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Connections in the Underworld: A Morphological and Molecular Study of Diversity and Connectivity among Anchialine Shrimp.

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

CONNECTIONS IN THE UNDERWORLD: A MORPHOLOGICAL AND
MOLECULAR STUDY OF DIVERSITY AND CONNECTIVITY AMONG
ANCHIALINE SHRIMP

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Robert E. Ditter

2020

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Robert E. Ditter, and entitled Connections in the Underworld: A Morphological and Molecular Study of Diversity and Connectivity among Anchialine Shrimp, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: November 10, 2020

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Florida International University, 2020

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DEDICATION

This dissertation is dedicated Peggy Sue Wayne who gave me courage and motivation to always follow my dreams, no matter how silly it may seem or what other people think. I would also like to dedicate this dissertation to Matt Inman and Jibbers Crabst who gave me the inspiration to tough it out and finish, even during times of crisis. Last, but not least I would like to dedicate this to all my teachers, my parents, everyone whose has shed blood that made this all possible and Ms. Aubree Zenone. Without their support, none of this would have been possible.

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ABSTRACT OF THE DISSERTATION

CONNECTIONS IN THE UNDERWORLD: A MORPHOLOGICAL AND
MOLECULAR STUDY OF DIVERSITY AND CONNECTIVITY AMONG
ANCHIALINE SHRIMP

by

Robert E Ditter

Florida International University, 2020

Miami, Florida

Professor Heather D. Bracken-Grissom, Major Professor

This research investigates the distribution and population structure of crustaceans endemic to anchialine systems in the tropical western Atlantic focusing on cave-dwelling shrimp from the family Barbouriidae. Taxonomic and molecular tools (genetic and genomic) are utilized to examine population dynamics and the presence of phenotypic hypervariation (PhyV) of the critically endangered species *Barbouria cubensis* (von Martens, 1872). The presence of PhyV and its geographic distribution is investigated among anchialine populations of *B. cubensis* from 34 sites on Abaco, Eleuthera, and San Salvador, Bahamas. Examination of 54 informative morphological characters revealed PhyV present in nearly 90% (n=463) of specimens with no identifiable geographic distribution. Updated range descriptions of *Macrobrachium lucifugum* Holthuis, 1974, *Parhippolyte sterreri* (Hart & Manning, 1981) and *B. cubensis* in the western Atlantic are provided with observations on the behavior of *B. cubensis* from over 10 years of work. Molecular phylogenetic analyses of a combination of two mitochondrial and five nuclear gene regions are used for molecular barcoding, to further investigate PhyV and to

re-examine the current classification of the family Barbouriidae. The results find no evidence of cryptic speciation, or genetic differentiation among individuals of *B. cubensis* (n=72). Morphological and molecular evidence supports synonymizing *Janicea* within *Parhippolyte* and recognition of the sub-families Calliasmatinae Ditter *et al.*, 2020 and Barbouriinae Christoffersen, 1987. Connectivity among anchialine systems and population structure of *B. cubensis* across the tropical western Atlantic is examined using a combination of next generation molecular tools. Genomic analyses suggest a single highly connected population of *B. cubensis* with low genetic diversity and a low effective population size, which conflicts with the generally accepted isolation paradigm of anchialine systems. Our findings support the value added to genomic studies by generating a partial draft genome constructed using *de novo* hybrid assembly of short read RADseq and Nanopore long read sequencing data through the investigation of the anchialine isolation paradigm.

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PREFACE

The following chapters have been or will be submitted for publication and are formatted according to journal specifications:

CHAPTER II

Ditter, R. E., Erdman, R. B., Goebel, A. M., and Bracken-Grissom, H. D. 2019.

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CHAPTER III

Ditter, R. E., Snyder, A. P., Schulman, S. E., and Bracken-Grissom, H. D. 2019. Notes on the behavior and first records of three enigmatic anchialine shrimps (Decapoda: Caridea: Barbouriidae, Palaemonidae) in the Bahamas. *Journal of Crustacean Biology*, 39(6): 684–688. [Reproduced with permission from copyright holder, Oxford University Press]

CHAPTER IV

Ditter, R. E., Mejía-Ortíz, L. M., and Bracken-Grissom, H. D. 2020. Anchialine adjustments: an updated phylogeny and classification for the family Barbouriidae Christoffersen, 1987 (Decapoda: Caridea). *Journal of Crustacean Biology*, 40(4): 401–411. [Reproduced with permission from copyright holder, Oxford University Press]

CHAPTER V

Ditter, R. E., Mejía-Ortíz, L. M., and Bracken-Grissom, H. D. In prep. Endangered cave shrimp reveal high connectivity despite an anchialine isolation paradigm across the tropical western Atlantic (Decapoda: Caridea: *Barbouria cubensis*).

CHAPTER I
INTRODUCTION

A common feature of the tropical western Atlantic are anchialine habitats with unique and endemic species (Iliffe, 2002). Anchialine communities exhibit characteristics of isolated populations, which allow them to function as natural laboratories (Edwards, 1996; Iliffe, 2002), making them ideal for studies regarding evolution, speciation and adaption (Sket, 1996; Dawson, 2005; Humphreys *et al.*, 2009; Christian *et al.*, 2010; Gonzalez *et al.*, 2011; Protas, *et al.*, 2011; Bishop & Iliffe, 2012; Iliffe, 2012; Becking *et al.*, 2013; Martin & Wainwright, 2013; Weese *et al.*, 2013). Anchialine ecosystems have provided insight into ancient communities prior to the dominance of vertebrates (Humphreys *et al.*, 2009; Becking *et al.*, 2013; Rose *et al.*, 2016). Often occurring in areas of high demand for tourism, anchialine habitats are being rapidly altered by development, pollution, and climate change (Wilson, 1985; Sket, 1999; Iliffe, 2002; Schawbe *et al.*, 2010; Iliffe *et al.*, 2015). Anchialine organisms are easily impacted by disturbances resulting from their endemism or restricted distributions, as a consequence of being high specialized for specific environmental conditions or low genetic diversity respectively (Humphreys *et al.*, 2009; Becking *et al.*, 2013). Conservation of these unique systems is increasingly urgent. As anchialine habitats are being destroyed the opportunity of substantial discoveries are quickly vanishing (Iliffe, 2002; Pérez-Moreno *et al.*, 2016).

The present uses traditional taxonomic and molecular techniques to examine genetic diversity, evolution, phylogeography, and the recent appearance of phenotypic hypervariation among anchialine shrimp within the family Barbouriidae to increase our understanding of anthropogenic impacts on anchialine ecosystems. To study these impacts, specimens collected were used in taxonomic and phylogenetic studies to examine population structure and potential sources of phenotypic hypervariation. The

current study resulted in an improved understanding of the evolutionarily relationships, estimates of population health, and an enhanced understanding of the connections and origins of anchialine organisms.

Advances in molecular techniques provide novel approaches and high-resolution information for studying non-model organisms and habitats with limited accessibility. With many prevalent anthropogenic threats and the delicate nature of anchialine habitats, the need to advance our understanding of these systems is clear. Next-generation molecular tools provide a scale of resolution previously not attainable and offer the ability to answer long-standing questions of the population dynamics and evolutionary processes underlying life in anchialine habitats. The study utilized a multitude of approaches to the study of non-model organisms, from traditional taxonomic methods to high resolution population analyses utilizing a partial draft genome assembled from a combination of Oxford NanoPore long read and Illumina short read genomic sequencing data.

Widespread phenotypic hypervariation in the enigmatic anchialine shrimp Barbouria cubensis (Decapoda: Barbouriidae)

Among anchialine crustaceans is a group of enigmatic shrimps from the family Barbouriidae Christoffersen, 1987 (Manning & Hart, 1984, De Grave *et al.*, 2009). Barbouriid shrimps have been described as having a tethyan distribution pattern along tropical latitudes (Manning & Hart, 1984). As a result of limited habitat availability, species of Barbouriidae have been placed on the IUCN's Red List (Iliffe, 1996). *Barbouria cubensis* is a species found exclusively in anchialine pools throughout the

tropical western Atlantic ranging from the Bahamas, Bermuda, Turks and Caicos, Cayman Brac and the Yucatán Peninsula (Mejía-Ortiz *et al.*, 2008).

Previous records have documented slight morphological variations for *B. cubensis* limited to the spines and length of the rostrum, ratio of length versus width of the scaphocerite, number of subdivisions of the second pereopods, length and spination of the appendix masculina and the body size of the species (Rathbun, 1912; Chace, 1972; Holthuis, 1973; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981; Hart *et al.*, 1985). Recent exploration of anchialine pools on the Bahamian islands of San Salvador, Eleuthera and Abaco from 2012–2015 has not only revealed the presence of extensive morphological variation in the previously documented variable characters, but also extends to an additional 50 morphological characters. On the basis of the high rate and extensive range of morphological variation that greatly deviates from any previous taxonomic records we described the presence of these phenotypes as phenotypic hypervariation (PhyV). Our objectives were to document PhyV exhibited by *B. cubensis* from three Bahamian islands, determine if PhyV is associated with geographic distribution, and to confirm the identity of morphologically variable specimens using molecular barcoding of two partial mitochondrial gene regions (16S and COI).

