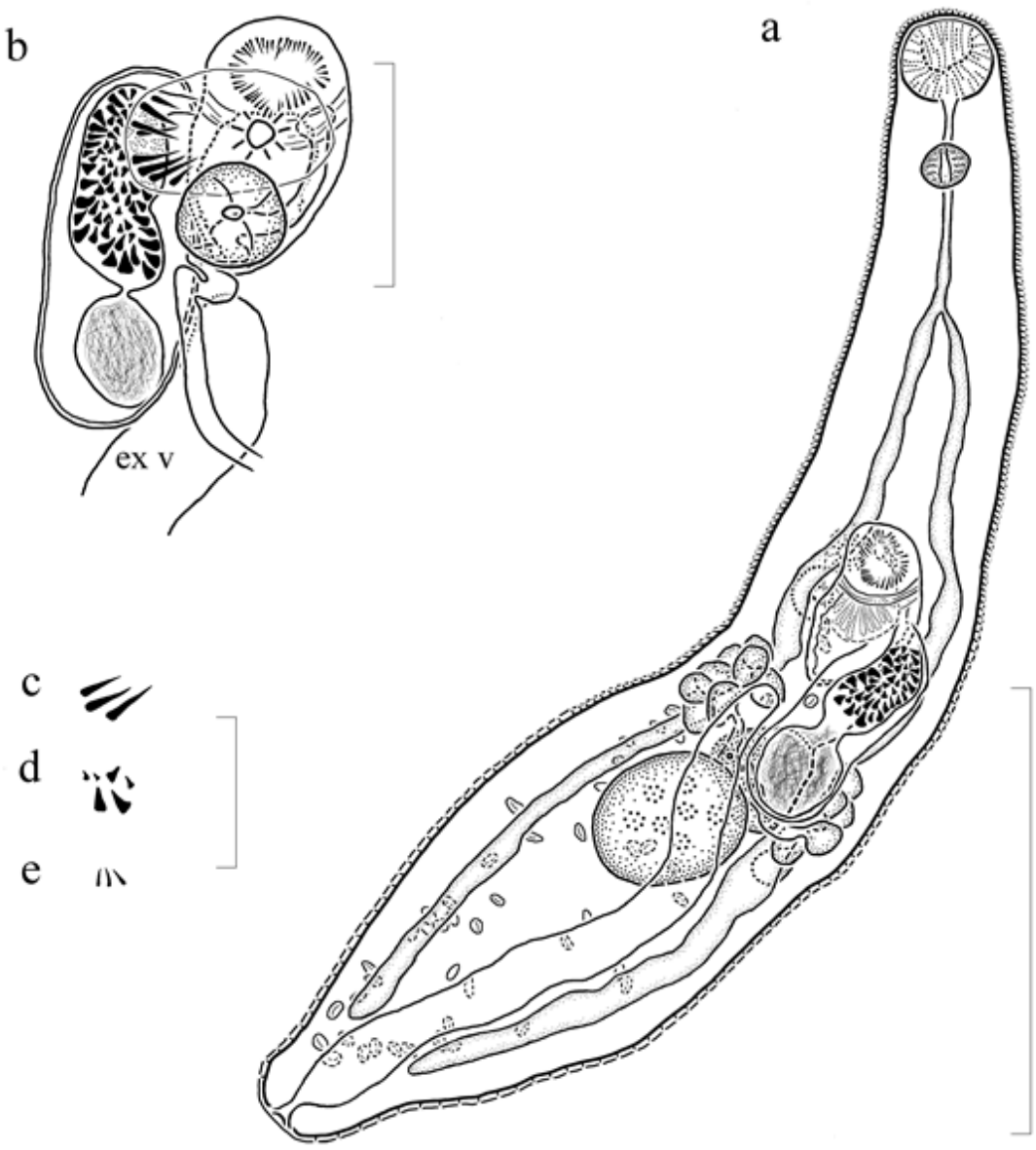

Figure 2.2 Genolopa vesca *from* Haemulon sciurus.

(a) Dorsal view, holotype, scale bar 400 µm; (b) ventral view, terminal genitalia and anterior extent of the excretory vesicle (ex v), note anterior portion of the terminal organ is a cross sectional view, scale bar 100 µm; (c) genital atrium spines, scale bar 50 µm; (d) cirrus spines, note the different sized spines, scale bar 50 µm; (e) anterior terminal organ spines, scale bar 50 µm.

Testis singular, subglobular to slightly elongate, median to submedian, dextral, 106 to 150 (106) long or 9% to 17% (9) of body length, 105 to 129 (105) wide. Post-

testicular space 194 to 375 (375) long or 22% to 32% (31%) of body length. Cirrus sac elongate, curving dextrally, dorsal to ventral sucker and ovary, terminating at ovarian level, 148 to 291 (193) long or 16% to 25% (16) of body length, 59 to 76 (70) wide (contents comprising internal seminal vesicle, pars prostatica, and cirrus); cirrus elongate, 86 to 102 (98) long or 8% to 10% (8) of body length, 27 to 45 (45) wide when not everted, spined; spines not evenly distributed, with larger spines posteriorly and exteriorly, 6 to 9 long, 5 to 7 wide at base; smaller spines anteriorly and interiorly, 5 to 7 long, 2 to 4 wide at base; seminal vesicle internal, unipartite, elongate to spherical, in posterior region of cirrus sac, 53 to 95 (75) long or 6% to 9% (6) of body length, 51 to 75 (75) wide. Genital atrium spined; spines forming a half ring-like structure located near where cirrus entering atrium, 35 to 43 long, 2 to 4 wide at base when cirrus not everting into genital atrium, more numerous in specimens than portrayed in Figure 2.2a,b. Genital pore median, 8 to 19 anterior to ventral sucker.

Ovary subglobular to triangular, never distinctly lobed, submedian, dextral, pre-testicular or overlapping anterior margin of testis, 72 to 89 (89) long or 7% to 9% (7) of body length, 83 to 95 (91) wide. Terminal organ subarcuate, distinct, muscular, bipartite, sinistral to cirrus sac, 120 to 146 (144) long or 11% to 15% (12) of body length, 46 to 79 (79) wide; posterior region muscular, unspined, blind; anterior portion separated by a muscular sphincter, opening into genital atrium, spined; spines uniform, 8 to 11 long, 1 to 3 wide at base. Mehlis' gland not observed. Seminal receptacle not observed. Laurer's canal not observed. Vitellarium comprising 2 lateral groups of 8 to 9 follicles at level of ovary; follicles 28 to 43 long, 22 to 29 wide, connecting as common vitelline duct, expanding to central, dorsal vitelline reservoir. Uterus voluminous, mostly intercecal,

extending 38 to 67 (67) or 4% to 7% (5) of body length from posterior end to genital atrium, descending in coils dorso-sinistral to testis from region of female complex, reaching posterior coiling extent, ascending in coils ventrally, sinistral to testis, entering terminal organ ventrally, slightly anterior to mid-level; post-testicular uterus occupying 123 to 305 (305) or 63% to 87% (81%) of post-testicular space, 14% to 28% (25%) of body length. Eggs operculate, non-filamented, tanned, 13 to 20 long, 6 to 11 wide when distal.

Excretory vesicle I-shaped, extending to level of internal seminal vesicle, curving sinistrally around cirrus sac, often obscured by voluminous egg-filled uterus, 1 specimen containing 1 concretion; excretory pore terminal.

2.3.1.2.3 Remarks

Genolopa vesca is most similar morphologically to *G. ampullacea* based on the presence, shape, and size of the genital atrium spines, size and shape of the terminal organ and cirrus spines, extent of the ceca, size and size ratios of the oral and ventral suckers, size of the pharynx, extent of the excretory vesicle, location and shape of the ovary and testis, and location of cecal bifurcation. *Genolopa vesca* may be differentiated from *G. ampullacea* by the amount of post-testicular space (22% to 32%) in *G. vesca* compared with the amount of post-testicular space (35% to 39%) in *G. ampullacea* and the amount of post-testicular space occupied by the uterus relative to body length (14% to 28%) in *G. vesca* n. sp. compared with *G. ampullacea* (30% to 36%). Additionally, the tegumental spines in the forebody (2 to 4 long, 1 to 3 wide at base) and hindbody (1 to 3 long, 2 to 3 wide at base) are smaller in *G. vesca* compared with those in the forebody (4

to 6 long, 1 to 3 wide at base) and hindbody (3 to 4 long, 2 to 3 wide at base) in *G. ampullacea*.

Genolopa vesca may be differentiated from *G. plectorhynchi* by the latter having bristle-like cirrus spines, a funnel-shaped oral sucker, a distinctly trilobed ovary, and larger eggs (26 to 29 long, 15 to 18 wide compared with 13 to 20 long, 6 to 11 wide). In addition, the illustration of the terminal genitalia of *G. plectorhynchi* appears to have spines in the genital atrium even though the presence of spines in the genital atrium was not stated in the description. The spines illustrated appear more similar in shape and size to those associated with the cirrus, so it is likely the spines in the genital atrium region in the illustration of *G. plectorhynchi* are from a partially extruded spiny cirrus.

Genolopa mugilis may be differentiated from *G. vesca* by the smaller sucker width ratio (1:0.53 to 1:0.58) in *G. mugilis* compared with the sucker width ratio (1:0.60 to 1:0.85) in *G. vesca* and the smaller genital atrium spine size (7 to 13 long) in *G. mugilis* compared with the larger genital atrium spines (35 to 43 long, 2 to 4 wide at base) in *G. vesca*. The genital atrium spines of *G. mugilis* are more dispersed throughout the genital atrium and appear similar to the cirrus spines in size and shape (9 to 11 long), whereas the genital atrium spines of *G. vesca* form a half ring-like structure of long bristles, near where the cirrus enters the genital atrium, that are distinct from the cirrus spines.

2.3.1.3 *Genolopa minuscula* Panyi, Curran, and Overstreet, 2020 (Figure 2.3)

2.3.1.3.1 Taxonomic Summary

Type host: *Anisotremus surinamensis* (Bloch, 1791), black margate, Haemulidae.

Type locality: Marathon, Florida (24°41′58.2432”N, 81°10′12.702”W).

Sites: intestine, pyloric ceca.

Specimens deposited: Holotype: USNM 1611641; 3 paratypes: USNM 1611642, 1611643, 1611644; 3 hologenophores: USNM 1611645, 1611646, 1611647.

Sequences deposited: Partial 28S rDNA, 4 identical replicates (1 submitted to GenBank: accession number MN984472); ITS2 rDNA, 1 sequence (1 submitted to GenBank: accession number MN984473).

Etymology: The specific epithet is a Latin feminine adjective meaning somewhat less in reference to the less extensive uterus in this species compared with the type-species.

http://zoobank.org/urn:lsid: F9EA40EE-C8B4-42C2-B641-73297EEE6EE7

2.3.1.3.2 Description (Based on 7 gravid, adult specimens and 1 non-gravid specimen, all mounted without pressure)

Body elongate, tapering slightly at both ends, widest near mid-body, 716 to 1373 (1356) long, 228 to 347 wide (335). Tegument spinose; spines larger and denser anteriorly, 4 to 6 long, 2 to 4 wide at base, smaller and less dense posteriorly, 2 to 3 long, 2 to 3 wide at base. Eyespot pigment absent. Oral sucker subspherical, subterminal, 76 to 103 (100) long or 7% to 9% (7) of body length, 78 to 107 (107) wide. Ventral sucker circular to subrounded, very weakly muscularized, near mid-body, 45 to 72 (64) long or 4% to 7% (4) of body length, 47 to 73 (73) wide. Sucker width ratio 1:0.57 to 1:0.79 (1:0.68). Forebody 296 to 614 (614) long or 30% to 51% (45%) of body length; hindbody 320 to 696 (696) long or 43% to 60% (51%) of body length. Prepharynx slightly more than half length of pharynx to shorter. Pharynx slightly elongate to spherical, 46 to 61 (61) long or 4% to 6% (4) of body length, 40 to 54 (53) wide. Esophagus length variable,

28 to 140 (111) long or 2% to 11% (8) of body length with cecal bifurcation closer to pharynx than ventral sucker, 134 to 284 (284) anterior to ventral sucker. Ceca blind, extending well into hindbody, terminating 100 to 136 (124) from posterior end or 8% to 12% (9%) of body length.

Testis single, subglobular to irregular, median to submedian, dextral, 97 to 204 (204) long or 11% to 16% (15) of body length, 62 to 142 (118) wide. Post-testicular space 163 to 411 (411) long or 22% to 31% (30%) of body length. Cirrus sac elongate, curving dextrally, dorsal to ventral sucker and ovary, terminating at level of or posterior to ovary, 156 to 299 (299) long or 16% to 27% (22) of body length, 54 to 90 (84) wide (contents consisting of internal seminal vesicle, pars prostatica, and cirrus). Cirrus elongate, 78 to 141 long or 9% to 12% (everted in holotype) of body length, 24 to 45 wide when not everted, spined; spines not uniform in size with larger spines posteriorly and exteriorly, 10 to 15 long, 6 to 9 wide at base; smaller spines anteriorly and interiorly, 3 to 8 long, 2 to 4 wide at base. Seminal vesicle internal, unipartite, elongate, in posterior region of cirrus sac, 34 to 122 (122) long or 5% to 10% (9) of body length, 25 to 71 (60) wide. Genital atrium spined; spines 28 to 36 long, 3 to 4 wide at base when cirrus not everting into genital atrium, more numerous than shown in Figure 2.3a,b. Genital pore median, 7 to 21 anterior to ventral sucker.

Ovary subglobular to triangular, never distinctly lobed, submedian, dextral, pre-testicular or slightly overlapping anterior of testis, 33 to 106 (83) long or 5% to 8% (6) of body length, 45 to 105 (97) wide. Terminal organ subarcuate, conspicuous, bipartite, sinistral to cirrus sac, 112 to 146 (146) long or 11% to 14% (11) of body length, 56 to 78 (64) wide; posterior portion muscular, unspined, blind; anterior region separated by a

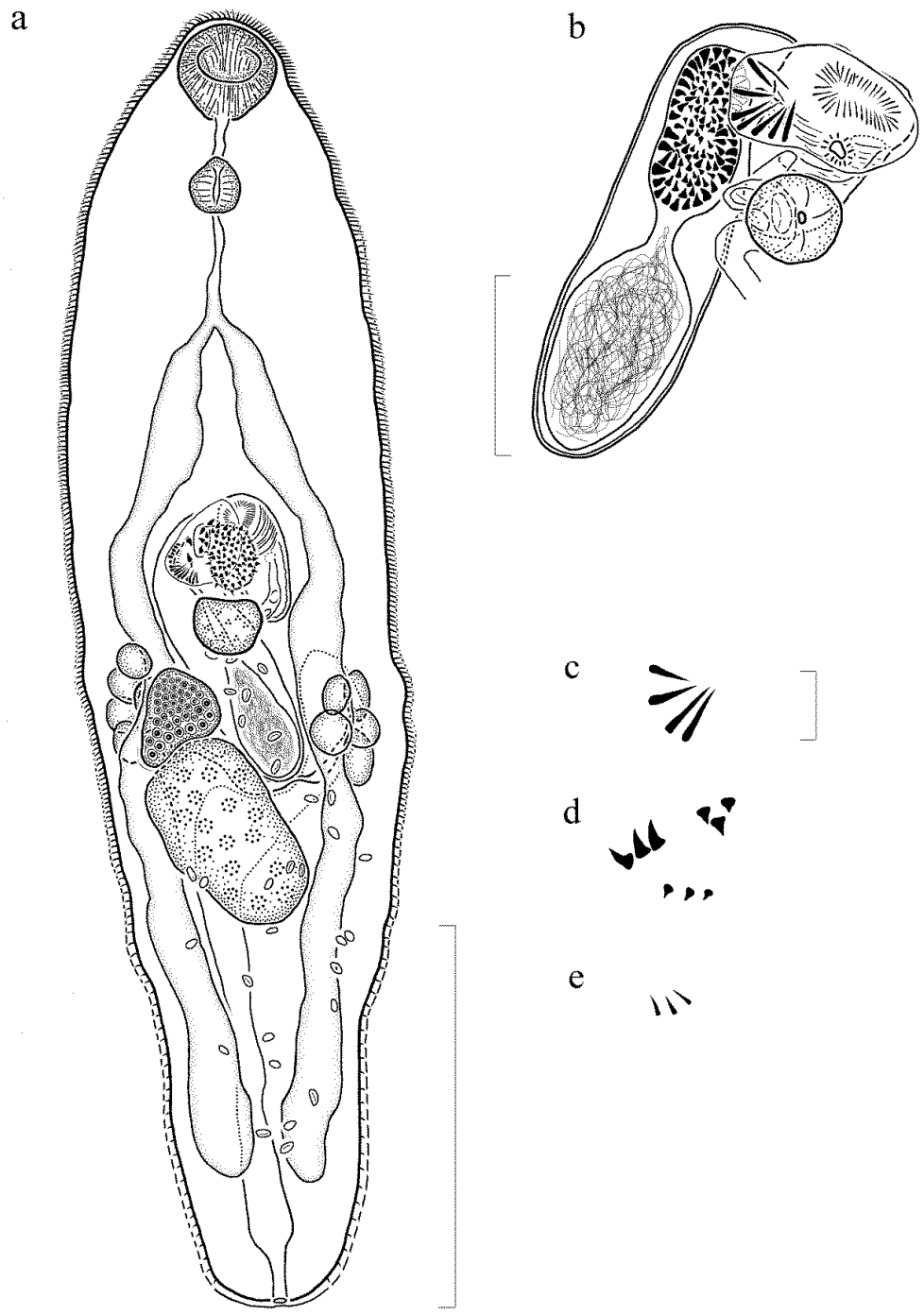

Figure 2.3 Genolopa minuscula *from* Anisotremus surinamensis.

(a) Ventral view, holotype, scale bar 400 µm, note cirrus everted; (b) ventral view, terminal genitalia, scale bar 100 µm, note anterior portion of terminal organ is a cross sectional view; (c) genital atrium spines, scale bar 40 µm; (d) cirrus spines, note the different sized spines, scale bar 40 µm; (e) anterior terminal organ spines, scale bar 40 µm.

muscular sphincter, opening into genital atrium, spined; spines uniformly spaced, 8 to 15 long, 1 to 2 wide at base. Mehlis' gland and female complex not observed. Seminal receptacle not observed. Laurer's canal opening dorsally, at ovarian level, dextral to ovary (observed in only 1 specimen). Vitellarium consisting of 2 lateral groups of 6 to 9 follicles at ovarian level; follicles 24 to 59 long, 14 to 40 wide, smaller in younger individuals, connecting with dorsal, common lateral duct, expanding dorsally as vitelline reservoir. Uterus voluminous, mostly intercecal, extending 98 to 202 (146) or 8% to 18% (11) of body length from posterior end to genital atrium, descending in coils from ovarian level, dorso-sinistral to testis, reaching posterior extent, ascending in coils ventrally, sinistral to testis, joining with terminal organ near mid-level; post-testicular uterus occupying 101 to 277 (264) or 41% to 76% (64) of post-testicular space, 12% to 21% (19) of body length. Eggs 14 to 23 long, 8 to 12 wide, typically 17 to 20 long, 9 to 11 wide when distal.

Excretory vesicle I-shaped, extending to ovarian level to posterior end of ventral sucker, occasionally curved in anterior half, usually obscured by eggs; excretory pore terminal.

2.3.1.3.3 Remarks

Genolopa minuscula is most morphologically similar to *G. vesca* and *G. ampullacea*. Similarities among the species include ovary and testis size, shape, location, the presence, size, and shape of the genital atrium spines, the size and shape of spines in the terminal organ, extension of the ceca well into the hindbody, extension of the excretory vesicle to the ovarian level or posterior edge of ventral sucker, and the oral sucker and ventral sucker size width ratios.

Genolopa minuscula may be differentiated from *G. vesca* by the latter having smaller tegumental spines in the forebody (2 to 4 long, 1 to 3 wide at base) and hindbody (1 to 3 long, 2 to 3 wide at base) compared with the size of tegumental spines in the forebody (4 to 6 long, 2 to 4 wide at base) and hindbody (2 to 3 long, 2 to 3 wide at base) of *G. minuscula*.

Genolopa minuscula may be differentiated from *G. vesca* and *G. ampullacea* by the slightly larger pharynx (46 to 61 long, 40 to 54 wide) and the slightly larger size of the "large" spines on the cirrus (10 to 15 long, 6 to 9 wide at base) in *G. minuscula* n. sp. compared with the pharynx (34 to 43 long, 33 to 40 wide) and "large" cirrus spines (6 to 9 long, 5 to 7 wide) in *G. vesca* and compared with the pharynx (36 to 40 long, 29 to 27 wide) and "large" cirrus spines (8 to 12 long, 3 to 7 wide at base) in *G. ampullacea.*

Genolopa minuscula may be further differentiated from *G. ampullacea* by the amount of post-testicular space (22% to 31%) in *G. minuscula* compared with the space (35% to 39%) in *G. ampullacea*, the amount of post-testicular space occupied by the uterus relative to body length (12% to 21%) in *G. minuscula* compared with the post-testicular space occupied by the uterus relative to body length (30% to 36%) in *G. ampullacea*, and the amount of post-testicular space occupied by the uterus relative to post-testicular space (41% to 76%) in *G. minuscula* compared with the post-testicular space occupied by the uterus relative to post-testicular space (83% to 92%) in *G. ampullacea*; all 3 features are relatively reduced in *G. minuscula* compared with *G. ampullacea*.

Genolopa minuscula may be differentiated from *G. plectorhynchi* by the latter having a funnel-shaped oral sucker, a distinctly trilobed ovary, larger eggs (26 to 29 long,

15 to 18 wide compared with 14 to 23 long, 8 to 12 wide), bristle-like spines on the cirrus, and no mention of a spiny genital atrium. However, the illustration of the terminal genitalia of *G. plectorhynchi* appears to have spines surrounding the genital atrium, but these spines appear to more closely resemble cirrus spines in shape and size. It is possible that the spines near the genital atrium in the illustration are from a partially extruded cirrus.

Genolopa mugilis may be differentiated from *G. minuscula* by the smaller size of the genital atrium spines (7 to 13 long) that are more evenly, widely dispersed throughout the whole genital atrium in *G. mugilis* compared with the larger genital atrium spines (28 to 36 long, 3 to 4 wide) that form a half ring-like structure of long bristles near where the cirrus enters the genital atrium in *G. minuscula* The range of the sucker width ratios slightly overlaps between *G. minuscula* (1:0.57 to 1:0.79) and *G. mugilis* (1:0.53 to 1:0.58).

2.3.2 Molecular

The trimmed multiple sequence alignment length of partial 28S rDNA fragments consisted of 1163 base pairs, including gaps. Masking revealed no ambiguous column, i.e., columns with confidence scores below the cut off value of 0.4, so no column was excluded in the phylogenetic analysis. BI analysis resulted in a recovered phylogeny (Figure 2.4) that is consistent with previously reported monorchiid phylogenies (Cribb et al., 2018; Wee et al., 2018, 2019). The recovered phylogeny indicates that the included species of *Genolopa* (all from western Atlantic Ocean) form a well-supported clade with *P. orthopristis* (see supplemental data below). This clade is separate from any species of *Lasiotocus, Parachrisomon,* or *Proctotrema*.