Notes on the behavior and first records of three enigmatic anchialine shrimps

(Decapoda: Caridea: Barbouriidae, Palaemonidae) in the Bahamas

A common feature of anchialine systems are the rare and often endemic species found within them (Edwards, 1996; Iliffe, 2005; Sket, 2005). The biodiversity within anchialine systems is presently being threatened by anthropogenic activity leading to habitat

destruction (Iliffe *et al.*, 1984; Culver & Sket, 2000; Iliffe & Kornicker, 2009). Among anchialine inhabitants *Barbouria cubensis* (von Martens, 1872) and *Parhippolyte sterreri* (Hart & Manning, 1981) are listed by the IUCN (2019) as critically endangered. It is imperative to study threatened anchialine species so that appropriate management efforts can be made to protect these enigmatic organisms. To this end, we reported on the first occurrence of *Barbouria cubensis*, *Parhippolyte sterreri* and *Macrobrachium lucifugum* Holthuis, 1974 in the anchialine pools of Acklins, Bahamas. We also discussed observations made for these species during collections as well as the behavior of *B. cubensis* in its natural habitat and in a laboratory setting over a 2-year period.

Anchialine adjustments: an updated phylogeny and classification for the family Barbouriidae Christoffersen, 1987 (Decapoda: Caridea)

Molecular methods can be especially useful to delineate species, especially when traditional taxonomic identification is not possible (e.g., metamorphic life stages). DNA barcoding is particularly useful for *B. cubensis* as PhyV has led to conflicted morphological identifications. Variances in genetic data can be sufficient to delineate species among morphologically similar individuals in taxa that form cryptic species complexes (Brian *et al.*, 2006; Bracken-Grissom *et al.*, 2014). For *B. cubensis*, where no geographic pattern of PhyV was detected, it is possible that other unconsidered barriers may exist. The family Barbouriidae has undergone substantial revisions in recent years (Christoffersen, 1989; Christoffersen, 1990). The evolutionary history of the family remains unclear and presently lacks a formal description (De Grave *et al.*, 2009; De Grave *et al.*, 2014). Recent collections have allowed us to include a greater number of

representative genera and gene regions than prior studies (Bracken *et al.*, 2009; Li *et al.*, 2011; Baeza, 2013; Aznar-Corman *et al.*, 2015). Our objective was to revisit the current classification and clarify the relationships among genera of Barbouriidae.

Endangered cave shrimp reveal high connectivity despite an anchialine isolation paradigm across the tropical western Atlantic (Decapoda: Caridea: Barbouria cubensis)

The family Barbouriidae are endemic to disjunct anchialine systems, nominating them as valuable species for investigating connectivity among vulnerable anchialine communities Dawson, 2005; Christin *et al.*, 2010; Becking *et al.*, 2013; Weese *et al.*, 2013; Rose *et al.*, 2016). Among them, *Barbouria cubensis* has the broadest distribution in the western Atlantic, however their dispersal abilities remain unknown (Bohonak, 1999; Kano & Kase, 2004; Oha & Paal, 2004; Santos, 2006; Turner *et al.*, 2008; Russ *et al.*, 2010; Onaga *et al.*, 2012).

The advent of next generation molecular techniques (e.g. RADseq) permits unprecedented resolution for a variety of long-standing questions regarding phylogeography and population structure (Davey & Blaxter 2010; Eaton & Ree, 2013; McCormack *et al.*, 2013; Timm & Bracken-Grissom, 2015). However, the effectiveness of population level analyses using RADseq data alone is limited in organisms that lack full genome sequences, because of its inability to calculate effective population size (N_E) and Linkage Disequilibrium (LD) (Ekblom & Galindo, 2011; Hoban *et al.*, 2016). Having a reference genome when conducting population analyses allows the calculation of N_E , LD, and allows the analysis of genomic structural variations (Calafell *et al.*, 2001; Li & Durbin, 2009; Istace *et al.*, 2017). Using a reference genome also greatly decreases the

number base calling errors, while it increases the accuracy and coverage depth of genomic data assembly (Austin *et al.*, 2017; Janser *et al.*, 2017; Tan *et al.*, 2017). The present study utilized a *de novo* hybrid assembly of short read and long read genomic sequences to construct a partial draft genome of *B. cubensis*.

The next-generation molecular approach used in the current study provides high resolution data and potential for answering questions surrounding population connectivity and genetic diversity (Fernandes *et al.*, 2011). We hypothesized that PhyV may be the result of low genetic diversity (*i.e.*, inbreeding) as morphological abnormalities have been correlated to low genetic diversity in other species (McMillan *et al.*, 2006; Duarte *et al.*, 2008; Lacy & Alaks, 2012). To test the isolation paradigm of anchialine cave systems and to further investigate the source of PhyV next generation molecular techniques are employed to investigate population dynamics and dispersal of the critically endangered *B. cubensis*, (Austin *et al.*, 2017; Janser *et al.*, 2017; Tan *et al.*, 2017).

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CHAPTER II

WIDESPREAD PHENOTYPIC HYPERVARIATION IN THE ENIGMATIC ANCHIALINE SHRIMP *BARBOURIA CUBENSIS* (DECAPODA: BARBOURIIDAE)

ABSTRACT

Classification and evolutionary relationships among anchialine shrimp from the family Barbouriidae Christoffersen, 1987, has long been a topic of debate amongst crustacean taxonomists. To date, no study has examined morphological or molecular variation among populations of these enigmatic shrimp. The present study documents and analyzes patterns of widespread morphological variation within populations of *Barbouria cubensis* von Martens, 1872, from anchialine pools on three Bahamian islands. Such extensive morphological variation confounds identification using classic taxonomical methods. Phenotypic variation is by no means a new topic, but studies of decapods are typically limited to isolated individuals or few morphological characters. Moreover, past studies of *B. cubensis* do not report extensive morphological variation, however we find that upwards of 90% of individuals are affected. Anomalous phenotypes are described in 54 morphological characters with no detectable pattern associated with geographic distribution. The term phenotypic hypervariation (PhyV) is used to describe morphological variation that greatly deviates from any previous taxonomic descriptions. Analysis of partial sequences of the 16S and COI mitochondrial genes confirm the identity of morphologically variable specimens as a single species (*B. cubensis*) without population structure across the tropical western Atlantic. The lack of genetic variation within proposed populations of *B. cubensis* suggests PhyV is not correlated with cryptic diversity. Morphological variation at this scale likely depends on recent changes either to their environment or genetic diversity.

INTRODUCTION

A common feature among many tropical islands in the tropical western Atlantic is the presence of seemingly disjointed anchialine pools, which are subterranean estuaries influenced by tidal flow (Stock, 1986; Bishop *et al.*, 2015; Pérez-Moreno *et al.*, 2016). Subsurface tidal flows can be strong enough to permit marine conditions in the landlocked pools via seawater exchange through conduits created by the dissolution of the underlying carbonate platform (Edwards, 1996; Mylroie & Carew, 2003). However, biological connectivity and diversity is largely restricted, which has led to the “ecological islands within islands” hypothesis (Edwards, 1996; Pérez-Moreno *et al.*, 2016). Most physical connections between anchialine pools and the surrounding ocean remain unexplored and form extensive spatially complex subterranean networks (Becking *et al.*, 2011; Mylroie & Mylroie, 2011; Bishop *et al.*, 2015). The biota found within these pools is often rare and endemic, with crustaceans representing over 80% of animal biodiversity in these systems (Iliffe, 2000; Iliffe, 2005; Sket, 2005; Iliffe, 2009). Among anchialine pool inhabitants is a group of enigmatic caridean shrimp from the family Barbouriidae that live within the caves and pools (Manning & Hart, 1984; De Grave *et al.*, 2009). Barbouriid shrimps are described as having a tethyan distribution pattern along tropical latitudes, mostly confined to land locked pools (Manning & Hart, 1984). The family Barbouriidae Rafineque, 1815 is comprised of *Barbouria* Rathbun, 1912, *Parhippolyte* Borradaile, 1900, *Janicea* Manning & Hart, 1984 and *Calliasmata* Holthuis, 1973, which contain a total of 11 species. Until the recent reclassification of *Calliasmata* (De Grave *et al.*, 2014), Barbouriidae was united by the presence of a unique subocular tooth on the carapace (Manning & Hart, 1984; Clark, 1989; Chace, 1997). Additionally, these shrimps

are described as having a mandible with a 3-jointed palp lacking incisor process, long slender pereopods, the first two pairs chelate with a multiarticulated carpus and merus, and an appendix masculina shorter or subequal to the length of the appendix interna on the endopod of the 2nd pleopod in males (Chace, 1972; Manning & Hart 1984; Christoffersen, 1987). Characters used to discriminate among members of Barbouriidae include; width of the cornea relative to the eyestalk, ratio of the length versus the width of the scaphocerite, teeth of the rostrum, arrangement of arthrobranchs and podobranchs, subdivision of the articles of pereopods three to five, ratio of the rostrum length versus height, number of setae of the appendix masculina, and shape of the terminal margin of the telson (Chace, 1972; Manning & Hart, 1984; Wicksten, 1996; Mejía *et al.*, 2008).

Barbouria cubensis is a common species found within anchialine pools throughout the tropical western Atlantic ranging from Bermuda, the Bahamas, Turks and Caicos, Cayman Brac and the Yucatán Peninsula of Mexico (Mejía *et al.*, 2008). Species within the genus *Barbouria* can most notably be identified by the presence of darkly pigmented cornea that are narrower than the eyestalk, and the lack of subdivision of the articles of pereopods three to five (Fig. 1) (Chace, 1972; Manning & Hart 1984). Previous records have documented minor morphological variation for *B. cubensis* but are limited to the length and teeth of the rostrum, ratio of length versus width of the scaphocerite, number of subdivisions of the articles of the second pereopod, length and spination of the appendix masculina and the body size (Rathbun, 1912; Holthuis, 1963; Chace, 1972; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981; Manning & Hart, 1984; Mejía *et al.*, 2008). However, recent examination of *B. cubensis* from anchialine pools on the Bahamian islands of Abaco, Eleuthera and San Salvador from 2012–2015

has revealed a wide range of morphological variation in these previously documented characters (Rathbun, 1912; Holthuis, 1963; Chace, 1972; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981; Manning & Hart, 1984; Mejía *et al.*, 2008), but also extends to an additional 50 morphological characters. For the purposes of this manuscript, we use the term phenotypic hypervariation (PhyV) to describe the morphological variation that deviates from previous taxonomic descriptions of *B. cubensis* (Rathbun, 1912; Holthuis, 1963; Chace, 1972; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981; Manning & Hart, 1984; Mejía *et al.*, 2008). To have a comparative framework for describing variation, we have defined “normal” to include all previous descriptions of morphological variation.