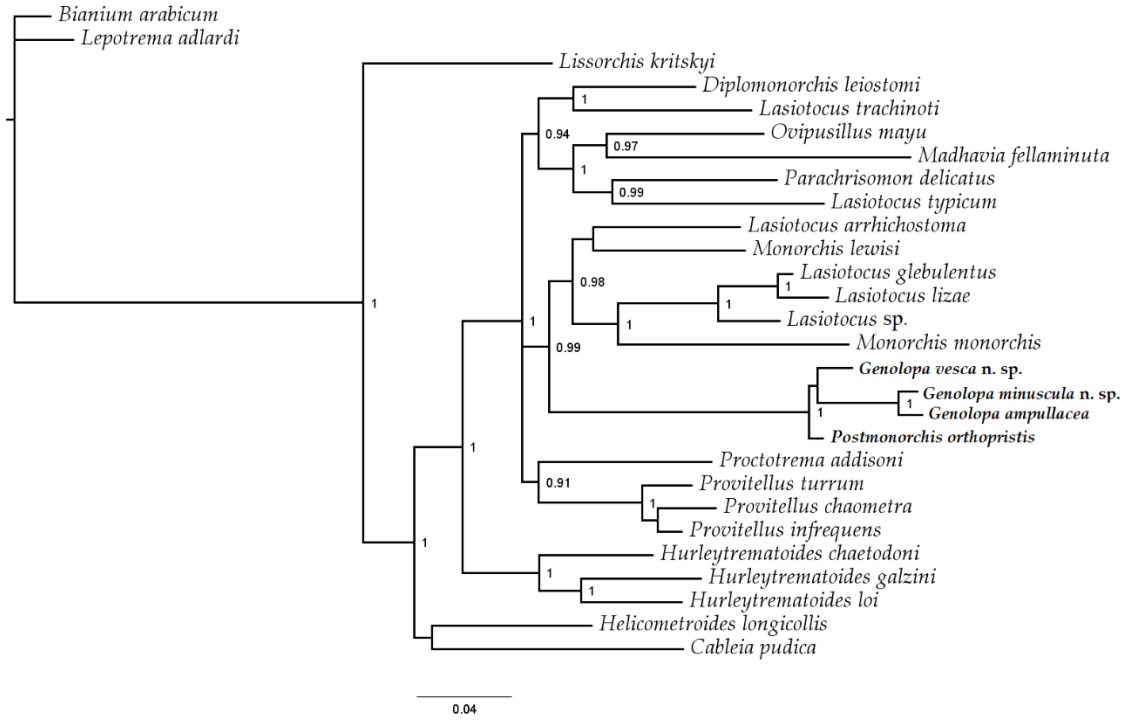

Figure 2.4 *Interrelationships among members of the Monorchiidae based on Bayesian inference analysis of partial 28S rDNA data.*

Bayesian inference posterior probabilities are shown at the nodes; support values < 0.85 are not shown. Genolopa–Postmonorchis clade highlighted with bold text.

Importantly, the recovered phylogeny supports that *Genolopa* is a distinct lineage from *Lasiotocus, Parachrisomon,* and *Proctotrema* at the generic level. Pairwise comparisons of variable sites of the partial 28S rDNA region among species of *Genolopa* and *Postmonorchis* are presented in Table 2.3. Sequences of *G. minuscula* and *G. ampullacea* differed by 1.6% (19 bp). Sequences of *G. vesca* and both *G. minuscula* and *G. ampullacea* differed by 4.9% (57 bp). The sequence of *P. orthopristis* differed the least with *G. vesca* by 2.1% (24 bp). Sequences of *P. orthopristis* and *G. minuscula* differed by 3.9% (45 bp), and sequences of *P. orthopristis* and *G. ampullacea* differed by 4% (47 bp).

Table 2.3 *Pairwise comparison among partial fragments (1163 base pairs long) of 28S rDNA from species of* Genolopa *and* Postmonorchis *in present study,*

Species	***G. minuscula***	***G. ampullacea***	***G. vesca***	***P. orthopristis***
P. orthopristis	45 (3.9)	47 (4.0)	24 (2.1)	—
G. minuscula	—	19 (1.6)	57 (4.9)	—
G. ampullacea		—	57 (4.9)	—
G. vesca			—	—

Shown as number of variable sites with (%) (above diagonal).

The recovered phylogeny does not support a distinction between *Genolopa* and *Postmonorchis*, suggesting the 2 genera do not represent distinct generic lineages or more taxa are necessary to elucidate this relationship. The *Genolopa–Postmonorchis* clade is closely affiliated with a clade consisting of species of *Monorchis* Monticelli, 1893 and some species of *Lasiotocus*. We also provide sequence data for 3 species of *Lasiotocus* that had no prior sequence data available (see supplemental data below). These new data further support that *Lasiotocus* is polyphyletic but at least some species of *Lasiotocus* are closely related to *Monorchis* (Cribb et al., 2018).

2.4 Discussion

The recovered phylogeny of the Monorchiidae (Figure 2.4) was constructed using publicly available partial 28S rDNA sequence data from all genera thus far plus new material supported by vouchers; it also includes taxa from the Indo-Pacific Ocean, Mediterranean Sea, North Sea, and western Atlantic Ocean. Prior to the present study, molecular data were available for only 2 monorchiid species from the northwestern Atlantic Ocean: *Diplomonorchis leiostomi* Hopkins, 1941 and *Hurlytrematoides*

chaetodoni (Manter, 1942) Yamaguti, 1954 (Olson et al., 2003; Andres et al., 2018). This study contributed novel molecular data from 6 additional northwestern Atlantic Ocean monorchiid species in 3 genera. The novel molecular data from species of *Genolopa* represent the first such available data for the genus, and novel molecular data from species of *Lasiotocus* represent the first sequence data available from northwestern Atlantic species of that genus. As expected, the northwestern Atlantic species of *Lasiotocus* herein included did not represent a monophyletic group as is apparent in this genus from other studies (Cribb et al., 2018; Wee et al., 2018, 2019); taxonomic and systematic problems among species of *Lasiotocus* will be the focus of a subsequent chapter.

Interestingly, the novel molecular data from *P. orthopristis* does not represent the first available data for the genus; however, I disagree with the generic classification of these sequences (GenBank accession no. KC603478 [Carella et al., 2013] and MF374321 [Mancini et al., 2018]) because 1 classification was made using the BLASTn tool with no morphological evidence derived from adult vouchers (MF374321), and the other was based on morphological examination of metacercariae, in which some of the key diagnostic features for *Postmonorchis* (uterus location and extent, spined cirrus, anteriorly spined terminal organ) are not yet manifested (KC603478). The 2 molecular data for *Postmonorchis* are publicly available partial 18S rDNA, complete ITS1, 5.8S rDNA, and ITS2, and partial 28S rDNA sequences. One is derived from metacercariae collected from the wedge clam in Italy (KC603478) (Carella et al., 2013), and the second is derived from the tissue of the European flat oyster in Italy (MF374321) (Mancini et al., 2018). The ITS2 sequences from both studies are identical; however, the published partial

28S rDNA sequences were too short to include with my analysis. I conducted a pairwise comparison of the ITS2 sequences from *Postmonorchis* sp. (KC603478) and my *P. orthopristis* material; there was a 26% bp difference between the 2, suggesting *Postmonorchis* sp. (KC603478) is not actually a species in *Postmonorchis*. My data are from morphologically identified adult material whereas *Postmonorchis* sp. (KC603478) data are based on metacercariae. This unidentified species may be included in analyses of the Monorchiidae once more ITS2 and 28S rDNA sequences become available.

The recovered phylogeny provides evidence suggesting *Genolopa* represents a distinct evolutionary lineage that is closely related to *Postmonorchis* and distinct from *Parachrisomon, Proctotrema,* and *Lasiotocus,* 3 genera to which *Genolopa* is morphologically similar and that have available molecular data (Olson et al., 2003; Searle et al., 2014; Atopkin et al., 2017; Wee et al., 2018). Similar to previous analyses, *Lasiotocus* is polyphyletic (Cribb et al., 2018; Wee et al., 2018, 2019). The present study contributed data from the type-species for *Genolopa* and *Postmonorchis,* but unfortunately, no molecular data are yet available from the type-species for *Parachrisomon, Proctotrema,* or *Lasiotocus.* Sequence data from the type-species is needed to determine the true lineage of *Lasiotocus*. Several attempts were made to collect *L. mulli* (type-species) but were unsuccessful. Ferrer-Maza et al. (2015) represents the most recent report of *L. mulli* collected in the Mediterranean Sea, but personal communication with the primary author revealed that the prevalence of *L. mulli* was very low in her study. Over 300 specimens of the definitive host were examined, and only 5 specimens of *L. mulli* were found.

The absence of molecular data from type-species for these related genera prevent us from making serious inferences regarding interrelationships among these 5 morphologically similar genera. Nevertheless, the novel molecular data from the species of *Genolopa* serve to confirm that the primary diagnostic features for the genus (the presence of spines in the genital atrium along with the presence of a bipartite, anteriorly spined terminal organ), as proposed by Manter (1942), serve reliably. Species in *Proctotrema* do not have spines in the genital atrium, and they have a unipartite terminal organ. Species in both *Lasiotocus* and *Parachrisomon* do not have spines in the genital atrium, and species in *Parachrisomon*, uniquely among these morphologically similar genera, have a vitellarium distributed well into the hindbody (Madhavi, 2008).

Despite the close similarity and phylogenetic relationship exhibited between *Genolopa* and *Postmonorchis*, there are several obvious morphologic differences between the genera (Jousson et al., 2000; Madhavi, 2008). Species of *Postmonorchis* are smaller and oval compared with species of *Genolopa* that are larger and elongate. The uterus is mostly intercecal, with portions overlapping the ceca, with only a small portion extending into extracecal space in species of *Genolopa*, whereas the uterus is mostly extracecal and overlapping the ceca with only a small portion extending into the intercecal space in species of *Postmonorchis*. Furthermore, the uterus extends from the cecal bifurcation to the testis, not posterior to the testis, in species of *Postomonorchis,* but the uterus extends from the genital pore to posterior to the testis in species of *Genolopa.* The size of the cirrus sac is larger relative to body size in species of *Postmonorchis* (approximately one third body size) compared with that in species of *Genolopa*. The testis is located at the posterior end in species of *Postmonorchis* but is located more medially in species of

Genolopa. Finally, and perhaps most importantly, species of *Genolopa* have spines in the genital atrium whereas species of *Postmonorchis* do not have spines in the genital atrium based on the original generic description and the description of the type-species by Hopkins (1941).

I accept *Genolopa* as a valid genus based on the evidence provided in this study. *Genolopa* currently contains 13 nominal species (Table 2.4). However, the authorities for only 3 of 13 species attempted to discuss or illustrated the presence of spines in the genital atrium in conjunction with an anteriorly spined, bipartite terminal organ in original descriptions: *G. ampullacea, G. plectorhynchi,* and *G. mugilis* (Linton, 1910; Manter, 1942; Knoff and Amato, 1991). Therefore, tentatively I do not agree with the placement of the other nominal species in *Genolopa* and consider them to be *incertae sedis* with a few violating the diagnosis of the family. The type materials from these species should be reexamined to determine whether genital atrium spines are present and if the terminal organ is bipartiate and spined anteriorly to confirm if these species represent acceptable species of *Genolopa.* The genital atrium spines described or illustrated in *G. plectorhynchi*, G. *brevicaecum* and *G. mugilis* resemble cirrus spines in size and shape. Consequently, type materials of *G. plectorhynchi*, *G. brevicaecum* and *G. mugilis* should also be examined to clarify if the genital atrium spines are from a partially extruded cirrus or are in fact spines associated with the genital atrium. Moreover, representatives of these confounding species should be sequenced to verify their generic status.

Table 2.4 *Nominal species of* Genolopa.

Species	Authority
Genolopa ampullacea *	Linton, 1910
Genolopa anisotremi **	(Nahhas and Cable, 1964) Yamaguti, 1971
Genolopa brevicaecum **	(Manter, 1942) Manter and Pritchard, 1961

Table 2.4 (continued).

Genolopa bychowskii **	Zhukov, 1977
Genolopa cheilini **	Nagaty and Abdel-Aal, 1972
Genolopa loborchis **	Wang, 1977
Genolopa lunulata **	Nagaty and Abdel-Aal, 1972
Genolopa magnacirrus **	Thatcher, 1996
Genolopa microsoma **	Lebedev, 1968
Genolopa mintungensis **	Wang, 1975
Genolopa mugilis	Knoff and Amato, 1992
Genolopa plectorhynchi	(Yamaguti, 1934) Hopkins, 1941
Genolopa pritchardae **	(Nahhas and Cable, 1964) Yamaguti, 1971

* Indicates type-species of the genus.

** Species considered *incertae sedis.*

Investigation of type material for *Genolopa longicaudata* Siddiqi and Cable, 1960 is also needed to clarify the validity of this species or its synonymy with *G. ampullacea*. Siddiqi and Cable (1960) described *G. longicaudata* and differentiated it from *G. ampullacea* based on the post-testicular space and length of the terminal organ. I do not believe these are the appropriate features to use to distinguish the 2 species, if indeed they represent 2 species, because these features can exhibit high levels of variability in monorchiids. At present, I tentatively accept the validity of *G. longicaudata* because I believe hindbody size and a more anteriorly located ventral sucker (based on observations of illustrations and scale measurements by Siddiqi and Cable (1960) serve to better differentiate *G. longicaudata*. Forebody and hindbody lengths are 18% and 77% of overall body length, respectively, in the illustration of *G. longicaudata*, or close to one fifth of the body length. Siddiqi and Cable (1960) stated that the ventral sucker of *G.*

longicaudata is approximately one fifth the body length from the anterior end. My observations of forebody and hindbody lengths of *G. ampullacea* range from 29 to 36% and 57 to 65% of body length, respectively (based on measurements of the type material and my newly collected material). The ventral sucker of *G. ampullacea* is located at approximately the anterior one third of the body length (Manter, 1942), showing these metrics are quite different between the species.

It is very difficult to decide on the validity of *G. ampullacea* from other reports without those reports having extensive descriptions, and very few do this; also, there is a need for molecular data. Many of these reports are not taxonomic papers; many are parasite community investigations of hosts from specific locations. No information about *G. ampullacea* other than host is provided in the reports by Manter (1947), Sogandares-Bernal (1959), Nahhas and Cable (1964), Rees (1970), Fischthal (1977), Centeno and Bashirullah (2003), and Bashirullah and Díaz (2015). The hosts listed from those reports (various grunt species) are consistent with known hosts for accurately identified specimens of *G. ampullacea*; however, without any additional information, the reports of the species unverified without vouchered specimens should be questioned. Overstreet (1969) noted some slight differences between his specimens of *G. ampullacea* and those by earlier works such as mostly smaller eggs, a distinctly trilobed ovary, and a pyriform oral sucker, but without a more detailed description of his specimens and molecular data, I am not sure if this is a valid report of *G. ampullacea*. Lozano et al. (2001) reported *G. ampullacea* from the Iberian Peninsula (new location) and a similar host species (another grunt), but the few measurements provided, such as body size, pharynx size, and cirrus sac size, are much larger indicating that these specimens are likely not *G. ampullacea*.

Similarly, the specimens of *G. ampullacea* described from Mosquera et al. (2014) had a distinctly trilobed ovary and much larger genital atrium spines suggesting they are likely not *G. ampullacea*.

To summarize, *Genolopa* has been provisionally considered synonymous with other genera (*Lasiotocus, Proctotrema, Parachrisomon, Proctotrematoides, Paraproctotrema,* and *Monorchicestrahelmins*) based on incomplete morphological data regarding terminal genitalia spination available for material. My phylogenetic analysis indicates that *Genolopa* likely represents a distinct lineage from those genera and is closely related to *Postmonorchis*, a genus ironically with which it has not been confused or associated as a close relative due to several distinct morphological differences. Therefore, like Madhavi (2008), I agree with Manter (1942) in believing that the combination of spines in the genital atrium and a bipartite, anteriorly spined terminal organ represent cornerstones for the generic diagnosis of *Genolopa*. I also acknowledge the need for morphologic investigation of the other species of *Genolopa* considered *incertae sedis* and the need for additional molecular data to determine if these features consistently determine species of *Genolopa* or possibly just form a western Atlantic clade and to better clarify interrelationships within the Monorchiidae.

2.5 Supplemental Molecular Data

Postmonorchis orthopristis Hopkins, 1941.

Host: *Haemulon flavolineatum* Desmarest, 1823, French grunt, Haemulidae.

Locality: Upper Matecumbe Key, Florida (24°53′52.36”N, 80°39′33.84”W).

Site: intestine.

Specimens deposited: USNM 1611660, 1611661 (2 vouchers); USNM 1611659 (1 hologenophore).

Sequences deposited: Partial 28S rDNA, 2 replicates, 1 hologenophore, 1 paragenophore, (hologenophore submitted to GenBank: accession number MN984475); ITS2 rDNA, 1 hologenophore (submitted to GenBank: accession number MN984475).

Remarks: My specimens agree well with the description by Hopkins (1941).

Lasiotocus trachinoti Overstreet and Brown, 1970.

Host: *Trachinotus carolinus* Linnaeus, 1766, Florida pompano, Carangidae.

Locality: Jacksonville, Florida (30°01′25.8”N, 81°19′21.9”W).

Sites: intestine, pyloric ceca.

Specimens deposited: USNM 1611664, 1611665, 1611666 (3 vouchers).

Sequences deposited: All paragenophores; partial 28S rDNA, 6 replicates (1 submitted to GenBank accession number MN984478); ITS2 rDNA, 6 replicates (1 submitted to GenBank: accession number MN984478).

Remarks: My specimens agree well with the description by Overstreet and Brown (1970).

Lasiotocus glebulentus Overstreet, 1971.

Host: *Mugil curema* Valenciennes, 1836, white mullet, Mugilidae.

Locality: Beaufort, North Carolina (34°41′03.5”N, 76°31′42.7”W)**.**

Sites: intestine.

Specimens deposited: USNM 1611662, 1611663 (2 vouchers).

Sequences deposited: All paragenophores; partial 28S rDNA, 4 replicates (1 submitted to GenBank: accession number MN984476).

Remarks: My specimens agree well with the description by Overstreet (1971).

***Lasiotocus* sp. unidentified**

Host: *Menidia menidia* Linnaeus, 1766, Atlantic silverside, Atherinopsidae.

Locality: Great Bay Estuary, New Jersey (39°31′11.0”N, 74°21.08.1”W).

Sequences deposited: Partial 28S rDNA, 2 replicates (1 submitted to GenBank: accession number MN984477).

Remarks: My specimens were in a condition too poor for species-level identification.

NOTE: This chapter has already been published: Panyi, AJ, Curran, SS, Overstreet, RM. 2020. Phylogenetic Affinity of *Genolopa* (Digenea: Monorchiidae) with Descriptions of Two New Species. *Diversity* 12(51); doi:10.3390/d12020051.

CHAPTER III - *LASIOTOCUS MINUTUS* (MANTER, 1931) THOMAS, 1959 DOES NOT REPRESENT A COMPLEX OF CRYPTIC SPECIES IN COASTAL FISHES FROM NORTH CAROLINA TO MISSISSIPPI

3.1 Introduction

Various definitions of cryptic species exist in the literature, but for the purposes of this thesis, cryptic species will be defined as groups of species with adults that are morphologically indistinguishable from each other but are genetically distinct (Pérez-Ponce De León and Nadler, 2010; Poulin, 2011; Bray and Cribb, 2015). Examples of cryptic digenean species, defined using the aforementioned definition, exist in the literature, such as in the transversotrematid genus *Transversotrema* Witenberg, 1944 (Hunter and Cribb, 2012). Additionally, Curran et al. (2013b) used molecular techniques to identify 2 cryptic species of *Homalometron* Stafford, 1904, a digenean genus in the Apocreadiidae from the southeastern United States. The cryptic forms occur in 2 separate but relatively close river systems, while *Homalometron armatum* (MacCallum, 1895) Manter, 1947 occurs in the upper Mississippi Basin and Great Lakes System. The authors were not confident enough to name the 2 cryptic forms of *Homalometron* from *H. armatum* using standard morphological techniques (Curran et al., 2013b). Later, Barger and Wellenstein (2015) investigated the identity of the 3 forms in the *Homalometron* species complex using multivariate analyses of morphometric characteristics and determined that the 3 species could in fact be differentiated statistically. The authors were able to find morphologic differences and subsequently named the 2 species that had been called *Homalometron* sp. A and sp. B by Curran et al. (2013b) as *Homalometron currani*

Barger and Wellenstein, 2015 and *Homalometron microlophi* Barger and Wellenstein, 2015, respectively.

There have also been investigations of cryptic species complexes in the Monorchiidae, such as in the genera *Hurleytrematoides* Yamaguti 1953, (McNamara et al., 2014) and *Monorchis* Monticelli 1893(Jousson et al., 2000; Jousson and Bartoli, 2002). McNamara et al. (2014) found cryptic speciation in 7 species of *Hurleytrematoides*, a monorchiid genus occurring throughout the Indo-Pacific, with species in the Chaetodontidae (butterflyfishes) and the Tetraodontidae (pufferfishes) serving as definitive hosts. Jousson et al. (2000) and Jousson and Bartoli (2002) reported a cryptic species complex of *Monorchis parvus* Looss, 1902, consisting of 2 species from distinct hosts, with a lack of detectable morphological distinction between the adults. One form was found in *Diplodus vulgaris* (Sparidae) and *Diplodus sargus* (Sparidae); the second form was found in *Diplodus annularis* (Sparidae). Jousson and Bartoli (2002) investigated *M. monorchis*, another related monorchiid suspected to be a cryptic species complex. One form infected *Spondyliosoma cantharus* (Sparidae) and *Diplodus puntazzo* (Sparidae), and the second form infected *Parablennius gattorugine* (Blenniidae). Sequence data indicated the 2 trematodes were different species, which was subsequently supported by morphological investigations. As a result of both methods, the species found in *P. gattorugine* was described and named as *Monorchis blennii* Jousson and Bartoli, 2002.

The northwestern Atlantic monorchiid fauna may support 1 potential complex involving *Lasiotocus minutus* (Manter, 1931) Thomas, 1959, which has a reported distribution from Massachusetts to Louisiana (Manter, 1931; Stunkard, 1981a; Smedley,

2000). Evidence for this suspected cryptic species complex comprising *L.* cf. *minutus* stems from the assumption that the species has an obligate relationship with its first intermediate host and maintains host specificity for the definitive host.

The intermediate and definitive hosts reported thus far for *L.* cf. *minutus* are all estuarine species. Estuarine species generally display more genetic diversity because their habitat is discontinuous compared with species that live within the open ocean because estuaries oftentimes restrict gene flow among populations (Bilton et al., 2002). For example, bays can act as dispersal barriers for larval stages (Bilodeau et al., 2005; Duvernell et al., 2008). One intermediate host of *L. minutus* of Smedley, 2000 has been reported as *Cyrenoida floridana* (Dall, 1896) in the Gulf of Mexico (Mississippi, Louisiana). Another intermediate host of *L. minutus* of Stunkard, 1981a has been reported as *Gemma gemma* (Totten, 1834) in the Atlantic Ocean (Massachusetts). Both are bivalves in the order Venerida but differ at the family level. *Cyrenoida floridana* is in the Cyrenoididae and *G. gemma* is in the Veneridae. Phylogeographic investigations by Hoos et al. (2010) and Zhang et al. (2014) showed that *G. gemma* has a sharp phylogeographic break between Maryland and New Jersey that splits the bivalve species into a southern and a northern population. Two different intermediate host species and genetically distinct populations within 1 intermediate host species provide evidence for *L.* cf. *minutus* representing a complex of cryptic species.