Molecular methods can be used to delimitate species, especially when the use of traditional taxonomic methods is not possible (Monaghan *et al.*, 2005; Clare *et al.*, 2007; Raupach *et al.*, 2010). This can be particularly useful for *B. cubensis* as PhyV confounds traditional methods of identification. In recent years, several studies have shown the benefit of using molecular methods when cryptic species complexes exist with extensive phenotypic variation (Lefébure *et al.*, 2007; Steinauer *et al.*, 2007; Trontelj *et al.*, 2009; Neiber *et al.*, 2012; Weese *et al.*, 2012; Cornils & Held, 2014). With molecular barcoding it is possible to identify specimens exhibiting PhyV and determine if PhyV is the result of cryptic diversification.

The objective of this study is to document PhyV exhibited by *B. cubensis* collected from three Bahamian islands, and to determine if PhyV is associated with geographic distribution or cryptic genetic diversity. Timely documentation of this species is imperative, as rapid human development, pollution and continued natural disturbances

(i.e., hurricanes) of tropical coastal areas is a formidable threat to these critically endangered animals. Fundamental studies of biodiversity are still needed to adequately protect endemic anchialine organisms.

METHODS

SAMPLE COLLECTION

Between June of 2012 and July 2015, 363 shrimps were collected from 25 anchialine pools on San Salvador, Bahamas (Fig. 2a). Suitable habitats were identified from previous records, Google Earth and information provided by island residents. Samples from this island were collected with hand nets or by baited minnow trap. For sites where conduits could not be located from land, snorkeling was utilized to locate the conduits and place minnow traps. In addition to the collection on San Salvador, 41 specimens from Abaco were donated by Dr. Craig Layman from three anchialine pools to the south of Little Harbour (Fig. 2b). Ninety-five specimens from Eleuthera were donated by Dr. Jocelyn Curtis-Quick from six anchialine pools distributed across the entire island (Fig. 2c). Tissue samples from Mayaguana, Bahamas and Mexican specimens were donated by Dr. Darryl L. Felder and Dr. Tom Iliffe respectively. A maximum of 30 shrimp were retained from each site per sampling effort. A total of 529 shrimp were examined for this study, however 463 were included in the final morphological analysis. Datum collected from 65 individuals determined to be juveniles, too heavily damaged or identified as *Parhippolyte sterreri* were excluded from analyses. Most individuals could not be identified to *Barbouria cubensis* based on taxonomic keys, however individuals were confirmed using DNA barcoding techniques. Specimens are deposited at the Oxford

University Museum of Natural History Zoology Collection (OUMNH.ZC) and the Florida International Crustacean Collection (FICC). Specimens archived within the FICC are cataloged with the prefix HBG.

MORPHOLOGICAL DATA COLLECTION

Fifty-four morphological characters were examined to document the extent of morphological variation in specimens collected from the Bahamas (see appendix, Tables AI–III). Initially, 14 morphological characters were chosen for examination because they represent common diagnostic characters in caridean taxonomy (Hobbs *et al.*, 1977; Hobbs, 1978; Manning & Hart 1984; Chace, 1996; Bauer, 2004; De Grave *et al.*, 2014). These include: three carapace characters, three rostral characters, two eye characters, three telson characters and three pereopod characters (Fig. 2). The presence of gills and exopods of the maxillipeds and pereopods were excluded in analyses because of inconsistency among previous records (Christoffersen, 1987; Wicksten, 1996). After PhyV was found during preliminary examinations of diagnostic characters, 13 additional characters believed to be conserved among carideans were included based on the advice of caridean taxonomic experts, Drs. Sammy de Grave and Raymond Bauer. These characters include five pereopod characters, two pleopod characters and six mouthpart characters. Twenty-seven additional characters diagnostic to the family Barbouriidae *sensu lato* were examined to document the extent of family and species-specific morphological variations across individuals (Rathbun, 1912; Chace, 1972; Hart & Manning, 1981; Manning & Hart, 1984; de Grave *et al.*, 2014). Lastly, the presence and position of sensory dorsal organs (SDO) were examined as they are believed to be present

in some form in most crustaceans (Laverack *et al.*, 1996; Laverack & Macmillan, 1999; Lerosey-Aubril & Meyer, 2013). Although the ultrastructure and function of SDOs can only be examined with the use of scanning electron microscopy (SEM) and histology their presence and external aspects can easily be found and described (Brandt, 1988; Lerosey-Aubril & Meyer, 2013). This character was examined for its potential as a new diagnostic character for the family Barbouriidae due to the recent reclassification of the genus *Calliasmata*, which lacks characters considered as synapomorphies for barbouriid shrimps (de Grave *et al.*, 2014).

MORPHOLOGICAL DATA PROCESSING

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was conducted to determine if there is any pattern in the distribution of PhyV across Abaco, Eleuthera and San Salvador, Bahamas. For the purposes of this manuscript we define the expected phenotype of *B. cubensis* to include the combination of all previous morphological descriptions (Rathbun, 1912; Holthuis, 1963; Chace, 1972; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981; Manning & Hart, 1984; Mejía *et al.*, 2008). Qualitative characters (see appendix, Tables A1–3) were coded for analysis, as follows: character states matching previous descriptions of *B. cubensis* were coded as 1 and characters states not matching *B. cubensis* were coded as 2. Values were selected to prevent artificial bias. Subset #1 was generated to maximize the number of individuals included in analyses, using data for 25 characters that was collected for all 463 specimens (see appendix, Table A1). Subset #2 was generated to maximize the number of characters included in analyses, using data for 50 characters for 121 specimens (see appendix, Table A2). Subset #3 was

generated to compare examine sexual dimorphism (see appendix, Table A3). All subsets included individuals from Abaco, Eleuthera and San Salvador, Bahamas. Data collected on the relative lengths of the segments of the antennule peduncle and pereopods was excluded, because the diagnosed states are estimated measures. Data on the arrangement of the gill complement was deemed unreliable to establish the expected state for *B. cubensis* and was also excluded due to inconsistencies in previous records and the possibility of inaccuracy due to damage (Christoffersen, 1987; Wicksten, 1996).

Normality of data was tested using the Shapiro-Wilk statistics in R version 3.3.1 (R Core Team, 2016). Where necessary, values were normalized using log-transformation (Brian, et al., 2006). Values for each character were averaged by locality to avoid any bias resulting from the differences in samples size (Baltanás, et al., 2002). Individuals were grouped by Longitude. A UPGMA tree was constructed using Euclidean distance between rows with 1000 bootstraps in SPSS ver. 22 (IBM, Armonk, NY). Based on the results of the UPGMA, no further analyses were conducted.

MOLECULAR DATA COLLECTION

To investigate the presence of cryptic speciation across the Bahamas, molecular data was collected from 69 specimens of *Barbouria cubensis*. Sampling localities on San Salvador Island were grouped into seven regions based on geographic distance and topographic features separating each region. Six individuals were randomly selected from each region, with the criteria individuals from each sampling site were included and that half of the individuals exhibit PhyV to ensure diversity. Two specimens were randomly selected from each sampling locality from Abaco and Eleuthera. Tissue was collected

using non-destructive methods to preserve specimen integrity, and preserved in 95-100% EtOH and stored at -20°C. All tissue samples from Mayaguana, Bahamas and the Yucatán Peninsula of Mexico were included. Additional sequences were acquired from GenBank.

Total genomic DNA was extracted from muscle tissue of the abdomen, antennule or the 3rd to 5th pleopod using DNeasy® Blood and Tissue Kits (Qiagen, Valencia, CA). For incomplete tissue digestions, 10µl of 10% DTT and 10µl Proteinase K was added, and samples were incubated until complete digestion was achieved. Total genomic DNA quality was visualized using 2% agarose gels and concentration was measured using the Qubit dsDNA HS Assay kit on the Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, CA) according to manufacturer's instructions.

Two partial mitochondrial genes were selected for their utility in decapod studies of identification (DNA barcoding), genetic diversity, and phylogeny (Bracken *et al.*, 2009; Beaza, 2013; De Grave *et al.*, 2014; Aznar *et al.*, 2015). The mitochondrial genes included the 16S large ribosomal subunit (~550 basepairs (bps)) and protein-coding cytochrome oxidase I (~600 bps, COI). The large ribosomal subunit (16S) was amplified with primers 16S-1471/1472 (Palumbi *et al.*, 1991; Crandall & Fitzpatrick 1996), COI was amplified with primers F/10, or LCO1490/HCO2198 (Folmer *et al.*, 1994; Bracken-Grissom *et al.*, 2014). Amplification was performed in 25µl volume reactions containing 12.5µl GoTaq DNA Polymerase (Promega, Madison, WI), 1µl forward and reverse primer for each gene, 9.5µl sterile H₂O and 1µl template DNA. The thermal cycling profile conformed to the following parameters: Initial denaturation for 5 min at 95°C followed by 35 cycles of 30 secs at 94°C, 45 secs at 48-56°C, 45 secs at 72°C and a final extension of 5 min at 72°C. PCR products were sent to GENEWIZ for amplicon purification and subsequent

sequencing (South Plainfield, NJ,). All sequencing data was visually inspected, quality trimmed, manually cleaned and assembled using Geneious 9.1.7 (Biomatters Ltd., Newark, NJ). Once assembled, sequences were aligned using MAFFT v7.308 (Katoh & Standley, 2013). To identify potential pseudogenes, we translated protein-coding sequences and checked for insertions and deletions, stop codons and identified the open reading frames, and compared sequences among conspecifics following the protocol of Song *et al.* (2008).

PHYLOGENETIC ANALYSES

Individual gene trees for 16S and COI were constructed alongside a concatenated dataset of 16S and COI sequences to investigate cryptic diversity in *Barbouria cubensis* (Table 1). Missing data were designated as a “?” in our alignment. To improve resolution, both genes were concatenated into a single alignment and partitioned by gene (Ahyong & O’Meally, 2004; Porter *et al.*, 2005; Robles *et al.*, 2007; Page *et al.*, 2008). We conducted a partition test of heterogeneity and incongruence length difference test to determine if the gene regions were appropriate to combine for analyses, as implemented in PartitionFinder 2.7.1 and PAUP* respectively (Swofford, 2002; Lanfear *et al.*, 2016). The model of evolution that best fit the individual data genes was determined by Partitionfinder 2.7.1. Independent models of evolution and parameters were partitioned in the Bayesian concatenated analysis.

The Maximum Likelihood (ML) analyses were conducted using RAxML 7.2.8 (Randomized Accelerated Maximum Likelihood, Stamatakis, 2014) with computations performed on the high-performance computing cluster at Florida International University.

Likelihood settings followed the General Time Reversible Model (GTR) with a gamma distribution and RAxML estimated all free parameters. Confidence in the resulting topologies was assessed using Rapid Bootstrapping and a search for the best-scoring tree with 1000 replicates (Felsenstein, 1996). Bayesian Inference (BI) analyses were performed using parameters selected by PartitionFinder 2.7.1 and conducted in MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001). A MCMC algorithm ran for 10,000,000 generations, sampling 1 tree every 1000 generations. Observation of likelihood scores allowed us to determine burn-ins and stationary distributions. Once split frequency in the Bayesian analysis reached < 0.01 a 50% majority-rule consensus tree was obtained from the remaining trees. Posterior probabilities for clades were compared for congruence between analyses, Bootstrap values >70 for RAxML and >0.90 for Bayesian are presented on the phylograms.

RESULTS

Over 90% of the 463 *B. cubensis* examined exhibit morphological variation beyond previous descriptions in one or more characters. PhyV exhibited in these shrimps often falls outside of characters prescribed to any genus of Barbouriidae or members of superfamily Alpheoidea (Table 1, Fig. 3: a–x). PhyV is present in individuals from all 34 localities sampled across Abaco, Eleuthera and San Salvador, Bahamas.

DIAGNOSIS OF PHENOTYPIC HYPERVARIATION IN *BARBOURIA CUBENSIS*

CARAPACE. Carapace is smooth; in some cases, a strong protuberance laterally or lateral grooves originating from the cardiac region are present (Fig. 3: f, g & l). While in most individuals the dorsal margin of the carapace forms a gradual rounded slope from the postorbital margin to the posterior margin, in some individuals the dorsal margin forms a distinct hump (see appendix, Fig. A1: a–c). Antennal and branchiostegal teeth are present in most specimens, but either could be absent. Some specimens lacking teeth the carina is present and multiple teeth originate from the carapace margin anterior to the carina (see appendix, Fig. A1: d–e). Approximately 18% (n=463) of individuals do not match previous descriptions of *B. cubensis* for these characters. Additionally, all specimens have at least two sensory dorsal organs (SDO) situated dorsally along the carapace (Lerosey-Aubril & Meyer, 2013). The first is allied posteriorly with the epigastric tooth and is present even when the epigastric tooth is absent. The second is medially situated along the dorsal margin of the carapace within the cardiac region and may have a smaller pair juxtapose and slightly posterior to the larger SDO (see appendix, Fig. A1: g–i).

ROSTRUM. Rostrum slender, typically reaching the middle of the second article of the antenular peduncle. However, it was common for the rostrum to be absent (Fig. 3: b, c & f), not reaching beyond the first article of the antenular peduncle or the eyestalk (Fig. 3: g) or reaching past the distal end of the second article (Fig. 3: d). The rostrum bears 4–7 total dorsal teeth, 3–4 along the postorbital margin, and 1–7 ventral teeth. However, the rostrum bears 0–15 dorsal (0–10 postorbital) and 0–8 ventral teeth.

Additionally, in some individuals the rostrum can have bifurcated spines, or is bifid or trifid (Fig. 3: c, g & h). Over 51% (n=463) of individuals exhibit character states inconsistent with the diagnosis of *B. cubensis*.

EYES. The eyes often have well-developed cornea that are narrower than the eyestalk (Manning & Hart, 1984). However, eyes with greatly reduced or absent pigmentation are present in some individuals (Fig. 3: j), or in some instances the cornea was subequal to or broader than the eyestalk (Fig. 3: l). Frequently a medial spine or tubercle is present along the interior margin of the eyestalk (Fig. 3: j & k). Just over 26% (n=463) of specimens exhibiting character states that are not prescribed to any species within Barbouriidae (see appendix, Fig. A1: j–l).

SCAPHOCERITE. The scaphocerite on average is approximately 3.1 times as long as wide. Some individuals exhibit lateral compressions in the anterior third of the scaphocerite (refer to appendix, Fig. A2: a), or exhibit asymmetry between the left and right scale. The length versus width ratio of approximately 66% (n=121) of individuals is greater than previously reported for species of *Barbouria*.

MOUTH PARTS. Variation was present in the maxilla and maxillipeds, predominantly in the shape of the caridean lobe. Epipods are present on the third maxillipeds. The distal margin of the epipods terminated in a single hook, or between two and four juxtaposed hooks. In some specimens a reduced incisor appeared to be present, but it could not be determined if the structure was a true incisor process. Variation was

also observed in the gill complements. However, this variation is not reported here due to inconsistent results and the possibility of inaccuracy due to damage.

PEREIOPODS. Pereiopods 1 and 2 chelate, but the finger may be shorter or longer than, or equal to the length of the palm. Additionally, the interior margins of the chelae may be straight or curved, and either strongly serrated or smooth. Pereiopod 2 exhibits a wide range of articulation from the ischium with 0–8 subdivisions, merus with 8–20 subdivisions and carpus with 20–40 subdivisions (refer to appendix, Fig. A2: b & c). The posterior 3 pairs of pereiopods are undivided but exhibit a wide range of spines running medially along the posterior margin. Spines may be absent or reduced to well-developed occurring either as pairs or alternating along the length of the segment. Epipods were present on the anterior four pereiopods, bearing between 1–4 hooks in varying positions similar to those on the third maxillipeds. Of the individuals in which pereiopods were examined, nearly all exhibit character states not assigned to *B. cubensis*.

ABDOMINAL PLEURA. The abdominal pleura of the somites typically match the previous descriptions (Manning & Hart 1984). It was found that for some specimens the first pleura may or may not overlap the carapace of the cephalothorax, and the first or third pleura may overlap the second (Fig. 3: n & p). The anterior three pleura may be square or have abnormal dentation along the margins (Fig. 3: n & p). In some instances, the pleura exhibit folding and can protrude laterally away from the abdomen (Fig. 3: o) similar to the findings of Fernandes *et al.* (2011). The fourth abdominal pleura ranged from obtusely to acutely round, or the posteroventral corner may be produced into a small

spine or tooth. The fifth and sixth abdominal pleura could lack spines (Fig. 3: n) or be produced into multiple small spines or teeth. Nearly 29% (n=463) of specimens exhibiting abnormalities in the pleura of the abdominal somites not described for any species within Barbouriidae.

PLEOPODS. The endopod of the male exhibits an appendix masculina that may be shorter by two-thirds or longer than the appendix interna and bearing 5–16 terminal setae arranged in a ring. Of the 140 individuals in which endopods were examined, 66 individuals were identified as male. Approximately 93% of male endopods exhibit character states outside of variation prescribed to *B. cubensis*.

TELSON. The telson ranged in shape from very slender and elongate, reaching well beyond the posterior margin of the uropods, to being short and stout not reaching beyond the posterior margin of the uropods (Fig. 3: r). Among many individuals with an elongate telson, the posterior third of the telson is laterally compressed (Fig 3B: v). Among individuals with an abnormally short telson, within the anterior third the telson becomes dorsoventrally compressed (Fig. 3: r). Alternatively, some individuals exhibit a telson with the medial third wider than the anterior and posterior third (Fig. 3: s) or could have a telson that is bifid (Fig. 3: t) or with a cavity anterior to the posterior margin (Fig. 3: x). The telson may bear 0–13 dorsal spines, and 0–23 terminal spines. Telson spines may be paired or unpaired. The terminal margin of the telson ranges in shape from being flat (Fig. 3: v) to pointed with an apical spine or tooth. The telson of 70% (n=463) of individuals could not be assigned as matching that of *B. cubensis*.

UROPODS. Exopods of the uropods are armed with 0–2 posterolateral teeth. It was found that few specimens possess additional teeth medially along the lateral margin of the exopod, or that the lateral margin may be straight or exhibit multiple curves.

DATA ANALYSIS

Statistical analyses were conducted to test the hypothesis that the distribution of phenotypic hypervariation is correlated with geographic location. All characters exhibit a normal distribution based on Shapiro-Wilk test. PhyV is present in specimens from Abaco, Eleuthera and San Salvador, Bahamas. No clustering or discernable distribution of PhyV is detected under all parameters in which UPGMA was conducted (Fig. 4).