There are many examples of animals that show either speciation or distinct genetic populations between the Atlantic Ocean and Gulf of Mexico, such as the horseshoe crab, black sea bass, blacktip sharks, and long squids (Avise, 2000; Herke and Foltz, 2002; Wise et al., 2004; Keeney et al., 2005; Soltis et al., 2006). Definitive hosts of

L. minutus have been reported as *Fundulus similis* (Thomas, 1959), *Fundulus grandis* and *Fundulus pulverus* (Smedley, 2000) in the Gulf of Mexico, and *Fundulus majalis, Fundulus heteroclitus,* and *Menidia menidia* (Manter, 1931; Stunkard, 1981a) in the Atlantic Ocean. The definitive host for the type material is *F. majalis* from Beaufort, North Carolina. A transition zone between *F. heteroclitus* and *F. grandis* exists in the Flagler Beach area of eastern Florida, just south of Jacksonville, Florida (Gonzalez et al., 2009). *Fundulus heteroclitus* exists from this transition zone northward up the Atlantic coast all the way to Newfoundland, Canada; *Fundulus grandis* exists from this transition zone southward and into the Gulf of Mexico. *Fundulus heteroclitus* has further differentiation along the Atlantic coast within the species. A transition zone exists in the Hudson Bay area that divides the species into genetically distinct northern and southern populations (Adams et al., 2006). Similarly, *F. grandis* has further differentiation along the Gulf of Mexico coast within the species. The population along the coast of western Florida between Tampa Bay and Mobile Bay is genetically distinct compared with the populations from the northwestern Gulf of Mexico (Williams et al., 2008).

Fundulus similis and *F. majalis* are distinct species of killifishes from distinct geographic areas, but a transition zone between these 2 species exists in northeastern Florida where the coastal salt marsh transitions from a *Juncus* - *Spartina* marsh to a mangrove dominated marsh (Duggins Jr. et al., 1995). *Menidia menidia* is another reported definitive host from the Atlantic Ocean, and *M. menidia* occupies 3 distinct phylogeographic regions (Mach et al., 2011). Mach et al. (2011) defined these regions as Florida to Massachusetts, Massachusetts to the Gulf of Maine, and the Gulf of Maine to

the Gulf of St. Lawrence, Canada. All the aforementioned definitive host species exist within the reported range of *L. minutus* (Manter, 1931; Smedley, 2000).

Reliance on numerous definitive host species with genetically distinct populations herein drives my evidence for the hypothesis that *L.* cf. *minutus* represents a complex of cryptic species; therefore, this study utilizes novel molecular sequence data in conjunction with modern conventional morphological techniques to assess specimens of *L.* cf. *minutus* from various geographic locations and hosts throughout the reported distribution range to determine if *L. minutus* constitutes a cryptic species complex. Additionally, molecular sequence data of *L. minutus* are provided for the first time.

3.2 Materials and Methods

3.2.1 Specimen Collection and Morphological Analysis

Various hosts (listed in the taxonomic summaries sections with specific localities) were sampled using cast nesting and minnow trapping from areas in North Carolina (August 2018), and Mississippi (January, May 2018). All specimen collection and preservation methods and terminology followed those described from Panyi et al. (2020).

Morphological comparisons of basic trematode structures, such as the oral sucker, ventral sucker, testis, ovary, uterus, eggs, ceca, body size, and vitelline follicles, among others (Jousson and Bartoli, 2002), in addition to the important monorchiid characteristics discussed previously in the morphology subsection of the introduction chapter, such as features of the terminal genitalia (Manter, 1931; Thomas, 1959; Overstreet, 1969; Overstreet and Brown, 1970; Fischthal, 1977; Madhavi, 2008) were conducted to determine if differences were present among specimens from various geographic locations and hosts. Measurements are provided as ranges in micrometers

(µm). The type series for *L. minutus* was borrowed from USNM (1321175) for comparison with the present material. The museum accession number is provided in parentheses.

3.2.2 Morphometric Analysis

Principle components analysis (PCA) and discriminant function analysis (DFA) were conducted on normalized data using PAST (Hammer et al., 2001) to determine if adult specimens of *L.* cf *minutus* from various locations and hosts in this study could be differentiated using morphometric data. The adult morphological features used in these morphometric analyses were total body length, body width (at maximum width), forebody, oral sucker length, oral sucker width, ventral sucker length, ventral sucker width, pharynx length, pharynx width, testis length, testis width, seminal vesicle length, seminal vesicle width, cirrus length, cirrus width, ovary length, ovary width, terminal organ length, cirrus sac length, cirrus sac width, post-testicular space length, and uterus distance into post-testicular space (all measurements in micrometers [µm]), following most of the features used in another investigation of a cryptic species complex of a monorchiid (Jousson et al., 2002). The following settings in PAST were applied to the analyses: matrix = ‘correlation,’ missing values = ‘iterative imputation,’ bootstrap n = ‘1,000.’ The ‘correlation’ setting indicates the data were normalized by dividing each variable by its respective standard deviation; any missing data were estimated using the recommended ‘iterative imputation’ setting (Ilin and Raiko, 2010); PCAs were bootstrap replicated (n = 1,000). Subsequently, DFAs were conducted on the same data sets to determine which morphological feature(s), if any, contributed most to variation among specimens collected from various hosts and locations and to determine if specimens from

the various hosts and locations could be correctly classified based solely on morphometric data.

3.2.3 Molecular Sequencing

All molecular sequencing methods followed those described from Panyi et al. (2020). Sequencing of the ITS2 rDNA region was successful for specimens from 1 geographic location and host only. Consequently, the present phylogenetic analysis is based on sequence data from the partial 28S rDNA region. The 5′ end of the partial 28S rDNA region was determined by annotation in the ITS2 Database using the 'Metazoa' model (Keller et al., 2009; Ankenbrand et al., 2015). Successfully generated sequence regions will be provided to GenBank. Although the ITS2 sequence was not used for phylogenetic analysis in this study, it will be made publicly available for use in future works.

3.2.4 Pairwise Comparison of 28S rDNA Region

Contiguous sequences were assembled using Sequencher™ version 5.0 (GeneCodes Corp., Ann Arbor, Michigan). Sequences of *L. minutus* from the different geographic locations and hosts were aligned and masked with the GUIDANCE2 web-server (http://guidance.tau.ac.il) (Landan and Graur, 2008; Sela et al., 2015) using the MAFFT alignment algorithm, 100 bootstrap repeats, 1,000 cycles of iterative refinement, and the *localpair* algorithm. Alignment (column) positions with confidence scores <0.4 were excluded from the subsequent pairwise comparison. The alignment was then trimmed on both ends to the shortest sequence and edited by eye in BioEdit (version 7.2.5) (Hall, 1999). A pairwise comparison was then conducted to determine if the sequences contained any base pair differences.

3.2.5 Phylogenetic Analysis of 28S rDNA Region

The alignment of newly generated geographical strains of *L.* cf. *minutus* was combined with available partial 28S rDNA sequences of some monorchiids and related species in GenBank (listed in Table 2.1) and those generated from the prior chapter of this thesis. Sequences were aligned and masked with the GUIDANCE2 web-server (http://guidance.tau.ac.il) (Landan and Graur, 2008; Sela et al., 2015) using the MAFFT alignment algorithm, 100 bootstrap repeats, 1,000 cycles of iterative refinement, and the *localpair* algorithm. Alignment (column) positions with confidence scores <0.4 were excluded from subsequent BI analysis. The alignment was untrimmed and edited by eye in BioEdit (version 7.2.5) (Hall, 1999). Nucleotides present in the alignment before the start of the 5' end of the 28S rDNA region were excluded from the phylogenetic analysis. Phylogenetic analysis was conducted using BI with MrBayes 3.2.7 software (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). The best nucleotide substitution model was estimated with jModeltest version 2.1.10 (Darriba et al., 2012) and both the Akaike information criterion (AIC) and Bayesian information criterion (BIC) predicted the GTR + I + Γ model as the best estimator. Therefore, the BI analysis was conducted using the closest approximation to this model. The BI analysis was performed using the following model parameters: "nst = 6," "rates = invgamma," "ngen = 5,000,000," "samplefreq = 500," "printfreq = 500," and "diagnfreq = 5,000." The values of the samples of the substitution model parameters were summarized using "sump." Tree and branch lengths were summarized using "sumt." The first 25% of trees were discarded using the following settings: "relburnin = yes," "burninfrac = 0.25." Nodal support was estimated by posterior probabilities. All other settings were left as default values. Two

species in the Lepocreadiidae and 1 species in the Lissorchiidae were included in the alignment, with *Bianium arabicum* Sey, 1996 serving as the outgroup for the analysis (Wee et al., 2018, 2019; Panyi et al., 2020; Wee et al., 2020). FigTree version 1.4.3 (Rambaut and Drummon, 2012) was used to visualize the phylogeny and Adobe® Photoshop® CS6 (Adobe Inc., San Jose, California) was used for subsequent editing.

3.3 Results

3.3.1 Morphological

Monorchiidae Odhner, 1911

Lasiotocus Looss in Odhner, 1911

3.3.1.1 *Lasiotocus minutus* (Manter, 1931) Thomas, 1959

3.3.1.1.1 Taxonomic Summary

Type host: *Fundulus majalis* (Walbaum, 1792), striped killifish, Fundulidae.

Type locality: Beaufort, North Carolina.

Specimen examined: USNM 1321175 (holotype).

3.3.1.1.2 Redescription of *Lasiotocus minutus* holotype (USNM 1321175)

Body small, slightly elongate to oval, widest in middle third of body, 696 long, 280 wide. Tegument spinose; spines denser anteriorly, 2 to 3 long, 2 to 3 wide at base, somewhat rounded at ends. Eyespot pigment absent. Oral sucker simple, subterminal, subglobular, wider than long, 77 long, 95 wide. Ventral sucker subspherical, in anterior third of body, wider than long, 68 long, 73 wide. Oral sucker to ventral sucker width ratio 1:0.77. Forebody 109 or 16% of body length. Hindbody 509 or 73% of body length. Pharynx spherical, 35 long, 37 wide; prepharynx very short, 14 long. Esophagus very

short, 11 long. Ceca extending well into hindbody, terminating 158 from posterior end or 23% of body length.

Testis single, elongate, median, slightly diagonal orientation, 184 long, 93 wide. Post-testicular space 118 long or 17% of body length. Cirrus sac elongate, sinistral, dorsal to ventral sucker, opening distally into genital atrium, following sinuous path to reach genital atrium, terminating at ovarian level, 226 long or 32% of body length, 63 wide (contents consisting of internal seminal vesicle, pars prostatica, and cirrus); cirrus narrow, elongate, 145 long, 19 wide, spines not observed**;** seminal vesicle unipartite, slightly elongate, in posterior region of cirrus sac, 86 long or 38% of cirrus sac length, 54 wide. Genital atrium unspined; genital pore slightly sinistral, opening immediately anterior to ventral sucker.

Ovary subglobular, not distinctly lobed, submedian, dextral, ventral to and slightly overlapping anterior margin of testis, 88 long, 86 wide. Terminal organ elongate, narrowing towards anterior end, bipartite, sinistral to cirrus sac, 136 long, 51 wide; posterior region vesicular, unspined, blind; anterior portion opening into genital atrium, spines not observed. Mehlis' gland median, anterior to testis, sinistral to ovary (mostly obscured). Seminal receptacle uterine. Laurer's canal not observed. Vitellarium comprising 2 lateral, non-follicular masses at level of seminal vesicle, 120 to 130 long, 55 to 69 wide. Uterus voluminous, both intercecal and extracecal, occupying entire post ovarian region of body, not entering forebody, extending to posterior end, overlapping gonads ventrally; post-testicular uterus occupying all of post-testicular space, 17% of body length. Eggs operculate, non-filamented, tanned, 18 to 21 long, 8 to 10 wide when distal.

Excretory vesicle I-shaped, obscured by voluminous egg-filled uterine area, with anterior extent not observed; excretory pore terminal.

3.3.1.1.3 Remarks

Observation of the holotype revealed that the specimen was mounted under extreme pressure. Fixation under pressure can result in distortion of the relative location of features, such as shifting the location of the genital pore more laterally, and the overestimation of sizes for some features, such as enlarging the oral sucker by 2 to 3 times its normal size (Panyi et al., 2020). Additionally, spines are not visible on the cirrus or in the terminal organ despite being reported in the original description of the specimen (Manter, 1931). Manter (1931) did not state his fixation methods for specimens in that paper, but as was common during that time period, he likely used AFA or some other acid during the fixation process, which creates slightly acidic conditions in the mounting medium that can lead to the degradation of hard structures over time. However, the tegumental spines on the type specimen are still present but appear to be rounded on the ends, which could be indicative of degradation. As a result of the artifact introduced to the morphological data of the type, the specimens of *L. minutus* collected from various locations and hosts in this study are not able to be directly compared with the morphological data from the type specimen.

3.3.1.1.4 Taxonomic summaries for specimens collected in this study

Host (present study): *Fundulus heteroclitus* (Linnaeus, 1766), mummichog, Fundulidae.

Locality: Beaufort, North Carolina (34°44’8.8044”N, 76°31’44.3994”W).

Site: intestine.

Specimens deposited: x vouchers: USNM XXX, XXX.

Sequences deposited: Partial 28S rDNA, 1 sequence, 2 hologenophores (1 submitted to GenBank, accession number: XXX); ITS2 rDNA, 2 identical replicates, 1 paragenophore, 1 hologenophore (1 submitted to GenBank, accession number: XXX).

Supplemental morphological data: based on 9 gravid, adult specimens, mounted without pressure.

Host (present study): *Fundulus grandis* (Baird and Girard, 1853), Gulf killifish, Fundulidae.

Locality: Fort Bayou, Ocean Springs, Mississippi (30°25’09.2”N, 88°49’39”W).

Site: intestine.

Specimens deposited: x vouchers: USNM XXXX, XXXX.

Sequences deposited: Partial 28S rDNA, 3 identical replicates, 3 paragenophores, 1 hologenophore (1 submitted to GenBank, accession number: XX).

Supplemental morphological data: based on 9 gravid, adult specimens, mounted without pressure.

Host (present study): *Fundulus similis* (Baird and Girard, 1853), longnose killifish, Fundulidae.

Locality: Weeks Bayou, Ocean Springs, Mississippi (30°23’53.9”N, 88°48’58.4”W).

Site: intestine.

Specimens deposited: x vouchers: USNM XXX, XXX.

Sequences deposited: Partial 28S rDNA, 2 identical replicates, 2 paragenophores (1 submitted to GenBank, accession number: XXX).

Supplemental morphological data: based on 5 gravid, adult specimens, mounted without pressure.

3.3.1.1.5 Remarks

Specimens of *L. minutus* from the 2 locations and 3 definitive hosts will be referred to in their separate groups as follows: *L. minutus* from *F. grandis* (MS), *L. minutus* from *F. similis* (MS), and *L. minutus* from *F. heteroclitus* (NC) throughout to avoid confusion.

No major, obvious morphological difference was seen when comparing specimens of *L. minutus* from the various hosts and locations. Upon closer examination of the morphological data, ovarian size of *L. minutus* from *F. heteroclitus* (NC) was generally smaller (43 to 79 long, 26 to 102 wide) compared with *L. minutus* from *F. similis* (MS) (53 to 103 long, 43 to 63 wide) and *L. minutus* from *F. grandis* (MS) (60 to 108 long, 63 to 115 wide). However, the ranges still overlapped. The cirrus sac width of *L. minutus* from *F. grandis* (MS) (40 to 67 wide) was slightly larger than the other 2 groups (30 to 45 wide and 20 to 37 wide), but there was still overlap in the ranges of measurements. The seminal vesicle width was slightly larger in *L. minutus* from *F. grandis* (MS) (31 to 58 wide), compared with the other 2 groups (26 to 35 wide and 15 to 34 wide), but there was still slight overlap among the ranges. Additionally, the extent of the uterus into the post-testicular space was less in *L. minutus* from *F. similis* (MS) (44% to 58% or 11% to 15% of body length) compared with the other groups (58% to 96% or 13% to 31% of body length and 72% to 100% or 19% to 26% of body length. The slight

differences in measurements of these features alone are not sufficient for establishing species differences.

3.3.2 Morphometric Results

Two PCAs were conducted on specimens of *L. minutus* from this study to determine if specimens could be differentiated morphometrically. One PCA investigated *L. minutus* and host species, *F. majalis* (NC) vs *F. similis* (MS) vs *F. heteroclitus* (NC) vs *F. grandis* (MS); the other investigated *L. minutus* and location, Gulf of Mexico (MS) vs western Atlantic Ocean (NC). In the PCA investigating *L. minutus* and host species, the type specimen of *L. minutus*, which was collected from *F. majalis* from NC, is represented by the red triangle (Figure 3.1). The type specimen was separated greatly from the rest of the individuals of *L. minutus*, which is most likely a result of the distortion of some features due to mounting under extreme pressure; therefore, it was excluded from the PCA.

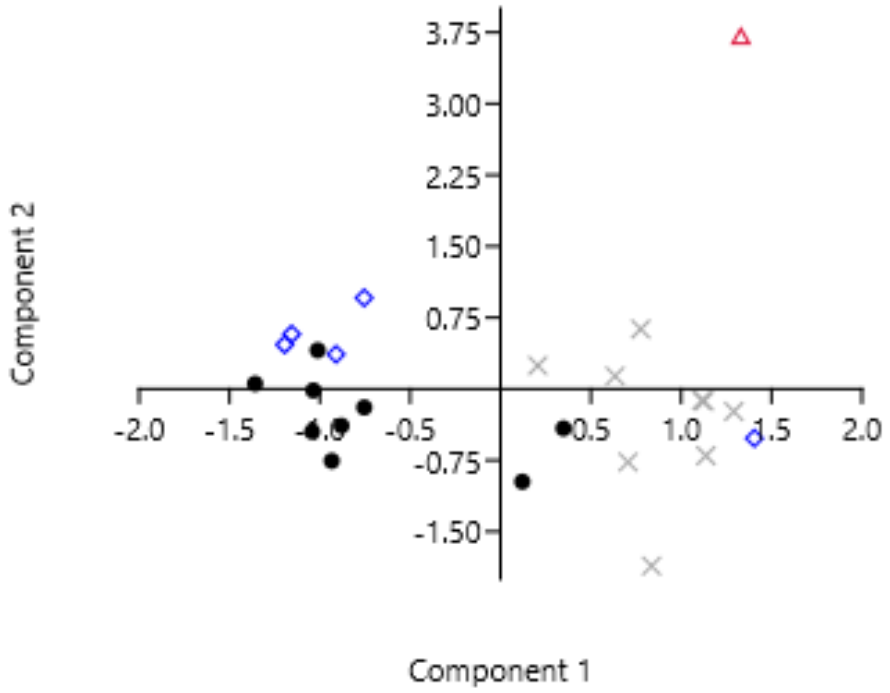

Figure 3.1 *Scatterplot based on the PCA of normalized morphometric data of individuals of* Lasiotocus minutus *from various hosts.*

Fundulus majalis (red triangle, museum specimen), *Fundulus similis* (blue diamond), *Fundulus grandis* (gray x's), and *Fundulus heteroclitus* (black dots).

Excluding the type specimen from *F. majalis*, the PCA explained 78.1% of the variance in the data set (65.7% by component 1, 12.4% by component 2) and resulted in a scatterplot without distinct separation among the specimens of *L. minutus* from the 3 different hosts (Figure 3.2). Overlapping of the 95% confidence interval ellipses can be seen among specimens from all hosts.

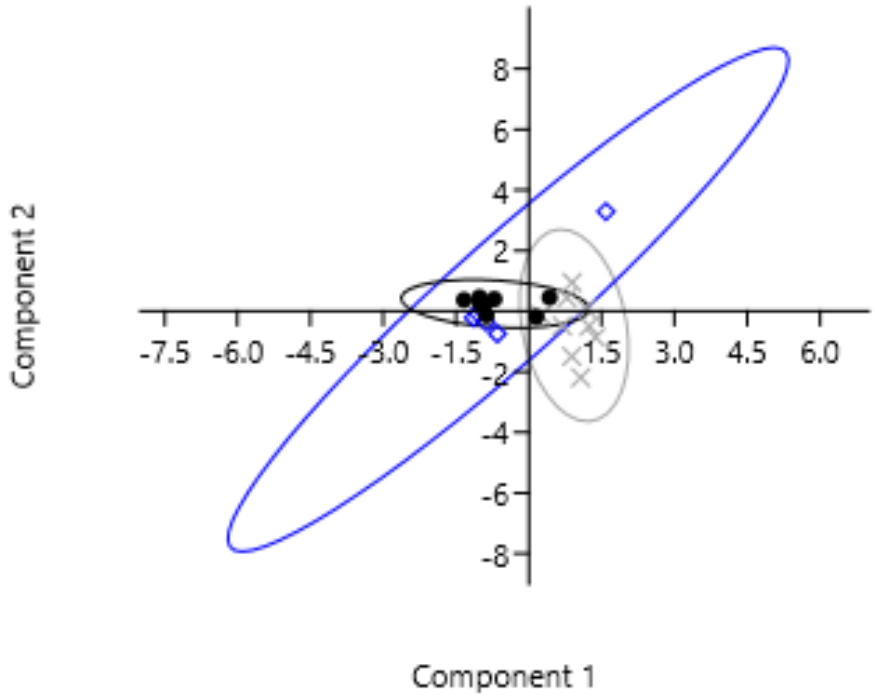

Figure 3.2 *Scatterplot based on the PCA of normalized morphometric data of individuals of* Lasiotocus minutus *from various hosts, not including the type specimen.*

Fundulus similis (blue diamond), *Fundulus grandis* (gray x's), and *Fundulus heteroclitus* (black dots). The ellipses drawn represent 95% confidence intervals to show potential overlap of the groups.

In the PCA investigating *L. minutus* and location, the same separation of the type specimen from other individuals was seen, so it was excluded from this analysis as well. After exclusion, the PCA explained 78.1% of the variance (65.7% in component 1, 12.4% in component 2) and resulted in a scatterplot without distinct separation between the specimens of *L. minutus* from the 2 locations (Figure 3.3). Overlapping of the 95% confidence interval ellipses can be seen between all the specimens from both locations. The DFA investigating *L. minutus* and host indicated the oral sucker width (+3.33), cirrus sac width (-2.35), and cirrus width (-2.09) contributed most to variation among groups. The DFA conducted on *L. minutus* and location showed that seminal vesicle width

(+5.50), cirrus width (-4.83), and cirrus sac width (-4.59) contributed most to variation between the groups. However, the DFAs investigating both *L. minutus* and location and host were unable to correctly classify any of the individuals.