CRYPTIC DIVERSITY

The combined 16S and COI tree included 70 individuals of *B. cubensis* as the ingroup taxa (Fig. 5). The species, *Parhippolyte misticia*, *Parhippolyte sterreri*, *Parhippolyte uveae* and *Janicea antiguensis* from the family Barbouriidae and *Lysmata amboinensis* from the family Lysmatidae were included as the outgroup taxa. For this dataset, 144 new sequences were generated including 72 new sequences for 16S, and 72 new sequences for COI (Table 2). Individual gene trees were without conflicting topologies. Results provided no support for cryptic speciation among *B. cubensis* (Fig. 5). Individuals identified as *B. cubensis* from all localities form a single polytomy, and we find no evidence for population structure across localities. All individuals in this data set that grouped with *B.*

cubensis included a wide range of PhyV, and no patterns relating to morphological variation were detected.

DISCUSSION

EXTENT OF PHENOTYPIC VARIATION IN *BARBOURIA CUBENSIS*.

Examples of phenotypic variation are abundant in the animal kingdom (Allegue *et al.*, 2017); however, the rate and extent of variation we report has never been documented in crustaceans (Agnalt *et al.*, 2013). Examples of “abnormal” morphologies of decapods have been reported in crabs, crayfish, penaeid prawns, and *Palaemon* shrimp (Béguer *et al.*, 2008; Duarte *et al.*, 2008). Reported abnormalities include: duplication and asymmetry of chelae, bifurcation of rostrum and telson, rostrum size, curve and tooth number or absence of rostrum, reversal of asymmetry, abnormal sexual appendages, carapace spines, pereopods, backwards folding and abnormal positioning of the abdominal epimera, and deformed telson and uropods (Aguirre & Hendrickx, 2005; Béguer *et al.*, 2008; Duarte *et al.*, 2008; Fernandes *et al.*, 2011). These records are not comparable to the morphological variation we report in this study because they are limited to one or few individuals and are typically limited to a single morphological character (Aguirre & Hendrickx, 2005; Béguer *et al.*, 2008; Fernandes *et al.*, 2011).

The 463 *B. cubensis* examined in this study represent a unique example of phenotypic hypervariation (PhyV). We define PhyV as morphological variation beyond the combined variation described in previous records of *B. cubensis* (Rathbun, 1912; Holthuis, 1963; Chace, 1972; Hobbs *et al.*, 1977; Hobbs, 1978; Hart & Manning, 1981;

Manning & Hart, 1984; Mejía *et al.*, 2008). Less than 7.5% (n = 463) of individuals are without PhyV and PhyV was present in all 54 characters examined in at least one individual. For each morphological character the frequency of PhyV present is between 6.9% (n=463) to 95.5% (n=121) of individuals. The presence of PhyV among shrimp sampled from Abaco, Eleuthera and San Salvador without any discernable pattern suggests PhyV is not associated with locality and is likely present among all populations of *B. cubensis* across the western Atlantic (Fig. 4).

Previous records indicate abnormal phenotypes typically occur at low rates in crustaceans (Béguer *et al.*, 2008) with one notable example. The highest rate of “deformities” is reported at 40% (n=1,578) and 58% (n=539) of individuals in *Palaemon longirostris*, which is far less than the observed 90% (n=463) in *B. cubensis* (Béguer *et al.*, 2008; Béguer *et al.*, 2010). Morphological “abnormalities” in *P. longirostris* is limited to four characters, which include cephalothorax anomalies, rostral “deformations” and pronounced bilateral dissymmetry of the scaphocerite and uropods (Béguer *et al.*, 2008). The nature of morphological variations of *P. longirostris* appears similar to those of *B. cubensis*, however PhyV in *B. cubensis* extends to 54 morphological characters compared to the four of *P. longirostris*. As recently as 1984, extensive variation has not been reported for *B. cubensis* by carcinologists, which includes records of specimens collected from localities sampled in this study (Hobbs, 1978; Manning & Hart, 1984). Similar to *P. longirostris*, PhyV of *B. cubensis* has likely appeared within the past 30 to 40 years (Hart & Manning, 1981; Manning & Hart, 1984; Béguer *et al.*, 2008). We do not believe that the appearance of PhyV is the result of increased sampling effort. Based on the numbers of specimens previously collected from sites included in this study if

aberrant individuals were present at the time of those collections they would have been reported, especially considering the high rate at which we find PhyV is present in this study.

Many of the shrimp examined in this study exhibited characters synonymous with *Barbouria cubensis*, *Barbouria yanezi*, *Parhippolyte sterreri* or *Janicea antiguensis* and that are not described as being shared by the four species (i.e. terminal margin shape and spination of telson). Additionally, some specimens exhibited morphological characters not present in any shrimp within the family Barbouriidae (Fig. 3). It is important to consider revisiting and sampling other species of barbouriid shrimp in the western Atlantic to determine if PhyV is limited only to *B. cubensis* or if it is common among all species.

POTENTIAL CAUSES OF PHENOTYPIC HYPERVARIATION

It is possible that phenotypic hypervariation may represent cryptic diversification as reported in atyid shrimp, if island populations are effectively isolated. *Caridina rubella* is reported to exhibit regional morphological variation in the length of the rostrum as either long or short (Weese et al., 2012). The two morphotypes of *C. rubella* correspond to two genetically divergent populations with two discrete COI haplotype networks as the result of cryptic speciation. We feel this is not comparable as the morphological variation of *C. rubella* occurs in a single character correlated to locality and resulting from speciation. In our phylogenetic analysis we included several individuals that exhibited severe PhyV along those with the individuals matching the normal *B. cubensis* phenotype. We found all individuals to be genetically identical with no population

structure attributed to morphological variation. Our findings provide some insight into the source of PhyV, as we can conclude that PhyV is not related to cryptic diversity.

Alternatively, PhyV may be caused by low genetic diversity due to severe inbreeding or population bottleneck, which can lead to an accumulation of deleterious mutations, increased expression of abnormal phenotypes and ultimately extinction (Creasey *et al.*, 2000; Fumagalli *et al.*, 2002, O'Grady *et al.*, 2006; Lampert *et al.*, 2007; Duarte *et al.*, 2008; Lacy & Alaks, 2012; Hedrick & Garcia-Dorado, 2016). Highly connected island populations are capable of inbreeding when species exhibit strong dispersal capabilities (Kano & Kase, 2004; Santos, 2006; Russ *et al.*, 2010). Previous records of *B. cubensis* only include two ovigerous females, in which abundant very small oocytes were found in the gonads (Hobbs, 1978). It is likely that these shrimps have extended planktonic development with strong dispersal capabilities similar to other caridean species that produce numerous small oocytes (Bauer, 2005; Russ *et al.*, 2010; Weese *et al.*, 2013). For anchialine organisms with planktotrophic larvae genetic connectivity occurring between populations has mostly been found to be limited to 600 km (Kano & Kase, 2004; Santos, 2006; Russ *et al.*, 2010; Weese *et al.*, 2013; Gonzales *et al.*, 2017). The shortest direct route between the Yucatan and the Bahamas is >1200 km, and the closest known population of *B. cubensis* to the Yucatan occurs ~700 km away in the Cayman Islands. In this case we would expect highly connected populations within the Bahamas, distinct from the Yucatan population (Kano & Kase, 2004; Santos, 2006; Russ *et al.*, 2010; Weese *et al.*, 2013; Gonzales *et al.*, 2017). Distinct isolated clades of *typhlatya* are reported from the Yucatan and the Bahamian archipelago (Hunter *et al.*, 2008). However, our phylogenetic analysis finds no population structure of *B. cubensis*

suggesting genetic connectivity is high across localities (Fig. 5). If a recent population bottleneck reduced genetic diversity, inbreeding among highly connected populations could maintain low genetic diversity. It is possible the markers used in this study lack sufficient resolution to determine population level structure and patterns that exist between PhyV and genetic diversity. If the PhyV is due to low genetic diversity, then management efforts may be required to restore genetic diversity to these critically endangered populations.

Multiple species of Barbouriidae have been reported inhabiting the same localities (Hart & Manning, 1981) and PhyV may indicate the presence of hybrid swarms due to interbreeding between these species (Wolf & Mort, 1986; Perry *et al.*, 2002; Cristescu *et al.*, 2010; McInerney *et al.*, 2014; Ribardire *et al.*, 2017). Hybrid swarm is characterized by major morphological variation between individuals in populations with interbreeding hybrid individuals that back-cross with parent types (Cockayne & Allan, 1926). The presence of intermediate character states between *B. cubensis* and *P. sterreri*, such as the terminal margin of the telson being pointed or nearly pointed and produced into a spine, and the number of spines on the postorbital margin of the rostrum support hybridization. Future studies using next generation sequencing techniques and fine-scale markers may provide further insights into the relationship between the abnormally rampant morphological variation and genetic diversity within Barbouriidae.

Deviations from perfect symmetry in bilaterally paired structures, or fluctuating asymmetry, may be used to evaluate developmental instability due to environmental factors, geographic location or as an indicator of environmental stress (Duarte *et al.*, 2008; Maia *et al.*, 2009; Klingenberg, 2015; Nishizaki *et al.*, 2015). It is noteworthy that

the presence of PhyV was commonly asymmetrical, especially in the situation of extra spines along the dorsal margin of the telson. However, most characters utilized in this study are either not paired structures (i.e., characters associated with the rostrum) or the PhyV expressed in paired structures were symmetrical (i.e., the number of subdivisions of segments of the second pereopod). Due to the lack of geographic pattern in the distribution of PhyV and insufficient symmetry measures we were unable to assess fluctuating asymmetry.

The anchialine pools in which *B. cubensis* are found are known to have strong turbulent flows through a complex series of subterranean passages in the karst landmass (Bishop *et al.*, 2015). Physical trauma during early development or following ecdysis can lead to extensive variation, possibly resulting from abrasion against the conduit walls due to strong tidal flows (Moncada & Gomes, 1980; Bohonak, 1999; Giménez, 2006; Luppi & Spivak, 2007; Follesa *et al.*, 2008; Vogt *et al.*, 2008). Similarly, damage due to parasitic infection can lead to abnormal phenotypes (Goodman & Johnson, 2011).

Environmental factors such as salinity, pH, temperature and contamination have been identified as causes of extensive variation in crustaceans (Smith & Palmer, 1994; Stibor & Lüning, 1994; Trussel, 1996; Trussel & Smith, 2000; Agrawal, 2001; Kappes & Sinsch, 2002; Chown *et al.*, 2007; Ituarte *et al.*, 2007; Reuschel & Schubart, 2007; Béguer *et al.*, 2008; Duarte *et al.*, 2008; Béguer *et al.*, 2010; Agnalt *et al.*, 2013). Routine migration of *B. cubensis* in and out of anchialine pools (across the pycnocline) regularly exposes them to acute changes in temperature, pH, salinity and dissolved oxygen levels (Bishop & Iliffe, 2012). Because of this lifestyle and no prior record of extensive variation for *B. cubensis*, it is unlikely PhyV is due to salinity, pH or temperature.

However, contamination such as lead remains a viable source of PhyV. If the source of PhyV has a non-genetic basis, then further study will be required to identify the source and implications for the conservation of endemic anchialine species in the western Atlantic.

CONCLUSION

We found that PhyV is present in populations of *B. cubensis* from Abaco, Eleuthera and San Salvador, Bahamas with no detectable pattern of geographic distribution or cryptic diversification. Future studies will use population genomic methods to investigate potential drivers of PhyV across western Atlantic populations of *B. cubensis*. It is important for us to further investigate the low level of genetic diversity and high levels of population connectivity suggested by our results (Fig. 5) with greater resolution through the use of next generation sequencing, such as restriction site associated DNA sequencing (Miller *et al.*, 2007; Davey *et al.*, 2011; Timm & Bracken-Grissom, 2015). We anticipate that further molecular and morphological studies with the addition of environmental data will identify the underlying cause sources of PhyV, which may be due to a complex combination of genetic and environmental factors.

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TABLES

Table 1. Common morphological characters used to discriminate among *Barbouriidae* spp. separated by prescribed character states for *Barbouria cubensis*, *B. yanezi*, *Parhippolyte sterreri* and *Janicea antiguensis*, and the most common, extremes and percentage of individuals exhibiting PhyV. * denotes percentages for which the character was examined for ≥ 121 individuals. Reproduced with permission from Magnolia Press.

	<i>Barbouria cubensis</i>	<i>Barbouria yanezi</i>	<i>Parhippolyte sterreri</i>	<i>Janicea antiguensis</i>	<i>Barbouria cubensis</i> sp. Examined		
					Most Common	Extremes	% PhyV
Carapace							
antennal tooth	present	present	present	present	present	absent / bifurcate	16.4%
branchiostegal tooth	present	present	present	present	present	absent / 4 spines	7.3%
Rostrum	simple	simple	simple	simple	simple	absent / trifid	7.1%
length vs antennular peduncle	past 1st article	end of 2nd article	scarcely past 1st article	end of 1st article	medial 2nd article	absent / end of 3rd article	25.9%
total dorsal teeth	4–7	3–7	3–4	3–4	5 ± 1.29	0 / 15	13.5%
postorbital dorsal teeth	3–4	2–4	1–2	1–2	3 ± 0.86	0 / 10	30.2%
ventral teeth	1–7	3–9	4–5	1	3 ± 1.61	0 / 8	9.1%
Eye							
eyestalk spine/tubercle	absent	absent	absent	absent	absent	absent / present	18.5%
cornea pigmentation	darkly pigmented	darkly pigmented	darkly pigmented	darkly pigmented	darkly pigmented	unpigmented	6.9%
cornea width vs eyestalk	narrower	narrower	broader	broader	narrower	absent/broader	16.6%
Scaphocerite							
length vs width	2.9X	2.47X	3X	4X	$3.04X \pm 0.28$	$2.6X / 4.73X$	87.0%*
Abdominal Pleura							
1st to 4th	rounded	rounded	rounded	rounded	rounded	acute / square	12.7%
5th & 6th	posteroventrally armed	posteroventrally armed	posteroventrally armed	posteroventrally armed	posteroventrally armed	rounded & unarmed	13.0%
Telson							
total dorsal spines	4	4	4	4	4 ± 1.23	0 / 13	20.8%
total posterior spines	6	6	6	6	6 ± 1.49	0 / 23	41.7%
terminal margin shape	broadly rounded	rounded	pointed	pointed	rounded	flat / pointed	13.0%
2nd Pereiopod							
ischium subdivisions	0	4	0–4	0	$3–4 \pm 1.39$	0 / 8	95.5%*
merus subdivisions	11–17	11	10	11–14	13 ± 3.21	8 / 20	20.1%*
carpus subdivisions	21–32	23–34	25–27	26–31	30 ± 4.44	20 / 40	40.3%*
Pleopod (male)							
appendix masculina vs interna (~ length)	shorter (~1/2–3/4)	shorter (~2/3)	subequal	longer	shorter (~2/3)	shorter (~1/3) / longer	10.0%*
appendix masculina terminal setae	5–7	11	14	8	10 ± 2.37	5 / 16	93.0%*

Table 2. Species used for phylogeny reconstruction for examining cryptic diversity within *Barbouria cubensis* (Dataset 1), showing taxon, collection locality, catalog number, and GenBank accession numbers for partial sequences of 16S, COI respectively.

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Taxon	Collection Locality	Catalog No.	16S	COI
<i>Barbouria cubensis</i> von martens, 1872	Abaco, Bahamas	HBG5588	MK501714	MK575421
		HBG5589	MK501715	MK575422
		HBG5632	MK501718	MK575425
		HBG5660	MK501719	MK575426
		HBG5680	MK501720	MK575427
	Eleuthera, Bahamas	HBG5687	MK501721	MK575428
		HBG3362	MK501710	MK575417
		HBG3363	MK501711	MK575418
		HBG3372	MK501712	MK575419
		HBG3373	MK501713	MK575420
		HBG5612	MK501716	MK575423
		HBG5621	MK501717	MK575424
	Mayaguana, Bahamas	HBG3187	MK501708	MK575415
		HBG3188	MK501709	MK575416
	San Salvador, Bahamas	OUMNH.ZC.2010-05-003	KF023098	-
		HBG1399	MK501653	MK575359
		HBG1764	MK501654	MK575360
		HBG1779	MK501655	MK575361
		HBG1780	MK501656	MK575362
		HBG1802	MK530650	MK575363
		HBG1815	MK501657	MK575364
		HBG1817	MK501658	MK575365
		HBG1819	MK501659	MK575366
		HBG1829	MK501660	MK575367
		HBG1832	MK501661	MK575368
		HBG1841	MK501662	MK575369
		HBG1842	MK501663	MK575370
		HBG1848	MK501664	MK575371
		HBG1857	MK501665	MK575372
		HBG1907	MK501666	MK575373
		HBG1924	MK501667	MK575374
		HBG1934	MK501668	MK575375
		HBG1935	MK501669	MK575376
		HBG1936	MK501670	MK575377
		HBG1937	MK501671	MK575378
		HBG1938	MK501672	MK575379
		HBG1939	MK501673	MK575380
		HBG1952	MK501674	MK575381
		HBG1956	MK501675	MK575382
		HBG1959	MK501676	MK575383
		HBG1993	MK501677	MK575384
		HBG2009	MK501678	MK575385
		HBG2018	MK501679	MK575386
		HBG2027	MK501680	MK575387
		HBG2028	MK501681	MK575388
		HBG2034	MK501682	MK575389
		HBG2035	MK501683	MK575390
		HBG2036	MK501684	MK575391
		HBG2056	MK501685	MK575392
		HBG2068	MK501686	MK575393
		HBG2113	MK501687	MK575394
		HBG2121	MK501688	MK575395
		HBG2123	MK501689	MK575396
		HBG2151	MK501690	MK575397
		HBG2167	MK501691	MK575398
		HBG2198	MK501692	MK575399
		HBG2201	MK501693	MK575400
		HBG2264	MK501694	MK575401

Table 2. (Continued)

Taxon	Collection Locality	Catalog No.	16S	COI
<i>Barbouria cubensis</i> von Martens, 1872	Yucatán Peninsula of Mexico	HBG2772 HBG2773 HBG2774 HBG2775	MK501704 MK501705 MK501706 MK501707	MK575411 MK575412 MK575413 MK575414
Outgroups				
<i>Janicea antiquensis</i> Chace, 1972	Cape Verde, Africa	OUMNH.ZC.2004-15-002	KF023112	-
<i>Parhippolyte sterreri</i> Hart & Manning, 1981	Iguana Cay, Bahamas San Salvador, Bahamas	MNHN-IU-2012-1057 HBG2189 HBG2274	KP725619 MK501722 MK501723	KP759480 MK575429 MK575430
<i>Parhippolyte uveae</i> Borradaile, 1900		MNHN-IU-2012-1001	KP725621	-
<i>Parhippolyte misticia</i> Clark, 1989			HQ315560	-
<i>Lysmata amboinensis</i> De Man, 1888	Hong Kong/ Bise Point, Okinawa, Japan	MSLH:CA23Lyamb UNML:32.9451	KF023091 -	- JF346249

SUPPLEMENTAL TABLES

Table S1. Morphological characters included in data subset 1t and 1b. Reproduced with permission from Magnolia Press.