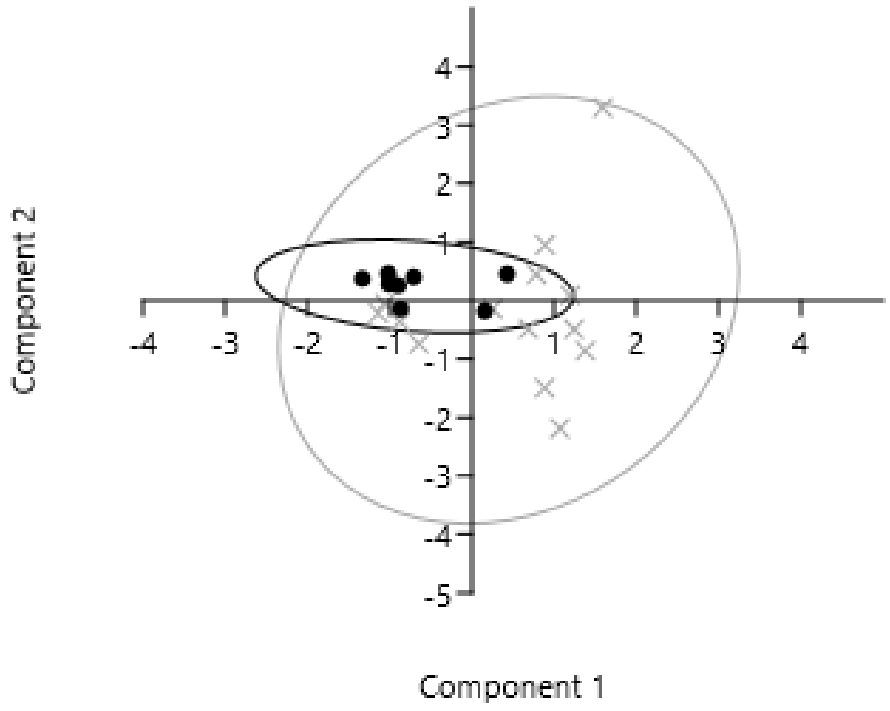

Figure 3.3 *Scatterplot based on the PCA of normalized morphometric data of individuals of* Lasiotocus minutus *from different locations, not including the type specimen.*

North Carolina (black dots) and Mississippi (gray x's). The ellipses drawn represent 95% confidence intervals to show potential overlap of the groups.

3.3.3 Molecular Results

3.3.3.1 Pairwise Comparison of 28S rDNA Region

Pairwise comparisons of variable sites of the partial 28S rDNA region were conducted among specimens of *L.* cf. *minutus* from definitive hosts in North Carolina (*F. heteroclitus*) and Mississippi (*F. grandis* and *F. similis*). The trimmed multiple sequence alignment (trimmed to the shortest sequence on both ends) of the 3 isolates consisted of

1330 base pairs, without any gaps. Masking revealed no ambiguous column, i.e., columns with confidence scores below the cut off value of 0.4, so no column was excluded from the pairwise comparisons. No site variation was found among the 3 isolates of *L.* cf. *minutus* from the various geographic locations and hosts.

3.3.3.2 Phylogenetic Analysis of 28S rDNA Region

The partial 28S rDNA fragments from the 3 *L.* cf. *minutus* isolates were aligned with the lepocreadiid, lissorchiid, and monorchiid sequences from this study and consisted of 1383 base pairs, including gaps. Masking revealed no ambiguous column, i.e. columns with confidence scores below the cut off value of 0.4, so no column was excluded in the phylogenetic analysis as a result of masking. BI analysis resulted in a recovered phylogeny (Figure 3.4) that is consistent with previously reported monorchiid phylogenies (Wee et al., 2019; Panyi et al., 2020; Wee et al., 2020).

The recovered phylogeny supports that specimens of *L.* cf. *minutus* from the 3 different groups of hosts and locations are the same at the species level and are sister to an unidentified species of *Lasiotocus* collected from *M. menidia* in New Jersey. Sequences of the partial 28S rDNA region of *L. minutus* and *Lasiotocus* sp. differed by 4.2% (56 bp, including gaps, 1351 total bp length). The well-supported clade of *L. minutus* and *Lasiotocus* sp. is sister to a well-supported clade consisting of 2 additional

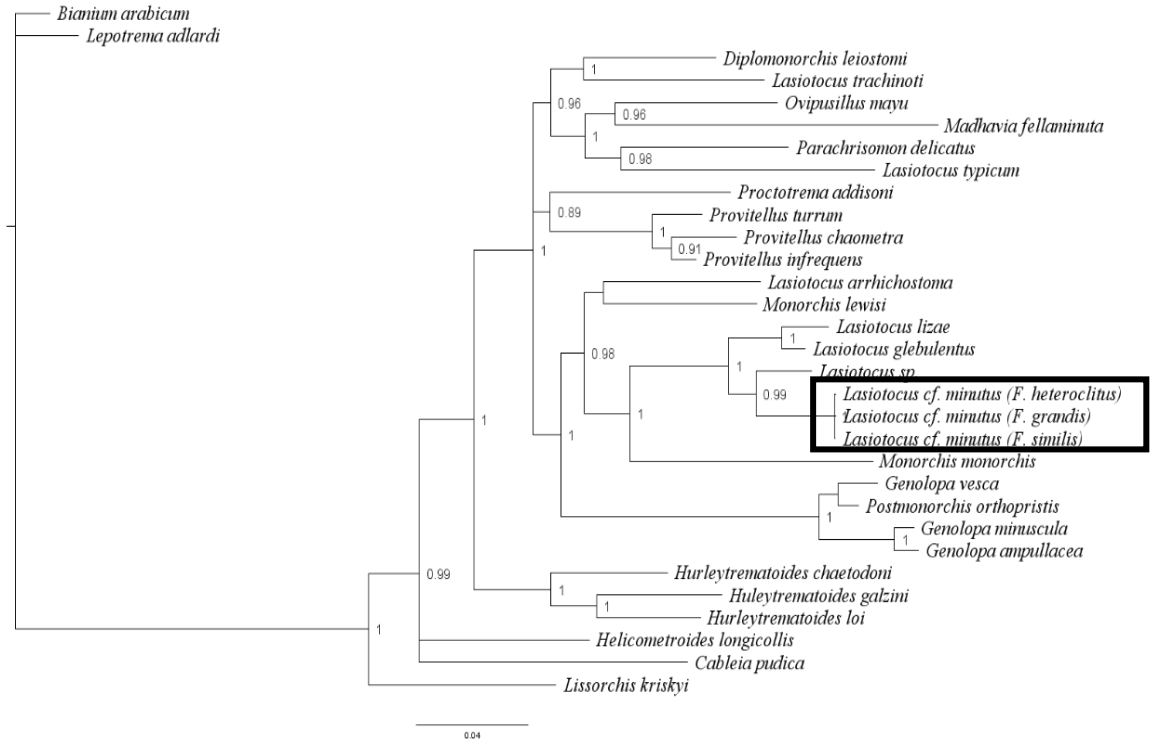

Figure 3.4 *Interrelationships among members of the Monorchiidae based on Bayesian inference analysis of partial 28S rDNA data.*

Bayesian inference posterior probabilities are shown at the nodes; support values <0.85 are not shown. Lasiotocus cf. minutus clade in black box with respective host species listed in parentheses.

species of *Lasiotocus*, *L. lizae* and *L. glebulentus*, which both parasitize mugilid fishes. Sequences of the partial 28S rDNA region of *L. minutus* and *L. glebulentus* differed by 5.2% (73 bp, including gaps, 1397 total bp fragment). All 4 of these species of *Lasiotocus* form a well-supported clade sister to *M. monorchis* collected from a sparid fish host. Sequences of the partial 28S rDNA region of *L. minutus* and *M. monorchis* differed by 11.4% (143 bp, including gaps, 1257 total bp length).

3.4 Discussion

The data presented herein do not support *L. minutus* as a complex of cryptic species. However, there are 2 major limitations of the dataset. First, only the partial 28S

region of the rDNA gene was successfully sequenced for specimens from all locations and hosts. Additional molecular data of the ITS1, ITS2, 18S, and cox1 regions could reveal genetic differences not seen in the more conserved partial 28S rDNA region. The ITS2 region of the rDNA gene is generally more variable than the 28S region, and mitochondrial DNA (mtDNA) is generally more variable than rDNA. It is important to note that the primers used in this study were not reliable for rDNA regions other than the partial 28S region. Sequences for the ITS2 region were only obtained for *L. minutus* from *F. heteroclitus* in North Carolina. Therefore, future works should consider using other available digenean primers to obtain the other targeted regions of the rDNA gene.

Second, only adult specimens of *L.* cf. *minutus* were obtained in this study. Successful collection and subsequent morphological and molecular investigation of earlier life stages from the intermediate hosts could reveal different life cycles throughout the various locations. *Gemma gemma*, a reported intermediate host from the Atlantic Ocean (Stunkard 1981a), was collected from Massachusetts, but no monorchiid cercaria nor metacercaria was found. Similarly, *C. floridana*, a reported intermediate host from the Gulf of Mexico, was collected on numerous occasions from locations reported by Smedley (2000), but no monorchiid metacercaria was found. Future works should target and prioritize collecting earlier stages in the life cycle of *L. minutus* to elucidate any differences in the life cycles between locations.

The morphometric analyses in this study did not include the type museum specimen of *L. minutus* of Manter (1931) collected from *F. majalis* in North Carolina because of the fixation methods used. Manter (1931) fixed his specimens under extreme cover slip pressure and very likely used acid for fixation. The use of acids in fixation can

damage/erode hard structures such as spines associated with various organs, which can be important diagnostic features in monorchiids. Also, fixation of specimens under cover slip pressure distorts soft tissue features such as body width, oral and ventral sucker size, cirrus sac size, and terminal organ size (Panyi et al., 2020). The PCA of *L. minutus* and hosts in Figure 3.1 clearly demonstrates the distortion of features that can occur from fixing a specimen under extreme compression because the red triangle mark representing the holotype was distinctly separate from the cluster of other individuals of *L. minutus*.

The DFAs indicated the features contributing the most to the variation among groups were seminal vesicle width (+5.50), cirrus width (-4.83), cirrus sac width (-4.59), oral sucker width (+3.33), cirrus sac width (-2.35), and cirrus width (-2.09). Close examination of the morphological data indicated slight differences between groups for ovarian size, seminal vesicle width, cirrus sac width, and the extent of the uterus into the post-testicular space. Both methods supported seminal vesicle width and cirrus sac width as features contributing to the variation among groups. However, the raw morphological data have ranges that overlap, and the features contributing to the most variation in the DFAs have relatively low loadings values compared with other studies that have incorporated similar analyses (Miller et al., 2010). Perhaps more importantly, the DFAs were unable to correctly classify the specimens into the correct group, providing more evidence that the morphometric data do not suggest distinct morphological species.

Regardless of the cryptic species status of *L. minutus*, the updated monorchiid phylogeny recovered in this study (Figure 3.4), now including *L. minutus*, further supports that *Lasiotocus* is a polyphyletic group, as seen in prior studies (Wee et al., 2018, 2019; Panyi et al., 2020; Wee et al., 2020). *Lasiotocus minutus* forms a well-supported,

monophyletic clade with some members of *Lasiotocus*: *Lasiotocus* sp. (collected from *Menidia menidia*) (sister taxon), *Lasiotocus glebulentus*, and *Lasiotocus lizae*. All the definitive fish hosts in this clade are euryhaline species, commonly associated with brackish, saltmarsh and/or estuarine habitats. *Lasiotocus glebulentus* and *L. lizae* are reported from mugilid species; *Lasiotocus* sp. is reported from an atherinopsid species; all specimens of *L. minutus* from this study are from fundulid species. The parasite - definitive host association in this group is most likely indicative of an ecological association. The fishes share a similar habitat type and exhibit omnivorous or detritivorous feeding behavior, putting them into contact with the likely bivalve intermediate hosts of these species of *Lasiotocus*.

To summarize, *L. minutus* has been hypothesized to be a complex of at least 2 cryptic species because of its vast distribution range and its various intermediate and definitive host species that can be further differentiated into distinct populations of those hosts. The presented morphological, morphometric, and phylogenetic data provide evidence that *L. minutus* does not represent a complex of cryptic species, but the data are incomplete to conclusively determine the cryptic species status of *L. minutus*. More morphological data are necessary from more individuals of *L. minutus* from additional locations and hosts (primarily the intermediate hosts) to get a better understanding of the potential variation of features. Additionally, more molecular data are needed to investigate if other rDNA or mtDNA gene regions are also identical or if they show variation in nucleotides that could be indicative of cryptic speciation.

CHAPTER IV – PHYLOGENETIC AFFINITY OF MONORCHIID TREMATODES FROM THE NORTHWESTERN ATLANTIC OCEAN WITH DESCRIPTIONS OF FOUR NEW SPECIES OF *LASIOTOCUS*

4.1 Introduction

Lasiotocus Looss in Odhner, 1911 is the most specious genus in the Monorchiidae with 49 nominal species and has a very intriguing history. The genus was essentially erected as a satirical, sarcastic jest at what Looss perceived as the inadequate rules governing zoological nomenclature at the time. Looss complained that he could simply state *Diplostomum mulli* Stossich, 1883 is the type-species for a new genus, choose the name *Lasiotocus*, without any formal generic diagnosis or illustration, and generic establishment was valid. As a result, many taxonomists have debated the rightful authority for *Lasiotocus* with both Looss (1907) and Odhner (1911) each receiving contrasting support for authorship. The current consensus is the original albeit "sarcastic" erection by Looss (1907) assigned a type-species for *Lasiotocus* but the action was not intended to erect the genus, and therefore, Looss (1907) is not accepted as the authority, despite Looss (1907) following the International Code of Zoological Nomenclature (ICZN) rules at the time as per Article 12 in regard to names published before 1931 (Dollfus, 1948; Manter and Pritchard, 1961; Bartoli and Prévot, 1966; Madhavi, 2008). Odhner (1911), anticipating a later work by Looss that would investigate the taxonomy of *L. mulli*, did not include a full description with illustrations. However, he included a limited diagnosis for *Lasiotocus* that provided several insights that indicated *L. mulli* was closely related to *Proctotrema bacillovatum* Odhner, 1911 and that the 2 genera were closely related within the same subfamily and family. The first full, detailed description

of *L. mulli* that included illustrations was completed by Dollfus (1948). Therefore, *Lasiotocus* Looss in Odhner, 1911 is the authority for the genus, and *Lasiotocus mulli* (Stossich, 1883), Looss in Odhner, 1911 is the consensus accepted authority for the type-species, originally collected from the red mullet (*Mullus barbatus* Linnaeus, 1758) in the Adriatic Sea.

Like the confused taxonomic history exhibited with species in *Genolopa* Linton, 1910, (highlighted in Chapter II), confusion and controversy have enshrouded the classification of species of *Lasiotocus*. Species of *Lasiotocus* have been variously moved into and out of several other genera that share morphologically similar traits. These genera include: *Genolopa*, *Monorchicestrahelmins*, *Parachrisomon*, and *Proctotrema*. Morphological differences among the genera are based on variation of the configuration of the terminal organ shape (unipartite vs. bipartite), terminal organ spination, genital atrium spination, and vitellarium shape and extent. Some of these now generic features were overlooked or undescribed in original descriptions and discovered upon reexamination of type material, e.g. *Proctotrema* (Bartoli and Prévot, 1966; Madhavi and Bray, 2018); other features were not originally considered as generic level features but became so later, e.g. *Parachrisomon* (Madhavi, 2008). The primary features that differentiate species of *Lasiotocus* from these other similar genera were summarized by Madhavi (2008) and consist of presence of an unspined genital atrium, bipartite terminal organ with spines in the anterior portion, and follicular vitellarium distributed in the middle of the body between the ventral sucker and gonadal zone. Species of *Genolopa* differ by having a spined genital atrium; species of *Proctotrema* have a unipartite terminal organ that is entirely spined; species of *Parachrisomon* have a vitellarium

composed of tubular acini that extend well into the hindbody, and species of *Monorchicestrahelmins* have a spined genital atrium and a unipartite, spined terminal organ (Madhavi, 2008; Madhavi and Bray, 2018).

Despite being the most speciose genus in the family, there are only 6 species of *Lasiotocus* with publicly available gene sequence data, none of which is from the type-species (Olson et al., 2003; Searle et al., 2014; Atopkin et al., 2017; Panyi et al., 2020). A great deal of morphological variation also exists among the nominal species of *Lasiotocus* (Manter, 1931; Thomas, 1959; Overstreet, 1971; Bartoli and Bray, 2004). Therefore, there is a need for broadening the molecular gene sequence data to represent more species in the genus, which will also address broader problems related to classifications for the entire family. Presently, most of the meager molecular data has been obtained from species from the Indo-Pacific Ocean. The data from this study will integrate new sequence data from representative species of *Lasiotocus* and other genera from the northwestern Atlantic Ocean into the worldwide database, which will better inform the informative morphological features for the group(s), add to the knowledge of monorchiids from the Atlantic Ocean, and allow us to obtain a better understanding of this cosmopolitan family.

This study describes and provides novel molecular data from 4 new species of monorchiids, placing them into the genus *Lasiotocus* based on the current generic diagnosis (Madhavi, 2008; Madhavi and Bray, 2018). The study also provides molecular data for *Diplomonorchis leiostomi* Hopkins, 1941, collected from the spot croaker (*Leiostomus xanthurus* Lacepède, 1802) in Morehead City, North Carolina, and for

Lasiotocus truncatus (Linton, 1910) Thomas, 1959, collected from the blue striped grunt (*Haemulon sciurus* Shaw, 1803) in the Florida Keys.

4.2 Materials and Methods

4.2.1 Specimen Collection and Morphological Analysis

Various hosts (listed in the taxonomic summaries sections with specific localities) were sampled using baited minnow trap, baited hook and line, or cast nest from areas in Florida (2010, 2017), Georgia (2007), Massachusetts (2017), and North Carolina (2018). All specimen collection and preservation methods and terminology followed those described from Panyi et al. (2020). The type series for several species of *Lasiotocus* were borrowed from USNM for comparisons with the present material: *Lasiotocus beauforti* (Manter, 1931) Thomas, 1959 (USNM 1337480), *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (USNM 1321176), *Lasiotocus mugilis* Overstreet, 1969 (USNM 1366949, 1366888), *Lasiotocus trachinoti* Overstreet, 1970 (USNM 1366395, 1366394), and *Lasiotocus truncatus* (Linton, 1910) Thomas, 1959 (USNM 1321279, 1321297, 1321277) (Salley et al., 1978). Data in parentheses refer to the museum (USNM) accession numbers.

4.2.2 Molecular Sequencing

All molecular sequencing methods followed those described from Panyi et al. (2020). Due to insufficient forward primers, sequencing of the ITS2 rDNA region was limited to only some of the species collected in this study. Consequently, the present phylogenetic analysis is based on sequence data from the partial 28S rDNA region. Although the ITS2 rDNA sequences were not used for phylogenetic analysis in this

study, they were used for pairwise comparisons and will be made publicly available for use in future works.

4.2.3 Pairwise Comparison of ITS2 and 28S rDNA Regions

Contiguous sequences were assembled using Sequencher™ version 5.0 (GeneCodes Corp., Ann Arbor, Michigan, USA). Sequences for pairwise comparisons were aligned and masked with the GUIDANCE2 web-server (http://guidance.tau.ac.il) (Landan and Graur, 2008; Sela et al., 2015) using the MAFFT alignment algorithm, 100 bootstrap repeats, 1,000 cycles of iterative refinement, and the *genafpair* algorithm for the ITS2 rDNA region and the *localpair* algorithm for the partial 28S rDNA region. The ITS2 rDNA alignment was then trimmed on both ends to the shortest sequence, excluding that for *L. lizae* because it had a much shorter sequence at the 3' end than the other species, and it was edited by eye using BioEdit (version 7.2.5) (Hall, 1999). The partial 28S rDNA alignment was trimmed to the shortest sequence and edited by eye using BioEdit (version 7.2.5) (Hall, 1999). Pairwise comparisons were then conducted, which entailed comparing aligned ITS2 sequences and partial 28S rDNA fragments in separate alignments and searching for base differences at particular sites.

4.2.4 Phylogenetic Analysis of the partial 28S rDNA region

The newly generated partial 28S rDNA sequence fragments derived from 4 new species of *Lasiotocus,* 1 newly generated sequence of *D. leiostomi,* and 1 newly generated sequence of *L. truncatus* were combined with available partial 28S rDNA sequences of some monorchiids and related species in GenBank (listed in Table 4.1). Sequences were aligned and masked with the GUIDANCE2 web-server (http://guidance.tau.ac.il) (Landan and Graur, 2008; Sela et al., 2015) using the MAFFT

alignment algorithm, 100 bootstrap repeats, 1,000 cycles of iterative refinement, and the *localpair* algorithm. Alignment (column) positions with confidence scores <0.4 were excluded from subsequent Bayesian inference (BI) analysis (Andres et al., 2018). Two alignments were created because some of the publicly available monorchiid sequences are relatively short, so I wanted to test if trimming the alignments to these short sequences had an impact on the phylograms. One alignment was trimmed to the partial 28S rDNA sequence of *Monorchis monorchis* (Stossich, 1890), Monticelli, 1893 and included a few species with shorter sequences in the alignment. The second alignment was trimmed to the shortest sequence on each end. Phylogenetic analyses were conducted using BI with MrBayes 3.2.7 software (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). The best nucleotide substitution models were estimated with jModeltest version 2.1.10 (Darriba et al., 2012), and both the Akaike information criterion (AIC) and Bayesian information criterion (BIC) predicted the GTR + I + Γ model as the best estimator. Therefore, the BI analyses were conducted using the closest approximation to this model. The BI analyses were performed using the following model parameters: "nst = 6," "rates = invgamma," "ngen = 5,000,000," "samplefreq = 500," "printfreq = 500," and "diagnfreq = 5,000." The values of the samples of the substitution model parameters were summarized using "sump." Tree and branch lengths were summarized using "sumt." The first 25% of trees were discarded using the following settings: "relburnin = yes," "burninfrac = 0.25." Nodal support was estimated by posterior probabilities. All other settings were left as default values. Two species in the Lepocreadiidae, 1 species in the Lissorchiidae, and 1 species in the Deropristidae were included in the alignments as a result of phylogenetic relationships shown in the literature (Olson et al., 2003; Pérez-

Ponce De León and Hernández-Mena, 2019; Sokolov et al., 2020), with *Bianium arabicum* Sey, 1996 (a lepocreadiid) serving as the outgroup for the analysis (Wee et al., 2018, 2019; Panyi et al., 2020; Wee et al., 2020). FigTree version 1.4.3 (Rambaut and Drummon, 2012) was used to visualize the phylogeny and Adobe® Photoshop® CS6 (Adobe Inc., San Jose, California) was used for subsequent editing.