Character (# of characters used)		prescribed state
Carapace (2)		Smooth
1	<i>suborbital tooth (antennal spine)</i>	Present
2	<i>branchiostegal tooth</i>	Present
Sensory Dorsal Organ (2)		
1	<i>in cardiac notch</i>	Undescribed
2	<i>posterior to epigastric spine</i>	Undescribed
Rostrum (4)		margins dentate
1	<i>length vs. antennular peduncle</i>	not past 2nd article
2	<i>dorsal rostral teeth (total)</i>	4 to 7
3	<i>postorbital rostral tooth count</i>	3 to 4
4	<i>ventral rostral teeth (total)</i>	1 to 7
Eye (2)		
1	<i>cornea pigmented</i>	pigmented cornea
	<i>cornea width vs. stalk width</i>	narrower than eyestalk
2	<i>eyestalk bearing spine/tubercle</i>	Absent
Abdomen (4)		
	<i>pleura 1 & 2</i>	rounded laterally
1	<i>pleura of 2nd somite</i>	overlapping 1st & 3 rd
	<i>pleura of 3rd somite</i>	rounded/obtuse
	<i>3rd posterolateral tooth</i>	Absent
2	<i>pleura of 4th somite</i>	Rounded
	<i>4th posterolateral tooth</i>	Absent
3	<i>angle of pleura of 5th somite</i>	Acute
	<i>5th posterolateral tooth</i>	Present
	<i>angle of pleura of 6th somite</i>	short and acute
4	<i>posterior ventral angle tooth</i>	Present
	<i>6th posterolateral tooth</i>	Present
Telson (4)		
1	<i>telson shape</i>	elongate & slender
2	<i>dorsal margin spines</i>	2 pairs of spines
3	<i>terminal margin shape</i>	blunted apex
4	<i>terminal margin spines</i>	3 pairs
	<i>length of terminal spines</i>	middle pair longest
Uropod (3)		
1	<i>length vs telson</i>	slightly longer
2	<i>outer margin shape</i>	exopod straight
3	<i>terminal tooth</i>	Present
	<i>inner movable spine</i>	Present
Antennular Peduncle (2)		
1	<i>basal segment spine/tubercle</i>	Absent
	<i>basal segment length</i>	Longest
	<i>stylocerite terminal spine</i>	Present
2	<i>2nd segment length vs. 1st</i>	Shorter
	<i>2nd segment length vs 3rd</i>	Longer
	<i>simple flagella</i>	Present
Pereiopod (2)		
1	<i>2nd articles subdivided</i>	Present
2	<i>3rd-5th articles not subdivided</i>	Absent

Table S2. Additional morphological characters used in data subset 2t and 2b. Reproduced with permission from Magnolia Press.

Character (# of characters used)		prescribed state
Scaphocerite (1)		
1	ratio of length vs. width	2.9X
Pereiopod (14)		
1st		
1	length vs scaphocerite	not past scaphocerite
2	chela finger length vs palm	Longer
	chela palm shape	Broad
3	upper dactylus, lower propodus	Convex
	cutting edge dactylus/propodus	Straight
4	carpus length vs chela	slightly longer
	merus vs carpus length	Subequal
5	subdivided segments	None
2nd		
6	merus+ischium vs carpus+chela	Equal
7	merus posterior margin setae	stiff & curved
8	articulation (carpus)	21-32
9	articulation (merus)	11-17
10	articulation (Ischium)	0 (4 minor subdivisions)
11	3rd - 5th	about same shape
12	3rd see Hobbs <i>et al.</i> , 1977	matches description
13	4th see Hobbs <i>et al.</i> , 1977	matches description
14	5th see Hobbs <i>et al.</i> , 1977	matches description
Pleopod (3)		
1st		
1	coupling hooks (male)	Absent
2nd		
2	2nd appendix masculina (male)	Present
3	Gender	male or female
Mouth Part (6)		
Mandible		
1	molar process	Stout
	incisor process	Absent
	3-jointed palp	Present
1st & 2nd Maxillae		
2 & 3	see Hobbs <i>et al.</i> , 1977	matches description
1st-3rd Maxilliped		
4-6	see Hobbs <i>et al.</i> , 1977	matches description

Table S3. morphological characters used to evaluate sexual dimorphism in place of characters used in data for 1st and 2nd pleopods. Reproduced with permission from Magnolia Press.

Character (# of characters used)		prescribed state
Pleopod (2)		
	2nd	
1	appendix masculina setae	5-9 or 5-7
2	appendix masculina vs interna	1/2 to 2/3

FIGURE CAPTIONS

Figure 1. *Barbouria cubensis*, **(top)** left lateral view of live specimen (HBG1911), **(bottom)** schematic drawing by R.E. Ditter of general morphological characters within Caridea. Reproduced with permission from Magnolia Press.

Figure 2. Map of Bahamian Islands with sites sampled from 2012–2016 **(a)** Abaco site codes and names labeled from North to South: ADS, Dripping Stones; ALP, Lora’s Pond; ARS, Runge’s Sinkhole **(b)** Eleuthera site codes and names labeled from North to South: EPC, Preacher’s Cave Blue Hole; EDM, Dump Pond; ETD, Too Deep Pond; ESS, Savannah Sound; ESP, Shrimp Pond; EMN, Mackery Nixon Pond **(c)** San Salvador site codes and names labeled from northern most moving clockwise: LHC, Light House Cave; SSL, South Stout’s Lake; MDP, Mermaid Pond; MNP, Merman Pond; PCC, Pigeon Creek Conduit; DCP, Dunk City Pond; WBH, Watling’s Blue Hole; BH2, Blue Hole #2; BH5, Blue Hole #5; RED, Redrum Pond; WLL, William’s Pond; LIL, Little

Lake; MJC, Major's Cave. *i*; site codes and names labeled from northern most moving clockwise: RHP, Reckley Hill Pond; PNP, Pain Pond; WDP, Wild Dilly Pond; OYP, Oyster Pond; SHP, Shrimp Holes. *ii*; site codes and names labeled from North to South: PTN, Plantation Pond; SPX, Small Pox Pond; RBH, Rolle's Blue Hole; LRP, Tilde Pond; BRP, Big Rob Pond; BDP, Big Drink Pond. Reproduced with permission from Magnolia Press.

Figure 3. Expected phenotypes for *B. cubensis* (left column) compared to examples of observed variable phenotypes; (**a–d**) teeth on rostrum and rostrum length, and the presence of antennal and branchiostegal spines (**e–h**) rostrum length, teeth and shape, antennal and branchiostegal teeth and abnormalities of carapace (**i–l**) eyes, cornea width versus eyestalk width, and the presence of spines on eyestalk. Images correspond to the following catalog numbers for specimen vouchers housed in the Florida International Crustacean Collection: a*, HBG1843; b, HBG2162; c, HBG2011; d, HBG5644; e*, HBG1843; f, HBG2139; g, HBG2095; h, HBG1935; i*, HBG1843; j, HBG 1952; k, HBG1776; l, HBG2217. Reproduced with permission from Magnolia Press.

Figure 3 con't. (**m–p**) shape and armament of pleura of the abdominal somites (**q–t**) telson shape, dorsal spines and terminal spines (**u–x**) shape and spines of terminal margin of telson. Yellow lines and arrows indicate expected characters, red lines and arrows indicate variable characters. * denotes examples of expected phenotypes for *B. cubensis*. Images correspond to the following catalog numbers for specimen vouchers housed in the Florida International Crustacean Collection: m*, HBG1843; n, HBG1932; o, HBG1934;

p, HBG1398; q*, HBG1843; r, HBG1936; s, HBG1937; t, HBG1970; u*, HBG1843; v, HBG1777; w, HBG1937; x, HBG2098. Reproduced with permission from Magnolia Press.

Figure 4. An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of morphological data in Subset #2 labeled with site codes corresponding to figure 2. Bootstrap values >90% are noted to the right of nodes. Reproduced with permission from Magnolia Press.

Figure 5. Bayesian (BI) phylogram for *Barbouria cubensis* (n = 70) based on a 16S & COI concatenated data set. BI posterior probabilities and Maximum Likelihood (ML) bootstrap values noted below branches. Values >0.7 for ML and >90% for BI are shown and represented by percentages. Vertical bars represent collection locality. AIB = Abaco, Bahamas, EIB = Eleuthera, Bahamas, MIB = Mayaguana, Bahamas, SSB = San Salvador, Bahamas, and YMx = Yucatán, Mexico. Catalog numbers represent tissue vouchers from the Florida International Crustacean Collection (FICC). Reproduced with permission from Magnolia Press.

SUPPLEMENTAL FIGURE CAPTIONS

Figure S1. Phenotypic hypervariation exhibited by *B. cubensis*; (**a–c**) variations in the shape and surface of the carapace, (**d–f**) additional and missing teeth without carina, (**g–h**) position of sensory dorsal organ associated with epigastric tooth and within the cardiac region (**j–l**) asymmetry in the presence of cornea pigmentation and a terminal spine or

tubercle on the eyestalk. Images correspond to the following catalog numbers for specimen vouchers housed in the Florida International Crustacean Collection: a, HBG1793; b, HBG1918; c, HBG2215; d & e, HBG1808; f, HBG1909; g–i, 1801 HBG; j–l, HBG1849. Reproduced with permission from Magnolia Press.