Table 4.1 *Partial 28S rDNA sequence data used in the phylogenetic analysis.*

Species	Host Species	GenBank Accession Number	Reference
Monorchiidae Odhner, 1911			
Allobacciger brevicirrus	*Scolopsis bilineata*	MK955781	Wee et al., 2020
Cableia pudica	*Cantherines pardalis*	AY222251	Olson et al., 2003
Diplomonorchis cf. *leiostomi* *	*Leiostomus xanthurus*	AY222252	Olson et al., 2003
Diplomonorchis leiostomi	*Leiostomus xanthurus*	**This study**	**This study**
Genolopa ampullacea *	*Haemulon flavolineatum*	MN984474	Panyi et al., 2020
Helicometroides longicollis *	*Diagramma labiosum*	KJ658287	Searle et al., 2014
Hurleytrematoides chaetodoni *	*Chaetodon striatus*	MH244116	Andres et al., 2018
Hurleytrematoides galzini	*Gnathanodon speciosus*	MK501988	Wee et al., 2019
Hurleytrematoides loi	*Gnathanodon speciosus*	MK501989	Wee et al., 2019
Lasiotocus arrhichostoma	*Diagramma labiosum*	KJ658289	Searle et al., 2014
Lasiotocus glebulentus	*Mugil curema*	MN984476	Panyi et al., 2020
Lasiotocus lizae	*Liza longimanus*	LN831723	Atopkin et al., 2017
Lasiotocus minutus	*Fundulus heteroclitus*	Chapter III	Chapter III
Lasiotocus minutus	*Funudulus grandis*	Chapter III	Chapter III
Lasiotocus minutus	*Fundulus similis*	Chapter III	Chapter III
Lasiotocus sp.	*Menidia menidia*	MN984477	Panyi et al., 2020
***Lasiotocus* sp. A**	*Fundulus similis*	**This study**	**This study**
***Lasiotocus* sp. B**	*Mugil curema*	**This study**	**This study**
***Lasiotocus* sp. C**	*Menidia menidia*	**This study**	**This study**
***Lasiotocus* sp. D**	*Haemulon sciurus*	**This study**	**This study**
Lasiotocus trachinoti	*Trachinotus carolinus*	MN984478	Panyi et al., 2020
Lasiotocus truncatus	*Haemulon flavolineatum*	**This study**	**This study**
Lasiotocus typicum	*Trachurus trachurus*	AY222254	Olson et al., 2003
Madhavia fellaminuta	*Upeneus tragula*	MG920219	Wee et al., 2018
Monorchis lewisi	*Acanthopagrus australis*	MF503309	Cribb et al., 2018
Monorchis monorchis *	*Diplodus vulgaris*	AF184257	Tkach et al., 2001
Ovipusillus mayu *	*Gnathanodon speciosus*	MF503310	Cribb et al., 2018
Parachrisomon delicatus	*Upeneus tragula*	MG920218	Wee et al., 2018
Postmonorchis orthopristis *	*Haemulon flavolineatum*	MN984475	Panyi et al., 2020
Proctotrema addisoni	*Diagramma labiosum*	KJ658291	Searle et al., 2014
Provitellus chaometra	*Gnathanodon speciosus*	MK501984	Wee et al., 2019
Provitellus infrequens	*Gnathanodon speciosus*	MK501985	Wee et al., 2019
Provitellus turrum *	*Pseudocaranx dentex*	AY222253	Olson et al., 2003
Lissorchiidae Magath, 1917			
Lissorchis kritskyi	*Minytrema melanops*	EF032689	Curran et al., 2006
Deropristidae Cable and Hunninen, 1942			
Skrjabinopsolus nudidorsalis	*Acipenser ruthensus*	MN700996	Sokolov et al., 2020

Table 4.1 (continued).

Lepocreadiidae Odhner, 1905			
Bianium arabicum	*Lagocephalus lunaris*	MH157076	Bray et al., 2018
Lepotrema adlardi	*Abudefduf bengalensis*	MH730015	Bray et al., 2018

*Indicates type-species of the genus.

4.3 Results

4.3.1 Morphological

Monorchiidae Odhner, 1911

Lasiotocus Looss in Odhner, 1911

4.3.1.1 *Lasiotocus* sp. A (Figure 4.1)

4.3.1.1.1 Taxonomic Summary

Type host: *Fundulus similis* (Baird and Girard, 1853), longnose killifish, Fundulidae.

Type locality: Cedar Key, Florida (29°08'16"N, 83°02'35"W).

Site: intestine.

Specimens deposited: Holotype: USNM XXXX; x paratypes: USNM XXXX, XXXX; 1 hologenophore: USNM XXXX.

Sequences: Partial 28S rDNA, 1 hologenophore (submitted to GenBank: accession number XXX; ITS2 rDNA, 1 hologenophore (submitted to GenBank: accession number XXX).

4.3.1.1.2 Description (Based on 14 gravid, adult specimens and 1 non-gravid specimen, all mounted without pressure)

Body elongate, slightly tapering at both ends, widest in middle third of body, 741 to 1052 (913) long, 197 to 285 (274) wide. Tegument spinose; spines larger anteriorly, 3 to 5 long, 1 to 3 wide at base, with some slightly rounded at distal end. Eyespot pigment

absent. Oral sucker simple, subterminal, circular, 91 to 177 (109) long, 85 to 199 (108) wide. Ventral sucker thickly muscularized, located approximately 1/3 of body length from anterior end, spherical to subspherical, 66 to 87 (81) long, 68 to 87 (81) wide. Oral to ventral sucker width ratio 1:0.6 to 1:0.8 (1:0.8). Forebody 196 to 294 (274) long or 26% to 31% (27%) of body length; hindbody 456 to 686 long or 60% to 66% of body length. Prepharynx very short if distinct, 0 to 20 (20) long. Pharynx subspherical, wider than long, 37 to 47 (46) long, 43 to 61 (55) wide. Esophagus half as long as pharynx to about as long as pharynx, 26 to 40 (35) long. Cecal bifurcation at midpoint between suckers. Ceca blind, extending to variable level in hindbody from testis to posterior extremity, but usually terminating near mid hindbody; termination 50 to 195 (195) from posterior end or 6% to 21% (21%) of body length.

Testis singular, median to submedian (dextral), subspherical to slightly elongated, smooth, 133 to 185 (173) long, 99 to 172 (138) wide. Post-testicular space 170 to 304 (250) long or 20% to 37% (27%) of body length. Cirrus sac elongate, curving dextrally, dorsal to ventral sucker and ovary, extending to testis level, usually to mid-level or posterior half of testis, 288 to 446 (265) long, 29% to 50% (29%) of body length, 42 to 70 (66) wide (containing internal seminal vesicle, pars prostatica, and cirrus). Cirrus elongate, 172 to 213 (179) long, 9 to 39 (39) wide (measured when not everted), spined; spines 4 to 8 long, 2 to 7 wide at base, usually 2 to 3 wide at base, somewhat larger on edges. Internal seminal vesicle unipartite, ovoid to elongate, in proximal region of cirrus sac, 65 to 148 (91) long, 35 to 63 (35) wide. Genital atrium inconspicuous, unspined; genital pore anterior to ventral sucker, median to slightly sinistral (usually slightly sinistral), 4 to 26 from anterior margin of ventral sucker.

Ovary subglobular to trilobed, submedian, dextral, overlapping anterior margin of testis, ventral to testis, 111 to 154 (154) long, 75 to 164 (129) wide. Terminal organ "Erlenmeyer flask-shaped," widest at posterior or blind end, narrowing toward anterior region, bipartite, sinistral to cirrus sac, 121 to 168 (191) long, 26 to 52 (43) wide; posterior region muscular, unspined, blind; anterior portion separated by a sphincter, opening into genital atrium, spined; spines 4 to 9 long, 1 to 3 wide at base. Mehlis' gland not observed. Uterine seminal receptacle present. Laurer's canal not observed. Vitellarium comprising groups of 22 to 49 tightly compacted, poorly differentiated follicular groups, 132 to 154 long, 49 to 99 wide, symmetrical to slightly asymmetrical, dorsal to gonads, mostly intercecal, concentrated at ovarian level, connecting as common lateral duct, meeting at central vitelline reservoir; vitelline reservoir as vertically linear pouch between vitellarium groups, ventral to cirrus sac, dorsal to ovary, 56 to 71 long (71), 31 wide. Uterus coiling, voluminous, extending from genital atrium to 38 to 149 (82) or 5% to 18% (9%) of body length from posterior end, proximal end not observed, ventral to and completely overlapping gonads, joining with terminal organ from dextral side, ventrally, posterior to anteriorly spined region; post-testicular uterus occupying 24 to 266 (172) or 14% to 88% (69%) of post-testicular space, 2% to 30% (19%) of body length. Eggs non-filamented, tanned, 17 to 23 long, 7 to 12 wide (measured from distal uterus).

Excretory vesicle I-shaped, usually extending to testis level, sometimes terminating posterior to testis, sometimes obscured by uterus; concretions absent; excretory pore terminal.

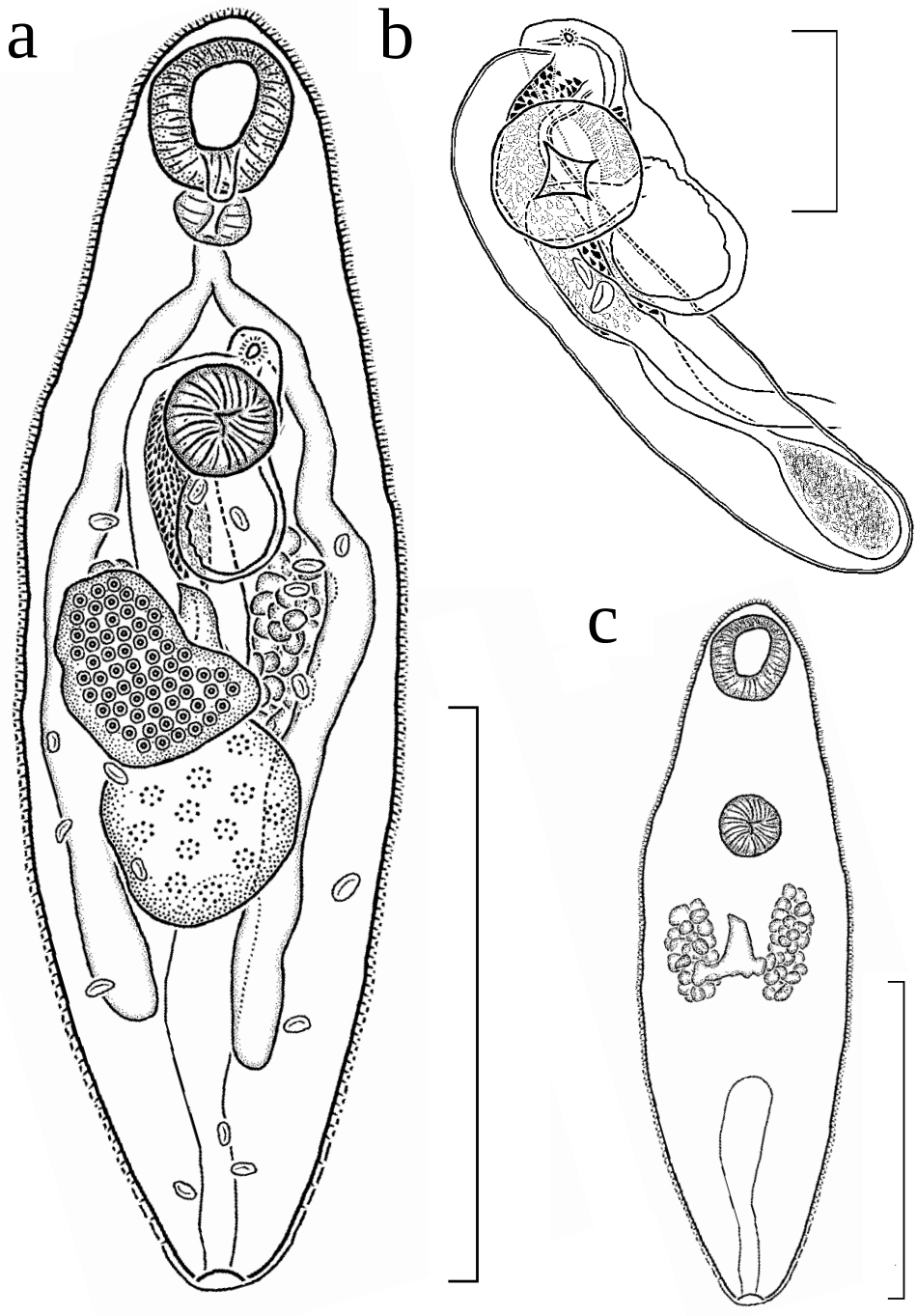

Figure 4.1 Lasiotocus *sp. A from* Fundulus similis.

(a) Ventral view, holotype, scale bar 400 µm; (b) ventral view, terminal genitalia, scale bar 100 µm; (c) ventral view, holotype, excluding all structures other than vitellarium, oral sucker, and ventral sucker, scale bar 400 µm.

4.3.1.1.3 Remarks

Prior to this study there were 49 accepted species of *Lasiotocus.* Three other named species, *Lasiotocus jagannathi* Ahmad and Gupta, 1985, *Lasiotocus polynemi* (Dutta, Hafeezullah, and Manna, 1994) Dove and Cribb, 1998 and *Lasiotocus rainai* Gupta and Jain, 1992, are considered *species inquirendae* (Madhavi and Bray, 2018). Additionally, Madhavi and Bray (2018) discussed *Lasiotocus sunderbanensis* (Dutta, Hafeezullah, and Manna, 1994) Dove and Cribb, 1998 as *incertae sedis* but I do not explicitly consider *L. sunderbanensis* as *incertae sedis,* so the species name is currently accepted; however, I do consider *L. sunderbanensis* as a *species inquirenda* because of the more posteriorly located gonads and the more extensive distribution of vitelline follicles that violate the generic diagnosis of *Lasiotocus*. Because of the current uncertainty of their taxonomic status, all 4 of the aforementioned species are included in the subsequent comparisons giving a total of 52 species for the comparisons.

Additionally, I consider 2 named species of *Lasiotocus* as *incertae sedis*: *Lasiotocus macrotrema* Wu, Lu, and Chen, 1999 and *Lasiotocus rohitai* Bilqees and Khan, 1990 because they both have features violating the generic diagnosis. *Lasiotocus macrotrema* does not have a spinous tegument and has an external seminal vesicle. The description of *L. rohitai* does not include a description nor an illustration of the cirrus or the terminal organ. An unspined cirrus and absence of the terminal organ would violate the generic diagnosis and potentially the familial diagnosis. Additionally, *L. rohitai* was reported from a freshwater cyprinid host in a lake in Pakistan; monorchiids occur in marine and estuarine fishes.

The remaining 50 species can be divided into 2 large groups, 1 group having a funnel-shaped oral sucker (21 species) and the other group having a typical circular or subspherical oral sucker (see Table 4.2). *Lasiotocus* sp. A belongs in the larger group having a circular or subspherical subterminal oral sucker. *Lasiotocus* sp. A lacks compact eyespot fragments, so it is easily distinguished from 5 of 29 species that have eyespot pigments: *Lasiotocus baiosomus* Kamegai, 1970, *Lasiotocus longicystis* Bartoli, 1965, *L. mulli*, *Lasiotocus oculatus* (Manter and Pritchard, 1961) Yamaguti, 1971, and *Lasiotocus trachinoti* Overstreet and Brown, 1970.

Table 4.2 *List of all nominal species of* Lasiotocus *grouped by oral sucker morphology.*

Species	**Authority**
With funnel-shaped oral sucker	
Lasiotocus accraensis	Fischthal and Thomas, 1969
Lasiotocus arrhichostoma	Searle, Cutmore, and Cribb, 2014
Lasiotocus asymmetricus	Fischthal, 1977
Lasiotocus attenuatus	Fischthal and Thomas, 1969
Lasiotocus beauforti	(Hopkins, 1941) Thomas, 1959
Lasiotocus cacuminatus	(Nicoll, 1915) Thomas, 1959
Lasiotocus chaetodipteri	Thomas, 1959
Lasiotocus costaricae	(Manter, 1940) Yamaguti, 1954
Lasiotocus cryptostoma	(Oshmarin, 1966) Mamaev, 1970
Lasiotocus guptai	Ahmad and Dhar, 1987
Lasiotocus haemuli	Overstreet, 1969
Lasiotocus himezi	Yamaguti, 1951
Lasiotocus longicaecum	(Manter, 1940) Manter, 1958
Lasiotocus longitestis	Durio and Manter, 1968
Lasiotocus longovatus	(Hopkins, 1941) Thomas, 1959
Lasiotocus macrorchis	(Yamaguti, 1934) Yamaguti, 1954
Lasiotocus maculatus	Madhavi, 1974
Lasiotocus overstreeti	Gupta and Gupta, 1990
Lasiotocus puriensis	Ahmad and Gupta, 1985
Lasiotocus sparisomae	Fischthal and Nasir, 1974
Lasiotocus truncatus	(Linton, 1910) Thomas, 1959

Table 4.2 (continued).

With circular or subspherical, subterminal oral sucker	
Lasiotocus baiosomus	Kamegai, 1970
Lasiotocus chichibu	Iwashita, Hirose, and Deguchi, 1995
Lasiotocus cynoglossi	Thomas, 1959
Lasiotocus elongatus	(Manter, 1931) Thomas, 1959
Lasiotocus ghanensis	Fischthal and Thomas, 1969
Lasiotocus glebulentus	Overstreet, 1971
Lasiotocus hastai	Madhavi, 1974
*Lasiotocus jagannathi***	Ahmad and Gupta, 1985
Lasiotocus lintoni	(Manter, 1931) Thomas, 1959
Lasiotocus lizae	Liu, 2002
Lasiotocus longicystis	Bartoli, 1965
*Lasiotocus macrotrema***	Wu, Lu, and Chen, 1999
Lasiotocus malasi	(Nagaty, 1948) Yamaguti, 1954
Lasiotocus minutus	(Manter, 1931) Thomas, 1959
Lasiotocus mugilis	Overstreet, 1969
*Lasiotocus mulli**	(Stossich, 1883) Odhner, 1911
Lasiotocus oculatus	(Manter and Pritchard, 1961), Yamaguti, 1971
Lasiotocus odhneri	(Srivastava, 1939) Thomas, 1959

*Indicates type-species of the genus.

**Indicates species of uncertain taxonomic status.

Lasiotocus sp. A can be differentiated from 5 of 24 remaining species (*Lasiotocus cynoglossi* Thomas, 1959, *L. jagannathi*, *L. sunderbanensis*, *Lasiotocus tropicus* (Manter, 1940) Bartoli and Bray, 2004, and *Lasiotocus typicus* (Nicoll, 1912) Bartoli and Bray, 2004) because *Lasiotocus* sp. A has a more typical arrangement of the vitellarium (restricted to the gonadal zone at approximately mid body), rather than a more extensive distribution extending anterior or posterior from this area, as seen in the aforementioned species. Additional differentiations can be made because *Lasiotocus* sp. A has a vitellarium in groups of very tightly compacted, small, almost indiscernible, numerous follicles, as opposed to large, conspicuous, less (4 to 9) numerous follicles described in 12 of 19 remaining species*: Lasiotocus chichibu* Iwashita, Hirose, and Deguchi, 1995,

Lasiotocus ghanensis Fischthal and Thomas, 1969, *Lasiotocus hastai* Madhavi, 1974, *Lasiotocus lintoni* (Manter, 1931) Thomas, 1959, *Lasiotocus malasi* (Nagaty, 1948) Yamaguti, 1954, *Lasiotocus odhneri* (Srivastava, 1939) Thomas, 1959, *Lasiotocus okinawaensis* Machida, 2011, *L. rainai*, *Lasiotocus sparui* (Shen, 1990) Machida, 2011, *Lasiotocus srivastavai* Mittal and Pande, 2007, *Lasiotocus synapturae* Fischthal and Thomas, 1969, and *Lasiotocus trifolifer* (Nicoll, 1915) Thomas, 1959.

Lasiotocus sp. A has an oral sucker to ventral sucker width ratio of 1:0.6 to 1:0.8, but 4 of 7 remaining species of *Lasiotocus* have an oral sucker to ventral sucker width ratio that is greater than 1:1: *L. elongatus*, *L. glebulentus*, *Lasiotocus lizae* Liu, 2002, and *L. polynemi*. Of the remaining 3 species in the group, *Lasiotocus* sp. A may be differentiated from 2 of these, *L. mugilis* and *Lasiotocus parvus* (Manter, 1942) Yamaguti, 1954, by egg size. *Lasiotocus mugilis* has smaller eggs (11 to 17 long, 9 to 10 wide) and *L. parvus* has larger eggs (25 to 28 long, 8 to 10 wide) compared with those of *Lasiotocus* sp. A (17 to 23 long, 7 to 12 wide).

Lasiotocus sp. A is most similar morphologically to *L. minutus*, the last remaining species in the group but differs in several important ways. *Lasiotocus* sp. A has a larger body size (741 to 1052 long, 197 to 285 wide) compared with *L. minutus* (350 to 630 long, 170 to 630 wide) and a longer forebody (26% to 31% of body length) compared with *L. minutus* (16% of body length). Most noticeably, *Lasiotocus* sp. A has a relatively larger cirrus sac extending to the testis level, usually to the posterior region of the testis, compared with other species of *Lasiotocus*.

4.3.1.2 *Lasiotocus* sp. B (Figure 4.2)

4.3.1.2.1 Taxonomic Summary

Type Host: *Mugil cephalus* (Linnaeus, 1758), flathead grey mullet, Mugilidae.

Type Locality: Sapelo Island, Georgia (31°23’49”N, 81°16’53”W).

Site: intestine.

Specimens deposited: Holotype: USNM XXX; x paratypes: USNM XXX; 3 hologenophores: USNM XXX, XXX, XXX.

Sequences: Partial 28S rDNA, 3 identical replicates (1 submitted to GenBank, accession number XXXX); ITS2 rDNA, 2 identical replicates (1 submitted to GenBank, accession number XXXX).

4.3.1.2.2 Description (Based on 4 gravid, adult specimens, measurements for features other than spines derived from only 3 of the specimens mounted without pressure)

Body elongate, anterior end rounded, sides tapering posteriorly, posterior end truncated, 748 to 920 long, 255 to 299 wide, widest near midbody. Body narrowing extremely towards anterior end in lateral view, not dorsoventrally compressed in posterior 2/3 of body in lateral view. Tegument spinose; spines larger on anterior half of body, 2 to 4 long, 1 to 3 wide at base (measured from anterior region). Eyespot pigment absent. Oral sucker simple, subterminal, subspherical, 72 to 85 long or 7% to 11% (10%) of body length, 76 to 116 wide, wider than long. Ventral sucker subspherical, 101 to 114 long or 11% to 14% (14%) of body length, 114 to 123 wide. Oral sucker to ventral sucker width ratio 1:1 to 1:1.6. Forebody 188 to 221 long or 22% to 28% of body length; hindbody 461 to 590 long or 61% to 64% of body length. Prepharynx absent or very

short, 0 to 7 long or 0% to 1% (0%) of body length. Pharynx spherical to subspherical, wider than long, 39 to 76 long or 4% to 9% (9%) of body length, 40 to 103 wide. Esophagus half length of pharynx to about as long as pharynx, 28 to 40 long or 3% to 5% (5%) of body length. Cecal bifurcation halfway between suckers. Ceca blind, extending to posterior extremity, terminating 49 to 101 from posterior end or 6% to 11% (8%) of body length.

Testis singular, subglobular to elongate, submedian, dextral, 64 to 134 long or 9% to 16% (16%) of body length, 81 to 110 wide. Post-testicular space 175 to 279 long or 24% to 30% (27%) of body length. Cirrus sac elongate, dextral, dorsal to ovary, terminating at posterior margin of ovary to anterior margin of testis, 281 to 289 long or 33% to 39% (33%) of body length, 53 to 78 wide (comprising proximal internal seminal vesicle, pars prostatica, and cirrus); cirrus elongate, entering genital atrium distally, 137 long or 16% (16%) of body length, 24 wide when not everted, spined; spines 7 to 9 long, 6 to 7 wide at base; seminal vesicle internal, unipartite, elongate, 94 to 139 long or 11% to 19% (11%) of body length, 51 to 73 wide. Genital atrium unspined. Genital pore slightly submedian, sinistral.

Ovary subglobular to triangular to u-shaped, median to submedian, dextral, pre-testicular, 71 to 106 long or 8% to 14% (9%) of body length, 65 to 114 wide. Terminal organ muscular, bipartite, sinistral to cirrus sac; posterior portion muscular, unspined, blind; anterior region opening into genital atrium, spined; spines 8 to 14 long, 2 to 5 wide at base. Mehlis' gland anterior to ovary, dextral to cirrus sac (observed in holotype only). Seminal receptacle not observed. Laurer's canal not observed. Vitellarium comprising groups of 54 to 75 tightly compacting small follicles at level of gonads, mostly intercecal,

dorsal, connecting as common vitelline ducts to median vitelline reservoir; vitelline reservoir narrow, shaped as elongate pouch extending anteriorly to female complex. Uterus highly coiling, voluminous, extending 21 to 132 from posterior end or 3% to 16% (16%) of body length to genital atrium, ventral to gonads, joining to terminal organ not observed; post-testicular uterus occupying 135 to 254 or 59% to 91% (59%) of post-testicular space, 15% to 28% (16%) of body length. Eggs non-filamented, tanned, 22 to 27 long, 7 to 10 wide when distal.

Excretory vesicle I-shaped, extending to posterior margin of testis to mid-level of testis, without concretions; excretory pore terminal.

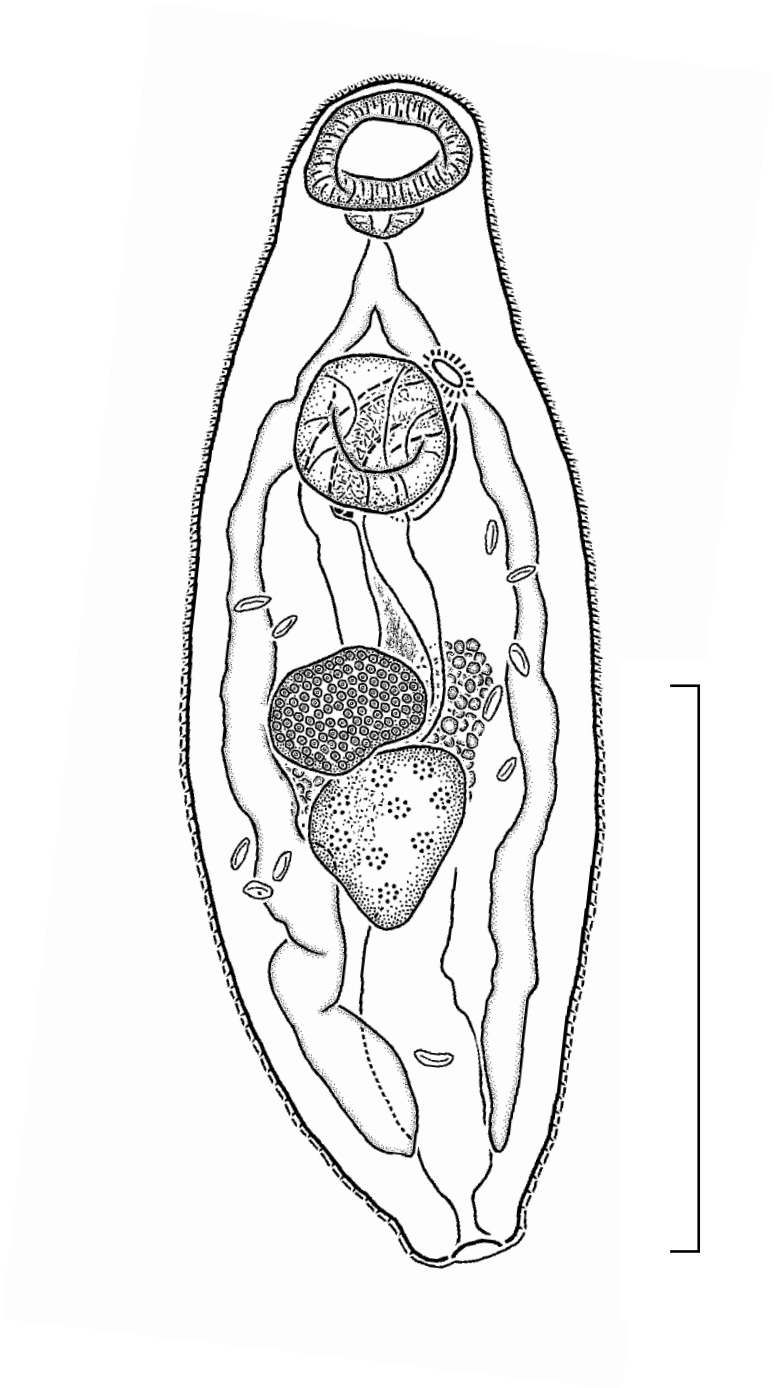

Figure 4.2 Lasiotocus *sp. B from* Mugil cephalus.

Ventral view, holotype, scale bar 400 µm.

4.3.1.2.3 Remarks

Lasiotocus sp. B, like Lasiotocus sp. A, has a subterminal, subspherical, non-funnel shaped oral sucker, allowing it to be distinguished from the 21 species of Lasiotocus with funnel shaped oral suckers (Table 4.2). Lasiotocus sp. B also differs from L. macrotrema by not having an external seminal vesicle and having a spinous tegument, and Lasiotocus sp. B differs from L. rohitai by having both a spined cirrus and terminal organ, neither of which are described nor illustrated for L. rohitai. Additionally, Lasiotocus sp. B lacks remnants of eyespot pigments, so it can also be distinguished from 5 of 30 remaining species of Lasiotocus that do have that feature: L. baiosomus, L. longicystis, L. mulli, L. oculatus, L. trachinoti.

Of the remaining 25 species, 5 species have an extensive vitellarium that extends well outside the gonadal region (L. cynoglossi, L. jagannathi, L. sunderbanensis, L. tropicus, and L. typicum), and 20 species resemble Lasiotocus sp. B by having the vitellarium restricted to the gonadal zone in the midbody. Twelve of these 20 species differ from Lasiotocus sp. B by having few (4 to 10), large, conspicuous, vitelline follicles (L. chichibu, L. ghanensis, L. hastai, L. lintoni, L. malasi, L. odhneri, L. okinawaensis, L. rainai, L. sparui, L. srivastavai, L. synapturae, and L. trifolifer), rather than smaller, more numerous, poorly discernible, tightly compact follicles. Lasiotocus sp. B is therefore most similar in morphology to L. elongatus, L. glebulentus, L. lizae, L. minutus, L. mugilis, L. polynemi, L. parvus, and Lasiotocus sp. A.

The oral sucker to ventral sucker width ratio is 1:1 or greater for Lasiotocus sp. B (1:1 to 1:1.6), which allows it to be differentiated from L. minutus that has an oral sucker to ventral sucker width ratio of 1:0.75. Lasiotocus sp. B is similar morphologically to L.

lizae and L. elongatus but differs in several ways. The eggs are smaller in both L. lizae (18 to 22 long, 8 to 10 wide) and L. elongatus (16 to 19 long, 7 to 9 wide) compared with the eggs of Lasiotocus sp. B (22 to 27 long, 7 to 10 wide). Additionally, L. elongatus has a unipartite terminal organ with smaller spines (4 long, 2 to 3 wide at base), compared with the bipartite terminal organ with larger anterior spines (8 to 14 long, 2 to 5 wide at base). Lasiotocus lizae also has a trilobed ovary and ceca that terminate at the ventral sucker level compared with Lasiotocus sp. B that has a subglobular to triangular to u-shaped ovary and ceca that terminate at the posterior extremity. Lasiotocus sp. B can be differentiated from L. polynemi by the gonads of the latter being located near the posterior extremity and the much longer esophagus (almost 5 times the length of the pharynx) of the latter. Lasiotocus sp. B can be morphologically distinguished from Lasiotocus sp. A by the latter having ceca terminating before the posterior extremity, having a smaller oral to ventral sucker width ratio (1:0.6 to 1:0.8), having smaller eggs (17 to 23 long, 7 to 12 wide), and having a cirrus sac terminating well into the testicular zone.

Lasiotocus sp. B is also morphologically similar to L. glebulentus but can be differentiated from the latter by not having ceca that reach the posterior extremity, having concretions in the excretory vesicle, and having some individuals with a unipartite terminal organ and some with a bipartite terminal organ. Lasiotocus sp. B may be differentiated from L. parvus by the latter having a smaller body size (300 long, 232 wide) compared with the body size of Lasiotocus sp. B (748 to 920 long, 255 to 299 wide), not having an esophagus, and having ceca that terminate at the ovarian level

compared with having an esophagus and the ceca terminating at the posterior extremity in Lasiotocus sp. B.

Lasiotocus sp. B is most similar morphologically to L. mugilis. The major difference between the 2 species is the smaller egg size of L. mugilis (11 to 17 long, 9 to 10 wide) compared with Lasiotocus sp. B (22 to 27 long, 7 to 10 wide). Additionally, L. mugilis has a slightly larger forebody (29% to 34% of body length) and the uterus occupying less of the post-testicular space (36% of body length) compared with the smaller forebody of Lasiotocus sp. B (22% to 28% of body length) and the uterus occupying more of the post-testicular space (59% to 91% of body length).

4.3.1.3 *Lasiotocus* sp. C A. Panyi and R. Heard (Figure 4.3)

4.3.1.3.1 Taxonomic Summary

Type host: *Menidia menidia* (Linnaeus, 1766), Atlantic silverside, Atherinopsidae.

Type locality: Plum Island Estuary, Massachusetts (42°47’23.5”N, 70°48’30”W).

Site: intestine.

Specimens deposited: Holotype: USNM XXX, x paratypes: USNM XXX; 2 hologenophores: USNM XXX, XXX.

Sequences: Partial 28S rDNA, 2 identical replicates (1 submitted to GenBank: accession number xxxx); ITS2 rDNA, 2 identical replicates (1 submitted to GenBank: accession number xxxx).

4.3.1.3.2 Description (Based on 3 gravid, whole adult specimens and 2 gravid hologenophores, all mounted without pressure)

Body oval to fusiform, tapering towards both ends, widest near mid-body, 341 to 436 (359) long, 111 to 138 (138) wide. Tegument spinose; spines denser anteriorly, 1 to 3 long, 1 to 2 wide at base (measured from near anterior end), absent towards posterior end. Eyespot pigment absent. Oral sucker subterminal, subspherical, 43 to 47 (47) long or 10% to 13% (13%) of body length, 42 to 45 (42) wide. Ventral sucker subspherical, near anterior third of body length, 35 to 42 (41) long or 8% to 12% (11%) of body length, 34 to 38 (38) wide. Oral sucker to ventral sucker width ratio 1:0.8 to 1:0.9 (1:0.9). Forebody 103 to 115 (115) long or 25% to 32% (32%) of body length; hindbody 197 to 289 (207) long or 57% to 66% (58%) of body length. Prepharynx absent or very short, 0 to 5 (5) long or 0% to 1% (1%). Pharynx spherical to dolliform, 18 to 20 (18) long or 4% to 6% (5%) of body length, 18 to 19 (18) wide. Esophagus short, 6 to 8 long or 1% to 3% of body length with cecal bifurcation at midpoint between suckers to slightly closer to ventral sucker. Ceca blind, extending to posterior margin of testis, terminating 151 to 214 (151) from posterior end or 42% to 49% (42%) of body length.

Testis singular, subspherical, median to submedian, dextral, 63 to 84 (76) long or 19% to 21% (21%) of body length, 49 to 57 (54) wide. Post-testicular space 99 to 191 (116) long or 29% to 44% (32%) of body length. Cirrus sac elongate, terminating at posterior end of ventral sucker or shorter, 74 to 105 (79) long or 21% to 24% (22%) of body length, 15 to 19 (19) wide comprising internal seminal vesicle, pars prostatica, and cirrus. Cirrus small, elongate, entering genital atrium distally 19 to 27 (19) long or 5% to 8% (5%) of body length, 7 to 11 (9) wide when not everted, spined; spines uniform in

size, triangular, 3 to 4 long, 1 to 2 wide at base. Internal seminal vesicle unipartite, ovoid, in proximal region of cirrus sac, 26 to 39 (31) long or 7% to 9% (9%) of body length, 17 to 23 (22) wide. Genital atrium unspined. Genital pore immediately anterior to ventral sucker, median to submedian dextral.

Ovary subglobular to triangular, median to submedian, dextral, mostly pre-testicular, ventral to testis, slightly overlapping anterior of testis, 38 to 50 (39) long or 10% to 12% (11%) of body length, 34 to 44 (43) wide. Terminal organ inconspicuous, bipartite, dorso-dextral to cirrus sac, 28 to 33 (28) long or 7% to 8% (8%) of body length, 13 (13) wide; posterior portion unspined, blind; anterior region opening into genital atrium, spined; spines uniform, triangular, 4 to 6 long, 2 to 4 wide at base. Mehlis' gland not observed. Seminal receptacle not observed. Laurer's canal not observed. Vitellarium consisting of groups of 9 to 13 tightly compact, poorly differentiated follicles, at pre-ovarian to ovarian level, connecting with lateral ducts to common vitelline reservoir; vitelline reservoir median, ventral to vitellarium. Uterus voluminous, loosely coiling, mostly intercecal with some extracecal, with uterine area extending 29 to 45 (29) or 7% to 13% (8%) of body length from posterior end to genital atrium**,** ventral to and overlapping gonads, joining with terminal organ not observed; post-testicular uterus occupying 54 to 160 (87) or 55% to 83% (75%) of post-testicular space, 15% to 37% of body length. Eggs 14 to 18 long, 5 to 9 wide when distal.

Excretory vesical I-shaped, short, 49 to 62 (49) long, not reaching to mid post-testicular space, without concretions; excretory pore terminal.

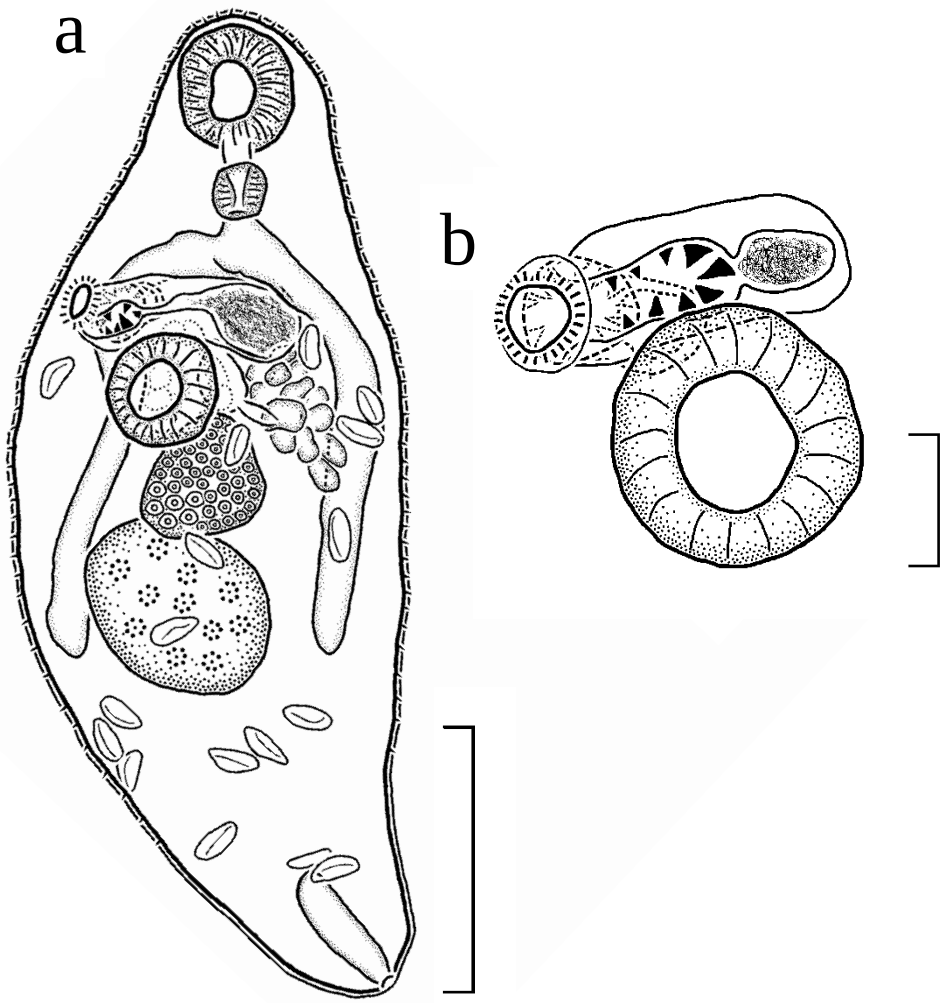

Figure 4.3 Lasiotocus *sp. C from* Menidia menidia.

(a) Ventral view, holotype, slightly lateral mount, scale bar 100 µm; (b) ventral view, terminal genitalia, from a non-gravid individual, scale bar 20 µm.

4.3.1.3.3 Remarks

Lasiotocus sp. C has a subterminal, subspherical, non-funnel shaped oral sucker and can therefore also be distinguished from the 21 species of *Lasiotocus* with funnel shaped oral suckers (Table 4.2). *Lasiotocus* sp. C also differs from *L. macrotrema* by not having an external seminal vesicle and having a spinous tegument, and *Lasiotocus* sp. C

differs from *L. rohitai* by having both a spined cirrus and terminal organ, neither of which are described nor illustrated for *L. rohitai. Lasiotocus* sp. C also lacks remnants of eyespot pigmentation and can therefore be distinguished from 5 of 31 remaining species of *Lasiotocus* that do have compact eyespots pigments mentioned in the prior 'remarks' sections. Additionally, *Lasiotocus* sp. C has a vitellarium restricted to the ventral sucker to gonadal zone at approximately the midbody, so *Lasiotocus* sp. C can be differentiated from 5 of 26 remaining species of *Lasiotocus* that have a vitellarium not restricted to this area only but extending further in the posterior half of the body: *L. cynoglossi*, *L. jagannathi*, *L. sunderbanensis*, *L. tropicus*, and *L. typicus*. *Lasiotocus* sp. C has a vitellarium in lateral groups of tightly compact, poorly differentiated, numerous, small follicles, which allows *Lasiotocus* sp. C to be differentiated from 12 of 21 remaining species of *Lasiotocus* that have conspicuous, large, less numerous (4 to 10) follicles: *L. chichibu*, *L. ghanensis*, *L. hastai*, *L. lintoni*, *L. malasi*, *L. odhneri*, *L. okinawaensis*, *L. rainai*, *L. sparui*, *L. srivastavai*, *L. synapturae*, and *L. trifolifer*.

The egg sizes of 3 of 9 remaining species of *Lasiotocus* are larger than the egg size of *Lasiotocus* sp. C (14 to 18 long, 5 to 9 wide), allowing differentiation to be made: *L. lizae* (18 to 22 long, 8 to 10 wide), *L. parvus* (25 to 26 long, 8 to 10 wide), and *Lasiotocus* sp. B (22 to 27 long, 7 to 10 wide). *Lasiotocus* sp. C can be differentiated from *L. elongatus* by the former having a smaller oral sucker to ventral sucker ratio (1:0.8 to 1:0.9) compared with the larger ratio of *L. elongatus* (1:1.3). *Lasiotocus* sp. C may be differentiated from *L. glebulentus*, *L. minutus*, *L. mugilis*, and *Lasiotocus* sp. A by having an excretory vesicle that is a short, saccular tube, not reaching the testicular level in the former. *Lasiotocus* sp. C can be differentiated from *L. polynemi* by having a short

esophagus, gonads in the midbody, and the cirrus sac not extending posterior to the ventral sucker compared with a longer esophagus (almost 5 times as long as the pharynx), gonads close to the posterior extremity, and the cirrus sac extending posterior to the ventral sucker in *L. polynemi*.

4.3.1.4 *Lasiotocus* sp. D (Figure 4.4)

4.3.1.4.1 Taxonomic Summary

Type host: *Haemulon sciurus* (Shaw, 1803), blue-striped grunt, Haemulidae.

Type locality: Long Key, Florida (24°47’26.93”N, 80°53’2.96”W).

Site: intestine.

Specimens deposited: Holotype: USNM XXX; 1 paratype: USNM XXXX; 1 hologenophore: USNM XXXX.

Sequences: Partial 28S rDNA, 1 sequence (submitted to GenBank, accession number XXXX).

4.3.1.4.2 Description (Based on 2 gravid, whole adult specimens and 1 gravid hologenophore, all mounted without pressure)

Body elongate, tapering posteriorly, flaring outward at anterior end, widest near mid to posterior third of body, 1218 to 1418 (1218) long, 183 to 260 (240) wide. Tegument spinose; spines denser anteriorly, 2 to 6 long, 1 to 4 wide at base (measured from near anterior end), rounded distally, smaller in oral sucker region, 1 to 4 long, 1 to 2 wide at base. Eyespot pigment absent. Oral sucker funnel shaped, flaring outward distinctly at anterior end, 129 to 161 (146) long or 11% to 12% (12%) of body length, 157 to 237 (221) wide, wider than long. Ventral sucker subspherical, near anterior third of body length, 65 to 85 (74) long or 5% to 6% (6%) of body length, 68 to 92 (82) wide,

wider than long. Sucker width ratio 1:0.37 to 1:0.43 (1:0.37). Forebody 363 to 535 (478) long or 37% to 39% (39%) of body length; hindbody 660 to 816 (660) long or 54% to 58% (54%) of body length. Prepharynx funnel-shaped, about half length of pharynx to length of pharynx, 23 to 46 (46) long or 3% to 4% (4%) of body length. Pharynx spherical, 39 to 48 (46) long or 3% to 4% (4%) of body length, 37 to 48 (44) wide. Esophagus more than twice length of pharynx, 100 to 166 (112) long or 9% to 12% (9%) of body length with cecal bifurcation about halfway between suckers. Ceca blind, extending beyond testis into hindbody, terminating 284 to 313 (284) from posterior end or 22% to 23% (23%) of body length.

Testis singular, subglobular to elongate, median, sometimes orienting diagonally, 136 to 151 (136) long or 11% to 12% (11%) of body length, 95 to 108 (108) wide. Post-testicular space 397 to 414 (397) long or 29% to 33% (33%) of body length. Cirrus sac elongate, dorsal to ovary, terminating at ovarian level, 202 to 252 (252) long or 16% to 21% (21%) of body length, 61 to 65 (62) wide containing internal seminal vesicle, pars prostatica, and cirrus. Cirrus elongate, 98 to 106 (98) long or 7% to 8% (8%) of body length, 20 to 30 (30) wide when not everted, spined; spine size not uniform, smaller anteriorly, 5 to 10 long, 2 to 4 wide at base, larger posteriorly, 11 to 13 long, 3 to 4 wide at base. Internal seminal vesicle unipartite, elongate, in proximal portion of cirrus sac, 74 to 89 (89) long or 5% to 7% (7%) of body length, 43 to 60 (55) wide. Genital atrium unspined. Genital pore median to slightly sinistral, 10 to 18 from anterior margin of ventral sucker.

Ovary subglobular, median to submedian, dextral, mostly pre-testicular, slightly overlapping anterior of testis ventrally, 74 to 103 (91) long or 7% to 8% (8%) of body

length, 59 to 80 (78) wide. Terminal organ muscular, bipartite with anterior and posterior regions divided by muscular sphincter, sinistral to cirrus sac; anterior region opening into genital atrium, spined; spines narrow, needle-shaped, 11 to 20 long, 2 to 4 wide at base; posterior region blind, spined; spines rose-thorn to triangular, 5 to 8 long, 2 to 4 wide at base. Mehlis' gland slightly overlapping ovary, dorso-dextral to ovary (observed in hologenophore). Seminal receptacle not observed. Laurer's canal not observed. Vitellarium consisting of lateral groups of 8 to 9 follicles at pre-ovarian to ovarian level; follicles 27 to 35 long, 30 to 36 wide, connecting with dorsal, common lateral duct, with central vitelline reservoir. Uterus voluminous, mostly intercecal and posterior to ovary except for coil ascending to terminal organ, extending 74 to 89 (74) or 6% to 7% (7%) of body length from posterior end to genital atrium, descending in coils mostly dorso-sinistrally to posterior extent, ascending in coils mostly ventro-dextral until testis level, further ascending sinistrally to gonads to terminal organ, not overlapping gonads, joining with terminal organ ventrally, at level of sphincter between anterior and posterior spined regions; post-testicular uterus occupying 325 to 331 or 80% to 82% (82%) of post-testicular space, 23% to 27% (27%) of body length. Eggs 13 to 17 long, 7 to 10 wide when distal.

Excretory vesical I-shaped, 422 long, extending dorsally to posterior margin of testis, without concretions; excretory pore terminal.

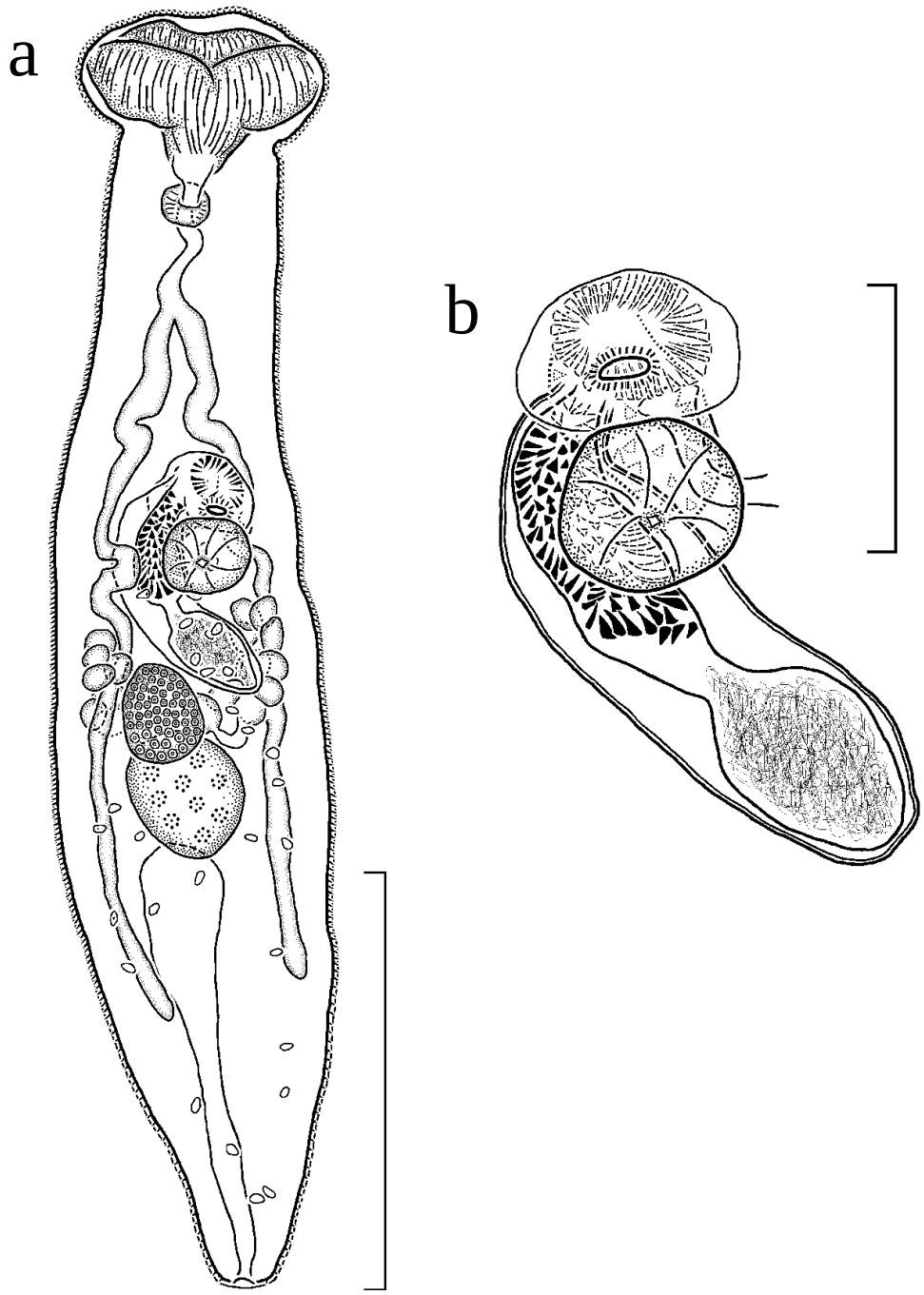

Figure 4.4 Lasiotocus *sp. D from* Haemulon sciurus.

(a) Ventral view, holotype, scale bar 400 µm; (b) ventral view, terminal genitalia, note anterior portion of terminal organ in cross sectional view, scale bar 100 µm.

4.3.1.4.3 Remarks

Lasiotocus sp. D has a funnel-shaped oral sucker and is therefore easily differentiated from 34 other species of *Lasiotocus* described as having subterminal, subspherical, non-funnel shaped oral suckers (Table 4.2. and *Lasiotocus* spp. A-C).

Lasiotocus sp. D can be differentiated from 13 of 21 remaining species with funnel-shaped oral suckers by egg size. Fifteen species, *Lasiotocus arrhichostoma* Searle, Cutmore, and Cribb, 2014, *Lasiotocus beauforti* (Hopkins, 1941), Thomas, 1959, *Lasiotocus cacuminatus* (Nicoll, 1915) Thomas, 1959, *Lasiotocus costaricae* (Manter, 1940) Yamaguti, 1954, *Lasiotocus cryptostoma* (Oshmarin, 1966), Mamaev, 1970, *Lasiotocus haemuli* Overstreet, 1969, *Lasiotocus himezi* Yamaguti, 1951, *Lasiotocus longicaecum* (Manter, 1940) Manter, 1958, *Lasiotocus longitestis* Durio and Manter, 1968, *Lasiotocus longovatus* (Hopkins, 1941) Thomas, 1959, *Lasiotocus macrorchis* (Yamaguti, 1934) Yamaguti, 1954, *Lasiotocus maculatus* Madhavi, 1974, and *Lasiotocus overstreeti* Gupta and Gupta, 1990, have larger eggs than does *Lasiotocus* sp. D, which has eggs measuring 13 to 17 long, 7 to 10 wide.

Of the remaining 8 species, *Lasiotocus* sp. D may be differentiated from 2 species by esophageal length. Both *Lasiotocus accraensis* Fischthal and Thomas, 1969 and *Lasiotocus chaetodipteri* Thomas, 1959 have an esophagus that is not quite as long as the pharynx, whereas *Lasiotocus* sp. D has an esophagus more than twice the length of the pharynx. *Lasiotocus* sp. D may be differentiated by *Lasiotocus asymmetricus* Fischthal, 1977 by the former having a vitellarium in symmetrical, opposite fields as opposed to asymmetrical fields as seen in *L. asymmetricus*. *Lasiotocus* sp. D can be differentiated from *Lasiotocus attenuatus* Fischthal and Thomas, 1969 and *Lasiotocus guptai* Ahmad

and Dhar, 1987 by the smaller oral to ventral sucker width ratio of *Lasiotocus* sp. D (1:0.37 to 1:0.43) compared with the larger ratios of *L. attenuatus* (1:0.45 to 1:0.56) and *L. guptai* (1:0.68 to 1:0.82). *Lasiotocus* sp. D can be differentiated from *Lasiotocus puriensis* Ahmad and Gupta, 1985 by the latter having a longer prepharynx (145 to 255 long), a larger oral to ventral sucker width ratio (1:0.6 to 1:0.7), a flask-shaped pharynx, ceca terminating at the posterior extremity, and less post-testicular space (20% to 27% of body length). *Lasiotocus* sp. D may be differentiated from *Lasiotocus sparisomae* Fischthal and Nasir, 1974 by the latter with ceca terminating at the testicular level, a ventral sucker embedded in the parenchyma, smaller body size (623 to 756 long, 185 to 237 wide), and smaller spines in the anterior region of the terminal organ (10 to 12 long, 2 to 3 wide at base) compared with the ceca terminating in the mid- post-testicular region, larger body size (1218 to 1418 long, 183 to 260 wide), and larger spines in the anterior region of the terminal organ (11 to 20 long, 2 to 4 wide at base) in *Lasiotocus* sp. D.

Lasiotocus sp. D is morphologically most similar to *L. truncatus*. *Lasiotocus* sp. D has a smaller oral to ventral sucker width ratio (1:0.3 to 1:0.4) and slightly smaller cirrus sac (16% to 21% of body length) compared with the ratio (1:0.4 to 1:0.7) and slightly larger cirrus sac (23% to 30% of body length) of *L. truncatus*. Most noticeably, *Lasiotocus* sp. D has an oral sucker that flares out more conspicuously anteriorly compared with the more gradual width increase anteriorly in *L. truncatus*. Also, the posterior portion of the terminal organ is unspined in *L. truncatus* compared with being sometimes spined in *Lasiotocus* sp. D, having similar size and shape of the cirrus spines.

4.3.1.5 *Lasiotocus truncatus* (Linton, 1910) Thomas, 1959

4.3.1.5.1 Taxonomic Summary

Type host: *Haemulon plumierii* (Lacepède, 1801), white grunt, Haemulidae.

Type locality: Dry Tortugas, Florida.

Specimens examined: USNM 1321291 (syntypes).

4.3.1.5.2 Redescription of *Lasiotocus truncatus* syntypes (USNM 1321291, based on 2 gravid, adult specimens)

Body elongate, tapering towards posterior end, widest near midbody, 935 long, 272 wide. Tegument spines not observed. Eyespot pigment absent. Oral sucker funnel shaped, gradually widening anteriorly, 138 long or 15% of body length, 157 wide. Ventral sucker approximately at midbody, weakly developed, subspherical, 61 to 66 long or 7% of body length, 60 to 65 wide. Sucker width ratio 1:0.41. Forebody 398 or 43% of body length; hindbody 464 or 50% of body length. Prepharynx funnel-shaped, shorter than pharynx, less than half length of pharynx. Pharynx subspherical, 36 long or 4% of body length, 34 wide. Esophagus 69 long or 7% of body length with cecal bifurcation about halfway between suckers. Ceca blind, extending well into mid hindbody, terminating 219 from posterior end or 23% of body length.

Testis singular, subglobular to subrectangular, submedian, dextral, 68 to 124 long or 7% to 13% of body length, 106 to 107 wide. Post-testicular space 255 long or 27% of body length. Cirrus sac dorsal, curving dextrally around or underneath ventral sucker, terminating at ovarian level, 154 to 216 long or 23% of body length, 46 to 52 wide consisting of internal seminal vesicle, pars prostatica, and cirrus. Cirrus elongate, 58 to 95 long or 6% to 10% of body length, 26 to 35 wide when not everted, spined; spines 6 to

8 long, 2 to 3 wide at base. Internal seminal vesicle unipartite, elongate, in proximal portion of cirrus sac, 96 long or 10% of body length, 44 wide. Genital atrium unspined, thickly muscular. Genital pore immediately anterior to ventral sucker.

Ovary subglobular, submedian, dextral, overlapping anterior margin of testis ventrally, 72 long or 8% of body length, 53 wide. Terminal organ bipartite, sinistral to cirrus sac; posterior portion unspined, blind; anterior region opening into genital atrium, spined; spines 10 to 16 long, 3 to 4 wide at base. Mehlis' gland antero-dextral of ovary (observed in 1 specimen only). Seminal receptacle not observed. Laurer's canal not observed. Vitellarium consisting of lateral groups of 7 to 8 follicles at ovarian level; follicles 21 to 24 long or 2% to 3% of body length, 25 wide, connecting transversally as common lateral duct, dorsal to ovary, in plane with testis, expanding submedian (dextral) as vitelline reservoir. Uterus voluminous, mostly intercecal, with some loops overlapping ceca, extending 46 or 5% of body length from posterior end to genital atrium, descending in coils ventrally from ovarian level to posterior extent, ascending in coils dorsally, ascending sinistrally of median line when anterior to testis, joining with terminal organ not observed; post-testicular uterus occupying 210 or 82% of post-testicular space, 23% of body length. Eggs when distal 13 to 17 long, 7 to 11 wide.

Excretory vesicle I-shaped, extending to posterior margin of testis, without concretions; excretory pore terminal.

4.3.1.5.3 Remarks

The syntypes of *L. truncatus* used in the redescription were from USNM 1321291. USNM 1321277 and 1321279 syntypes were also examined, but the specimens were in too poor condition for morphological data collection because of extreme

constriction or extreme pressure. Both specimens used for the redescription were in relatively poor condition because 1 specimen was missing the anterior region and was a slightly lateral mount, and the other specimen was ripped in half with both pieces mounted on the slide. Despite these issues, measurements could still be taken for most features and provided more detailed information than is currently available from Linton's (1910) original description and Manter's (1940) supplemental data from material he collected.

4.3.1.5.4 Taxonomic summaries for specimens collected in this study

Host (present study): *Haemulon flavolineatum* (Desmarest, 1823), French grunt, Haemulidae.

Locality: Lower Matecumbe Key, Florida (24°50'42.7"N, 80°44'53.4"W).

Site: intestine.

Specimens deposited: 1 hologenophore: USNM XXXX; 4 vouchers: USNM XXX, XXX, XXX, XXX.

Sequences deposited: Partial 28S rDNA, 1 sequence (submitted to GenBank, accession number XXX); ITS2, 1 sequence (submitted to GenBank, accession number XXX).

4.3.1.5.5 Remarks

My specimens agree well with the description by Linton (1910), supplemental data provided by Manter (1940), and the above redescription. The specimens from this study are of utmost importance because of the poor condition of the available syntypes due to maceration of specimens and because the specimens were fixed using acid (Linton, 1910), which, as stated beforehand, can lead to the loss of hard parts such as spines.

Manter (1940) also used an acid in his fixation method and mounted specimens under pressure. Therefore, the specimens from this study will be the first that use modern fixation techniques, heat-killed and mounted without pressure, for use by future taxonomists.

4.3.1.6 *Diplomonorchis leiostomi* Hopkins, 1941

4.3.1.6.1 Taxonomic summary of specimens collected in this study

Host: *Leiostomus xanthurus* (Lacepède, 1802), spot croaker, Sciaenidae.

Locality: Morehead City, North Carolina (34°42’40.8”N, 76°44’14”W).

Site: intestine.

Specimens deposited: 1 hologenophore: USNM XXX; 1 voucher: USNM XXX).

Sequences deposited: Partial 28S rDNA, 1 sequence (submitted to GenBank, accession number XXX).

4.3.1.6.2 Remarks

My specimens agree well with the description of *Diplomonorchis leiostomi* by Hopkins (1941).

4.3.2 Molecular

4.3.2.1 Phylogenetic Analyses and Pairwise Comparisons

Two alignments of partial 28S rDNA fragments were generated for analyses in this study. One alignment consisted of 1284 base pairs, including gaps, and was trimmed to the partial 28S rDNA sequence of *M. monorchis* for the phylogenetic analysis, meaning shorter sequences were included to retain as many informative sites as possible (Figure 4.5). A second alignment consisted of 792 base pairs, including gaps, and was

trimmed to the shortest sequence on each end for phylogenetic analysis (Figure 4.6). Masking revealed no ambiguous column, i.e., columns with confidence scores below the cut off value of 0.4, so no column was excluded in the phylogenetic analysis as a result of masking. BI analysis resulted in recovered phylogenies (Figure 4.5, 4.6) that are mostly consistent with previously reported monorchiid phylogenies, where slight variations occur in topology, support values are poor (Wee et al., 2018, 2019; Panyi et al., 2020; Wee, Cutmore et al., 2020).

The recovered phylogenies provide further evidence that *Lasiotocus* is polyphyletic. Representative species of *Lasiotocus* are found in 4 separate clades, 3 of which are clades with representative species from other genera, *Diplomonorchis*, *Monorchis, Allobacciger* Hafeezullah and Siddiqi, 1970, and *Parachrisomon*.

The 2 recovered phylogenies for this study are shown for comparison to determine if any major topology or support value changes occur after removing approximately 500 base pairs from the dataset. Although not the focus of this study, *Cableia pudica* Bray, Cribb, and Barker, 1996 does not form a well-supported clade with its sister group, the rest of the representative species of the Monorchiidae. A second difference between the 2 phylogenies, albeit not the focus, is the well-supported *Genolopa* clade as sister to a group containing a well-supported clade of *Diplomonorchis* – *Lasiotocus* and a well-supported clade of *Ovipusillus* – *Parachrisomon* – *Lasiotocus* – *Madhavia* in Figure 4.6. However, the support value is poor for the relationship between the *Genolopa* clade and the sister group, and the entire group of all 3 clades forms a polytomy.

One clade containing a species of *Lasiotocus, L. arrhichstoma – Monorchis lewisi* Cribb, Wee, Bray, and Cutmore, 2018 changes topology between the 2 phylogenies. In Figure 4.5, the 2 species form a somewhat supported clade (0.86) that is somewhat sister (0.82) to a group containing a well-supported clade of *Allobacciger* spp., and a clade of the remaining species of *Lasiotocus* and *M. monorchis*. In Figure 4.6, *L. arrhichostoma* is sister to the clade of *Allobacciger* spp., but the support value is poor. *Monorchis lewisi* is in the clade of remaining species of *Lasiotocus* and *M. monorchis* but forms a polytomy with these groups. Additionally, in Figure 4.6, *M. monorchis* is sister to the clade of *Lasiotocus* spp. A-C, *L. glebulentus, L. lizae, L.* sp. unknown, and *L. minutus*. However, the support value is poor, so it is not much of a change from the polytomy it forms with that group in Figure 4.5. Finally, in Figure 4.6, the support value between the clade of *Lasiotocus* sp. A – *L. minutus* and *Lasiotocus* sp. C – *Lasiotocus* sp. unknown decreases from 0.99 to 0.88. Although some slight differences in topology occur between the 2 phylogenies, the support values are not very good where those changes occur (<0.90); therefore, pairwise comparisons and interrelationships will be discussed based on the shorter, completely trimmed alignment represented by Figure 4.6.

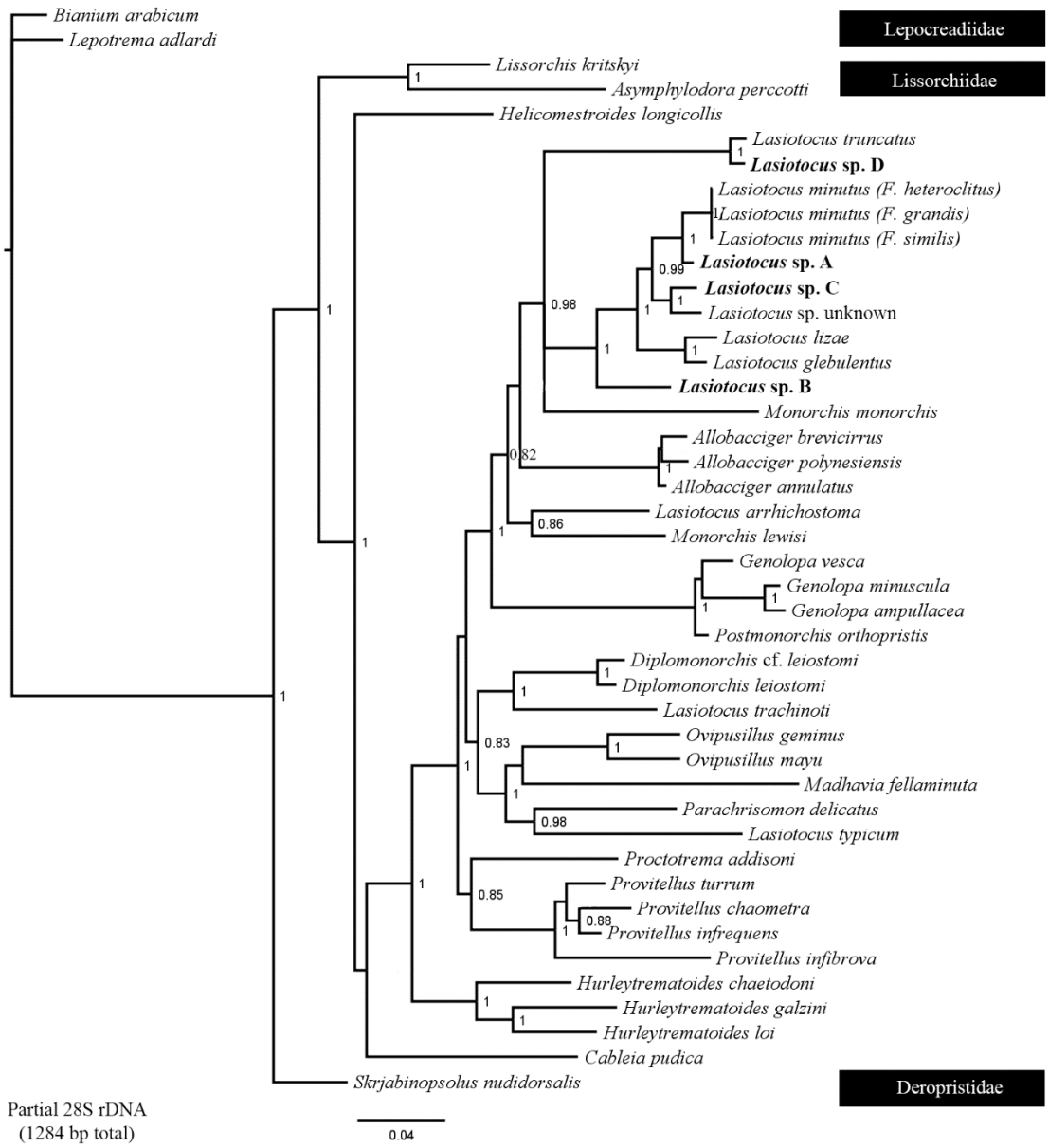

Figure 4.5 *Interrelationships among members of the Monorchiidae based on Bayesian inference analysis of partial 28S rDNA data.*

Alignment trimmed to length of Monorchis monorchis (1284 bp fragment length), not shortest sequence (Lasiotocus sp. D). Bayesian inference posterior probabilities are shown at the nodes; support values < 0.80 are not shown. Non-monorchiid taxa are shown with their respective family listed in black boxes. Newly described species of Lasiotocus from this study are higlighted with bold text. Host species for specimens of L. minutus listed in parentheses.

Figure 4.6 *Interrelationships among members of the Monorchiidae based on Bayesian inference analysis of partial 28S rDNA data.*

Alignment trimmed to shortest sequence (792 bp fragment length). Bayesian inference posterior probabilities are shown at the nodes; support values < 0.80 are not shown. Non-monorchiid taxa are shown with their respective family listed in black boxes. Newly described species of Lasiotocus from this study are higlighted with bold text. Host species for specimens of L. minutus listed in parentheses.

Three of the new species of *Lasiotocus* described in this study (*Lasiotocus* spp. A-C) fall within a larger, well-supported clade consisting of some representative species of *Lasiotocus*: *L. glebulentus, L. lizae, Lasiotocus* sp. unknown, and *L. minutus*. *Lasiotocus* sp. A is most closely related to *L. minutus,* forming a well- supported clade that is sister to another well-supported clade consisting of *Lasiotocus* sp. C and *Lasiotocus* sp. unknown. These sister groups form a clade that is sister to a well-supported clade of *L. glebulentus* and *L. lizae*. *Lasiotocus* sp. B is sister to all of them, with *M. monorchis* sister to *Lasiotocus* sp. B but poorly supported. *Lasiotocus* sp. D forms a well-supported clade with *L. truncatus* that forms a polytomy with the clade consisting of *Lasiotocus* spp. A-C - *M. monorchis* and *M. lewisi*.

Pairwise comparisons of variable sites of the partial 28S rDNA and ITS2 rDNA regions among *Lasiotocus* spp. A-D are presented in Table 4.3. ITS2 rDNA sequences were not obatined for all species, represented by "NA" in Table 4.3. *Lasiotocus* sp. A is most closely related to *L. minutus*; partial 28S rDNA sequences of the 2 differed by 2.3% (18 bp), and ITS2 rDNA sequences differed by 0.9% (3 bp). *Lasiotocus* sp. C is most closely related to *Lasiotocus* sp. unknown, and partial 28S rDNA sequences of the 2 differed by 3.2% (25 bp). *Lasiotocus* sp. A and *Lasiotocus* sp. C differed by 4.9% (39 bp) in the partial 28S rDNA region and 4.3% (15 bp) in the ITS2 region. *Lasiotocus* sp. D is mostly closely related to *L. truncatus*, and partial 28S rDNA sequences of the 2 differed by 1.8% (14 bp). *Lasiotocus* sp. B and *L. glebulentus* differed by 8.2% (65 bp) in the partial 28S rDNA region and 14.4% (50 bp) in the ITS2 region; *Lasiotocus* sp. B and *Lasiotocus* sp. C differed by 9.1% (72 bp) in the partial 28s rDNA region and 12.9% (45

bp) in the ITS2 rDNA region. *Lasiotocus glebulentus* and *L. lizae* differed by 3.0% (24 bp) in the partial 28S rDNA region and 2.3% (8 bp) in the ITS2 rDNA region.

Table 4.3 *Pairwise comparisons among fragments of partial 28S rDNA (792 base pairs long) and ITS2 rDNA (348 base pairs long) from* Lasiotocus *spp. A-D.*

Species	***Lasiotocus* sp. A**	***Lasiotocus* sp. B**	***Lasiotocus* sp. C**	***Lasiotocus* sp. D**
***Lasiotocus* sp. A**	—	39 (11.2)	15 (4.3)	NA
***Lasiotocus* sp. B**	72 (9.1)	—	45 (12.9)	NA
***Lasiotocus* sp. C**	39 (4.9)	72 (9.1)	—	NA
***Lasiotocus* sp. D**	127 (16.0)	112 (14.1)	125 (15.8)	—

Data are shown by number of base pair differences with percentage in parentheses. ITS2 rDNA data are above the diagonal; 28S rDNA data are below the diagonal. NA represents unobtained sequence data.

I also provide sequence data for *Diplomonorchis leiostomi* collected from the type-host and type-locatliy of the species. *Diplomonorchis leiostomi* sequence data from this study differed from *Diplomonorchis* cf. *leiostomi* (AY222252) sequence data publicly available that was collected from a different geographic location. The 2 partial 28S rDNA sequences differed by 2.4% (19 bp).

4.4 Discussion

Lasiotocus is polyphyletic, as is apparent from the recovered phylogenies of the Monorchiidae (Figures 4.5, 4.6) presented in this study, which include the molecular data from 4 new species of *Lasiotocus*, the first molecular data for *L. truncatus*, and molecular data for *D. leiostomi* from the type-host and type-locality, and from prior works (Wee et al., 2018, 2019; Panyi et al., 2020; Wee et al., 2020). Most publicly available molecular data from the Monorchiidae are from the Indo-Pacific Ocean, so this study contributes

substantially to the data available from monorchiids in the northwestern Atlantic Ocean by providing novel molecular data from 6 monorchiid species from 2 genera, *Lasiotocus* and *Diplomonorchis*. Prior to this study, publicly available sequence data existed for 9 monorchiids from the northwestern Atlantic Ocean; now data are available for 15 monorchiids. Despite this additional data, an important problem remains. The type-species, *L. mulli*, has not been sequenced, and I was unsuccessful in obtaining specimens, despite orchestrating several attempts by several colleagues in both the eastern and western Mediterranean Sea. Personal communication with the primary author of the most recent paper reporting *L. mulli* in the Mediterranean Sea revealed a very low prevalence of the species in her study (Ferrer-Maza et al., 2015); only 5 specimens of *L. mulli* were found from examination of over 300 specimens of *Mullus barbatus*, the type-host.

Without sequence data of the type-species, it is impossible to know the true lineage of the genus, and the recovered phylogenies show 5 possibilities. However, based on morphological data from the supplemental data of *L. mulli* from Dollfus (1948), Bartoli and Prévot (1966), and Bartoli and Bray (2004), I hypothesize that *L. mulli* is most closely related to *L. trachinoti* based on the spination pattern in the terminal organ as the key feature. The original description by Stossich (1883) is short, vague, and does not include an illustration, nor does that by Odhner (1911). Odhner (1911) merely comments on the egg size, suckers, elongated testis, and the terminal genitalia, which he merely states are exactly like that of *Monorchis* Odhner, 1911; another incomplete description that lacks illustrations. The terminal organ spines in both *L. mulli* and *L. trachinoti* are divided into 2 sections in the anterior region, with an unspined portion in the middle separating them. No other described species of *Lasiotocus* has this feature.

Additionally, both species have distinct, compact eyespots, an elongated testis, the same general body shape, vitellaria shape and location, and sucker ratios.

A great deal of morphological variability exists within the current array of nominal species of *Lasiotocus*. Approximately half are described as having funnel-shaped oral suckers and the other half do not. Some have conspicuous, compact eyespots; some have dispersed, inconspicuous pigmentation, while others do not any have eyespot pigmentation at all. Some species have gonads closer to the posterior extremity, while others have gonads in the middle to posterior third of the body. Other species have vitellaria in restricted fields at the ventral sucker to gonadal level, symmetrical or asymmetrical, and others have more extensive vitellaria towards the posterior end of the body; the vitellaria can be in masses or in poorly differentiated groups of small, numerous follicles or they can be in fewer, larger, distinct follicles. In some species the excretory vesicle extends into the forebody, and, in other species, it is a very short sac, terminating well posterior to the testis. The current generic diagnosis describes the terminal organ as bipartite with only the anterior region having spines, but some species descriptions report spination in the posterior portion as well.

Based on the clades containing species of *Lasiotocus* from the recovered phylogenies, some hypotheses about the informative features can be made. *Lasiotocus* spp. A-C form a clade with other species of *Lasiotocus* that all have vitellarium as masses or as poorly differentiated, very small, numerous follicles in the ventral sucker to gonadal zone and without a funnel-shaped oral sucker. *Lasiotocus* sp. D and *L. truncatus* form a well-supported clade, and both species have funnel-shaped oral suckers, vitellaria in conspicuous, large, less numerous follicles, restricted to the gonadal zone. However, *L.*

arrhichostoma also has a funnel-shaped oral sucker and is found in a different clade, so the funnel-shaped oral sucker alone is not a suitable morphologic differentiation.

Lasiotocus arrhichostoma forms a somewhat supported clade with *M. lewisi* in Figure 4.5, and the partial 28S rDNA region differed by 10.7% (85 bp) between the 2 species. This difference is less than what is seen among *Lasiotocus* sp. D and *Lasiotocus* spp. A-C. *Monorchis* is another genus that is morphologically different from *Lasiotocus*, so members of the 2 have not been confused often, if ever. Three important morphological differences between the 2 genera are the small, oval body shape, the vitellaria in the forebody, and a V-shaped or Y-shaped excretory vesicle in *Monorchis* and the elongated body shape, the vitellaria in the ventral sucker to gonadal zone, and an I-shaped excretory vesicle in *Lasiotocus*. However, *M. lewisi* has an I-shaped excretory vesicle, unlike what is reported in the generic diagnosis, as discussed by Cribb et al. (2018). Morphological variation in species of *Monorchis* and the possibly polyphyletic relationship in the recovered phylogenies and from previous studies suggest the current nominal species of *Monorchis* belong to more than 1 genus, especially because the type-species, *M. monorchis*, which has a V-shaped excretory vesicle, is included. *Monorchis monorchis* is in a separate clade than *M. lewisi* and represents the true lineage of the group as the type-species. One feature in common between *M. lewisi* and *L. arrhichostoma* is the I-shaped excretory vesicle extending to the ventral sucker or even more anteriorly into the forebody. Many differences, however, exist between the 2 such as body shape, vitellarium location, oral sucker shape, esophagus, and several others (Searle et al., 2014; Cribb et al., 2018).

In Figure 4.6, *L. arrhichostoma* formed a poorly supported clade with *Allobacciger* spp, and the partial 28S rDNA region between *L. arrhichostoma* and *Allobacciger annulatus* Wee, Cutmore, Sasal, and Cribb, 2020 differed by 10.1% (80 bp). This difference is closer than what is seen among *Lasiotocus* sp. D and *Lasiotocus* spp. A-C. *Allobacciger* is another genus that is distinctly different from *Lasiotocus* based on a few key features (Madhavi, 2008). *Lasiotocus* has 1 testis, whereas *Allobacciger* has 2 symmetrical testes. The ceca terminate in the anterior half of the body in *Allobacciger* and are short and inflated, as opposed to the ceca in *Lasiotocus* that terminate throughout the hindbody and are long and slender. Additionally, *Allobacciger* has the vitellarium in the forebody, at the pharyngeal level and a V-shaped excretory vesicle, whereas *Lasiotocus* has vitellarium at the level of the ventral sucker to gonadal level and an I-shaped excretory vesicle.

However, of the 3 species of *Allobacciger* represented in the phylogenies, none represents the type-species (*Allobacciger macrorchis* Hafeezullah and Siddiqi, 1970) and all species differ morphologically from the generic diagnosis of Madhavi (2008). These 3 species all have ceca extending into the hindbody and have I-shaped excretory vesicles. As a result, Wee et al. (2020) provided an amended diagnosis for *Allobacciger* including variations of the aforementioned morphological features among others observed in their new species. Wee et al. (2020) believed the excretory vesicle of the type-species material was erroneously described as V-shaped. The authors also discussed potential variability in the spination of the cirrus (unspined in the type-species) and shape of the terminal organ as possibly unipartite instead of bipartite in *Allobacciger ditrematis* (Wang, 1982) Madhavi, 2008. A few morphological features in common between *Lasiotocus* and

Allobacciger are the *usually* spined cirrus and bipartite, anteriorly spined terminal organ, usually with a muscular sphincter separating the 2 regions. Similar to *Lasiotocus*, without sequence data from the type-species, the true lineage of *Allobacciger* remains unknown.

Interestingly, *L. trachinoti* forms a well-supported clade with *D. leiostomi* and *D.* cf. *leiostomi*. The partial 28S rDNA region differed by 10.5% (83 bp) between *L. trachinoti* and *D.* cf. *leiostomi.* This difference is less than what is seen among *Lasiotocus* sp. D and *Lasiotocus* sp. A-C, which are more similar morphologically to each other yet form separate clades. *Diplomonorchis* and *Lasiotocus* are 2 genera that have not been confused because they have distinct morphological differences. The most obvious difference being *Diplomonorchis* has 2 testes, whereas *Lasiotocus* has 1 testis. Additionally, *Diplomonorchis* has a small, oval body shape; *Lasiotocus* has a more elongated body shape.

The current taxonomic key to the entire Monorchiidae uses the number of testes (1 vs. 2) as the first character to differentiate genera of the subfamily Monorchiinae, to which *Lasiotocus, Diplomonorchis, Allobacciger, Ovipusillus* Dove and Cribb, 1998, and *Madhavia* Wee, Cutmore, and Cribb, 2018, all belong (Madhavi, 2008; Wee et al., 2018; Wee et al., 2020). Because the genera in the Monorchiinae with 2 testes (*Diplomonorchis, Allobacciger, Ovipusillus,* and *Madhavia*) represented in the phylogenies in this study and a study by Wee et al. (2020) do not form a well-supported, separate clade together, this potentially provides evidence that the number of testes does not hold as much weight in morphological differentiation as has been assumed and may be an example of convergent evolution. A second example of testes number suspected as not being as informative morphologically, more likely representing a convergent trait, is

from the Lepocreadiidae, a group relatively closely related but distinct from the Monorchiidae (Bray et al., 2019). Most lepocreadiid genera have 2 testes; however, a few other genera, with publicly available molecular data, have multiple testes such as *Multitestis* Manter, 1931, *Neomultitestis* Machida, 1982, *Deraiotrema* Machida, 1982 (Bray et al., 2019). As was seen with the Monorchiidae, the lepocreadiid genera with multiple testes did not form a single, well-supported clade in the recovered phylogeny of Bray et al. (2019). These examples raise questions about the use of testes number as a synapomorphic feature. Another current example of using testes as a synapomorphic feature for differentiation is in the Lissorchiidae, the family sister to the Monorchiidae. In the Lissorchiidae, testes number (1 vs. 2) is used to determine which subfamily within each genus is classified (Madhavi, 2008). Very few lissorchiid species with representative sequence data exist, so it is not possible to investigate testes number as a real feature at this point, but future works on the lissorchiids should consider this question.

Lasiotocus typicus and *Parachrisomon delicatus* (Manter and Pritchard, 1961) Madhavi, 2008 form a well-supported clade, which is not as surprising because the 2 genera are very similar morphologically, with aspects of the vitellarium being the differentiating feature. The partial 28S rDNA region differed by 12.8% (101 bp), which is slightly less than what is seen among species of *Lasiotocus* sp. D and *Lasiotocus* spp. A-C, which form separate clades and are more similar morphologically. Madhavi (2008) established *Parachrisomon* as a new genus, and 3 species formerly classified as *Lasiotocus* were transferred to it: *Parachrisomon albulae* (Overstreet, 1969) Madhavi, 2008, *Parachrisomon decapteri* (Nahhas and Cable, 1964) Madhavi, 2008, and *P.*

delicatus. Species in *Parachrisomon* have a vitellarium shaped as lateral, tubular acini extending well into the hindbody; in contrast, species in *Lasiotocus* predominantly have a vitellarium shaped as globular follicles, restricted to the ventral sucker and gonadal level in the midbody. Additionally, some species of *Parachrisomon* have a Y-shaped excretory vesicle. *Lasiotocus typicus* has a vitellarium shaped as globular follicles as opposed to tubular acini (Bartoli and Bray, 2004), and *P. delicatus* has vitellaria described as elongated follicles (Manter and Pritchard, 1961). Both are in the same region of the body, but *L. typicus* has more numerous follicles (Bartoli and Bray, 2004). Additionally, both have a short excretory vesicle. However, the type-species of *Parachrisomon, P. decapteri*, does not yet have publicly available sequence data, so we do not know the true lineage for the genus. Although *L. typicus* does not have elongated vitelline follicles, it is similar to *P. decapteri* and *P. delicatus* in having a short excretory vesicle and very long esophagus (more than 2 times the length of the pharynx).

Diplomonorchis leiostomi, the type-species of *Diplomonorchis*, was described from *Le. xanthurus* (type-host) in Beaufort, North Carolina (type-locality), but the publicly available molecular data for the species were collected from *Le. xanthurus* from Ocean Springs, Mississippi (Olson et al., 2003). The novel molecular data provided in this study for *Diplomonorchis leiostomi* were collected from the type-host and type-locality. Although identified as the same species, the partial 28S rDNA fragments were not the same between the 2 geographical locations, causing me to question the identification of *D.* cf. *leiostomi* collected from Mississippi. The partial 28S rDNA region differed by 2.4% (19 bp), suggesting these are 2 separate species, possibly cryptic. Sequencing reactions of *D.* cf. *leiostomi*, collected for this study from Mississippi, failed,

which prevented me from being certain they were the same as those collected from this locality in Olson et al. (2003). An attempt was made to obtain the vouchered specimen of *D.* cf. *leiostomi* from Olson et al. (2003), but a global pandemic caused by an outbreak of SARS-CoV-2 occurred during data analysis and writing of this thesis that prevented shipping of the specimen from the Natural History Museum, London. Specimens of *D. leiostomi* collected in this study from North Carolina agree well with the original description of Hopkins (1941) and are from the type-host and type-locality, so the sequence data in this study likely belongs to the true *D. leiostomi*. Future works can obtain these specimens from USNM, the specimen of Olson et al. (2003) from the Natural History Museum, London, and the type specimen of *D. leiostomi* from USNM to solve this problem.

Without the molecular data from the type-species of *Lasiotocus*, serious inferences regarding the interrelationships of the represented species of *Lasiotocus* and nomenclatural changes cannot be made. However, the data provide us with further evidence that *Lasiotocus* is polyphyletic and shed some light into potential morphological features that may be informative for differentiation among these groups. The data suggest that combinations of terminal organ spination patterns, oral sucker shape, vitellaria shape, size, and distribution, and excretory vesicle shape and size may be key features for differentiation among clades containing species of *Lasiotocus*. Another feature to reconsider in the diagnosis of *Lasiotocus* is the presence of spines in the posterior portion of the terminal organ. For example, *Lasiotocus* sp. D does have a bipartite terminal organ, but 1 individual had spines in the posterior region that were different in both size and shape from the spines in the anterior region of the terminal organ. The generic diagnosis

of *Lasiotocus* by Bartoli and Prévot (1966) included this phenomenon. In terms of variability in the partial 28S rDNA region, *Lasiotocus* spp. A-C have 2% to 9% variability among each other and with other species in that respective clade, whereas *Lasiotocus* sp. D has 14% to 16% variability with species in the *Lasiotocus* spp. A-C clade. *Lasiotocus* sp. D and *L. truncatus*, both in the same clade, have 1.4% variability. These within-clade species variabilities in the partial 28S rDNA region are similar to those seen for other clades with congeners of monorchiid genera, e.g., *Genolopa*, *Allobacciger* (Panyi et al., 2020; Wee et al., 2020).

It is impossible to establish a benchmark or genetic ruler that determines the amount of variability in the partial 28S rDNA region or any DNA gene region that definitively separates genera within a family or species within a genus. However, the differences between the species of *Lasiotocus* in clades with species from other genera, e.g., *Lasiotocus – Monorchis, Lasiotocus – Parachrisomon,* have partial 28S rDNA variability similar to partial 28S rDNA variability between monophyletic clades of monorchiid species from 1 genus compared with others (Wee et al., 2020). This suggests that although these species of *Lasiotocus* (*Lasiotocus – Monorchis, Lasiotocus – Parachrisomon*) form clades with species from other genera in the current data set, it could be an artifact of missing taxa that cause these apparent close relationships. More molecular and morphological data from more species, particularly type-species, are needed to more thoroughly understand these relationships and re-evaluate the current classification of species and genera in the Monorchiidae, particularly in *Lasiotocus*.

CHAPTER V – SUMMARY

Many unknowns and questions remain regarding the interrelationships among monorchiids. Much of the recent morphological and molecular work has been conducted in the Indo-Pacific Ocean, with very few works including monorchiids from the Atlantic Ocean after 1980 (Olson et al., 2003; Andres et al., 2018). In response to the relative dearth of knowledge and molecular representation of the Atlantic monorchiid fauna, the main goal of this thesis was to investigate aspects of taxonomic and phylogenetic interrelationships among monorchiids from the northwestern Atlantic Ocean. I was able to provide morphological and molecular data of monorchiid species from 4 genera, *Genolopa, Lasiotocus, Diplomonorchis* and *Postmonorchis*, provide supplemental morphological data and novel molecular data for 3 type-species, *G. ampullacea, D. leiostomi* and *P. orthopristis,* provide novel molecular data for 5 other named species, and provide novel morphological and molecular data for 6 new species. The molecular data for species of *Genolopa* and *Postmonorchis* are the first representatives for their genera, from morphologically identified and vouchered adult specimens.

Using conventional morphological and molecular techniques, I was able to answer the question of whether *Genolopa* represents a lineage within the Monorchiidae, provide data on the type-species, and describe 2 new species of *Genolopa*. Confusion has existed surrounding the correct classification of some monorchiids into *Genolopa, Lasiotocus*, *Parachrisomon*, and *Proctotrema* as a result of incomplete original descriptions leading to ignorance of informative generic-level features and inappropriate fixation techniques leading to opposing interpretations of features by various taxonomists. Using both the morphological and molecular data obtained during this work,

I concluded that the features relating to components of the terminal genitalia (spiny genital atrium in conjunction with a bipartite, anteriorly spined terminal organ) are key features in the generic diagnosis to differentiate species of *Genolopa* from species in morphologically similar genera, with the phylogenetic analysis supporting *Genolopa* as a lineage distinct from *Lasiotocus, Parachrisomon,* and *Proctotrema*.

Cryptic speciation is another important topic in trematode taxonomy and systematics, with examples and suspected examples existing within the Monorchiidae along with many other families. One such suspected complex of cryptic species was *L. minutus*. I used combined morphological, morphometric, and molecular approaches to investigate if *L. minutus* represented a complex of cryptic species throughout its extensive range and various intermediate and definitive host species. I was able to obtain specimens from only definitive hosts, from only 2 geographic locations, and from only 1 rDNA gene region (28S rDNA region). The 3 analyses did not show any differences among specimens of *L. minutus* from the various hosts and locations, suggesting it is not a complex of cryptic species. However, more data are required to come to a well-supported conclusion about the cryptic species status of *L. minutus* such as data from more DNA regions (both rDNA [at least ITS2] and mtDNA), from more geographic locations, and from the various intermediate hosts.

Finally, I described 4 new species of *Lasiotocus* and provided novel molecular data for them in this work. Phylogenetic analyses provided further evidence that *Lasiotocus* is polyphyletic. I cannot know the true lineage without the sequence data of the type-species. However, based on the various interrelationships observed, some hypotheses can be made about the real synapomorphic features within some groups in the

Monorchiidae, e.g., 2 areas of spines in the anterior region of the terminal organ and distinct eyespots in *L. mulli* and *L. trachinoti* and vitelline follicle size and number (numerous, smaller, poorly differentiated follicles vs. few, larger, distinct follicles), and features that are likely convergent, not demonstrating synapomorphy, e.g., number of testes (1 vs. 2).

No obvious cophyly (coevolution between parasite and host) exists within the current recovered phylogeny between monorchiids and definitive hosts. However, 1 monophyletic clade consisting of *Lasiotocus* sp. B, *L. glebulentus*, *L. lizae*, *Lasiotocus* sp. A, *L. minutus*, *Lasiotocus* sp. C, and *Lasiotocus* sp. unknown provides evidence of an ecological association between those monorchiid species and their definitive hosts. The 7 aforementioned monorchiids are found in euryhaline fish hosts that inhabit brackish, estuarine (often saltmarsh) habitats and exhibit omnivorous or detrivorous feeding behavior that likely puts them in direct trophic interaction or close contact with the bivalve intermediate hosts.

Although this thesis provides a great amount of new information on monorchiids, there is still much more work to be done. Future works should target more monorchiid species from the northwestern Atlantic Ocean to continue documenting the biodiversity that exists in this region of the world and to gather more data to help clarify evolutionary relationships among already described species. The molecular data obtained can be expanded to include more rDNA regions and to include mtDNA regions, as well. Additionally, intermediate hosts can be targeted to improve our understanding of monorchiid life cycles, as we currently have data on the life cycles of less than 20 monorchiid species. This information can also contribute to investigations of cryptic

species complexes to have a multifaceted approach involving life cycle data, morphological data, morphometric data, and molecular data.

APPENDIX INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICES OF COMMITTEE ACTION

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	18101102
PROJECT TITLE:	Collection, Maintenance, Life Histories, Taxonomy, and Experimental Studies with Parasites, Disease-Causing Agents, and Host Biology of Fishes
PROPOSED PROJECT DATES:	10/2018 – 09/2021
PROJECT TYPE:	Renewal (replaces 15101503)
PRINCIPAL INVESTIGATOR(S):	Robin Overstreet
DEPARTMENT:	Coastal Sciences
FUNDING AGENCY/SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Designated Review Approval
PROTOCOL EXPIRATON DATE:	September 30, 2021

Jake Schaeffer, PhD
IACUC Chair

October 20,2018
Date

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