Figure S2. Phenotypic hypervariation exhibited by *B. cubensis*; (a) asymmetrical lateral compression of scaphocerite, and the absence of the rostrum and antennal tooth while the carina are present (b & c) dorsal and ventral view of the ischium of the 2nd pereopod with six subdivisions (d) lateral extrusions along carapace, (e) bifid rostrum, antennal and branchiostegal spines absent and carapace not smooth (f) rhizocephalan parasite, (g–l) additional examples of PhyV in character located on the cephalothorax. Images correspond to the following catalog numbers for specimen vouchers housed in the Florida International Crustacean Collection: a, HBG1907; b & c, HBG1904; d, HBG2227; e, HBG2011; f, HBG2083; g, HBG1816; h, HBG1872; i, HBG1882; j, HBG2095; k, HBG1982; l, HBG1793. Reproduced with permission from Magnolia Press.

Figure S3. Phenotypic hypervariation exhibited by *B. cubensis*; (a & b) abnormal shape and armament of the pleura of the abdominal somites, (d–l) additional examples of PhyV in characters located on the telson and uropods. Images correspond to the following catalog numbers for specimen vouchers housed in the Florida International Crustacean Collection: a, HBG2093; b, HBG1398; c, HBG1399; d, HBG1793; e, HBG1802; f, HBG1791; g, HBG1777; h, HBG1395; i, HBG1954; j, HBG1982; k, HBG1855; l, HBG1853. Reproduced with permission from Magnolia Press.

FIGURES

Figure 1.

Figure 2.

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Figure 3.

Figure 3 con't.

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Figure 5.

SUPPLEMENTAL FIGURES

Figure S1.

Figure S2.

Figure S3.

CHAPTER III

NOTES ON THE BEHAVIOR AND FIRST RECORDS OF THREE ENIGMATIC
ANCHIALINE SHRIMPS (DECAPODA: CARIDEA: BARBOURIIDAE,
PALAEMONIDAE) IN THE BAHAMAS

ABSTRACT

Barbouria cubensis (von Martens, 1872), *Parhippolyte sterreri* (Hart & Manning, 1981) (Barbouriidae) and *Macrobrachium lucifugum* Holthuis, 1974 (Palaemonidae) are recorded for the first time from anchialine systems on Acklins Island, Bahamas. Seventy-two individuals of *B. cubensis*, 29 of *P. sterreri*, and nine of *M. lucifugum* were collected in July 2017 from five sites across the island. We also report on observations of behavior for all three species during collection and of *B. cubensis* monitored in a laboratory setting over a two-year period.

INTRODUCTION

Anchialine systems are comprised of landlocked bodies of water with subterranean connections to the surrounding ocean that permit tidally driven water exchange (Bishop *et al.*, 2015). Members of the family Barbouriidae Christoffersen, 1987 are globally distributed along tropical latitudes and are mostly endemic to anchialine systems (De Grave *et al.*, 2014). The family consists of four genera, *Barbouria*, *Calliasmata*, *Janicea*, and *Parhippolyte*. *Macrobrachium* Spence Bate, 1868, is a species-rich genus of the family Palaemonidae Rafinesque, 1815 widely distributed in bodies of freshwater across tropical and subtropical latitudes (Holthuis, 1980; Vera-Silva *et al.*, 2016). Some palaemonids, such as *M. lucifugum* Holthuis, 1974, are sometimes found in anchialine systems and coastal freshwater sink holes (Hobbs, 1994; Komai & Fujita, 2005).

It is common for different species of Barbouriidae to inhabit the same locality (Hart & Manning, 1981). Such cohabitation has previously been observed in anchialine caves in Bermuda and in Exuma and San Salvador islands in the Bahamas (Hart &

Manning, 1981; Botosaneanu & Iliffe, 1999; Ditter *et al.*, 2015). *Barbouria cubensis* von Martens, 1872 and *Parhippolyte sterreri* Hart & Manning, 1981 are anchialine species that lack many troglomorphic characteristics associated with cave dwelling organisms such as the absence of pigmentation, reduced or absent eyes, cuticular structural reduction, and the elongation of sensory and ambulatory appendage (Holthuis, 1963; Turk *et al.*, 1996; Lamoreux, 2004; Bishop & Iliffe, 2012; Friedrich, 2013; Pérez-Moreno *et al.*, 2017).

Barbouria cubensis has been reported from Cuba and the islands of Abaco, Exuma, Mayaguana, Grand Bahama, and San Salvador in the Bahamas, Providenciales in the Caicos Islands, Bermuda, Jamaica, Cayman Brac in the Cayman Islands, and in the Yucatan Peninsula (Hobbs *et al.*, 1977; Manning & Hart, 1984; Manning *et al.*, 1985; Bishop & Iliffe, 2012; Ditter *et al.*, 2015). *Parhippolyte sterreri* is known to occur in Bermuda, the Yucatán Peninsula, and Andros, Exuma, Grand Bahama, and San Salvador, Bahamas (Hart & Manning, 1981; Manning *et al.*, 1985; Kensley, 1988; Wicksten, 1996; Ditter *et al.*, 2015; M. Brooks, unpublished data). Despite their abundance across the tropical western Atlantic, little is known about the biology of these two species, with only anecdotal observations of their behavior reported. There is so far only one study comparing physiological differences between populations of *B. cubensis* (Bishop & Iliffe, 2012). *Macrobrachium lucifugum* has previously been reported from Bonaire, Cuba, Curaçao, the Dominican Republic, Grand Bahama, Jamaica and Puerto Rico (Holthuis, 1974; Chace, 1975; Hobbs, 1994; De Grave & Fransen, 2011).

We report on the first occurrence of *Barbouria cubensis*, *Parhippolyte sterreri*, and *Macrobrachium lucifugum* in the anchialine pools of Acklins Island, Bahamas. In

addition to these first records, we discuss observations made for these species during collection as well as the behavior of *B. cubensis* in its natural habitat and in a laboratory setting.

MATERIALS AND METHODS

SPECIMEN COLLECTION

Seventy-two specimens of *B. cubensis*, 29 of *P. sterreri*, and nine of *M. lucifugum* were collected on Acklins Island, Bahamas (22.3658° N, 74.0535° W) in July 2017. Specimens were collected from Big Pond, Harbour Hill Cave (“Darling’s Cave”), Harbour Hill Cenote, Flamingo Pond, Nibbles Cave, Red Lantern Cave, and Student Pond (Fig. 1).

Specimens were captured using a baited minnow trap deployed near the mouth of the conduit while snorkeling, or by hand. Specimens were preserved in 95-100% ethyl alcohol or RNAlater (Sigma-Aldrich, St. Louis, MO, USA) on site or kept alive until they could be properly processed. Specimens were transferred to the Florida International University Crustacean Collection (FICC) located on the Biscayne Bay campus, North Miami, FL, USA after preservation.

Barbouria cubensis was captured from Big Pond, Flamingo Pond, Nibbles Cave, Red Lantern Cave, and Student Pond (Fig. 1), *P. sterreri* from Flamingo Pond, and *M. lucifugum* was from Harbour Hill and Red Lantern cave, juveniles observed in a freshwater cenote (Harbour Hill Cenote) 150 m north of the cave entrances. We noted that *B. cubensis* collected alongside *M. lucifugum* were smaller in size than those found at other sites.

BEHAVIORAL OBSERVATIONS

Previously collected from San Salvador, Bahamas, 10 *B. cubensis* were used for behavioral observations. Eight individuals were paired and placed into four 19 l (5 gal.) aquaria and two were isolated in two separate aquaria to serve as the control groups. The six aquaria were placed in a light controlled environment. Each aquarium contained one limestone shelter to mimic cave substratum to recreate the natural habitat as best as possible. Each aquarium was equipped with bubble filters, and timers were utilized to control the day/night cycles as follows: 2 h of indirect light, 8 h of direct light, 2 h of indirect light, and 12 h of dark. Red LED lights were installed to observe specimens during the night cycle.

Water was changed every other day using fresh filtered seawater from Biscayne Bay. Water quality parameters were monitored daily to maintain optimal water quality. Specimens were fed frozen brine shrimp daily. Noteworthy behavior was digitally recorded using a GoPro Hero2 and any exuvia recovered were preserved in 70% ethyl alcohol.

RESULTS AND DISCUSSION

The presence of *B. cubensis*, *P. sterreri*, and *M. lucifugum* on Acklins Island, Bahamas represents minor range extensions for all three species (Fig. 2). *Barbouria cubensis* has the widest distribution on Acklins, being found in all sampling sites except for Harbour Hill Cave. As on San Salvador Island, *P. sterreri* is present only found on the southern portion of the island, possibly indicating that this species is a recent arrival due to its limited distribution on the island compared to *B. cubensis* (Ditter *et al.*, 2015).

Parhippolyte sterreri is present in other anchialine pools on southern Acklins Island, but these sites were not sampled. *Macrobrachium lucifugum* is limited to the northeastern portion of the islands (Fig. 1). Juvenile *M. lucifugum* were only observed in one freshwater cave (Fig. 1a), and adults were only collected in two marine caves (Fig. 1b, c). Many species of *Macrobrachium* are amphidromous, migrating between freshwater and saltwater habitats as part of their life history (Bauer & Delahoussaye, 2008). Harbour Hill and Red Lantern caves are the only localities found in close proximity to a non-ephemeral surface freshwater feature on Acklins Island. It is likely that the distribution of juvenile and adult *M. lucifugum* indicates subterranean connections between these localities because of its amphidromous life history. Alvarez *et al.* (2004) reported observing *B. cubensis* from Jumbey Hole cave; however, the cave was found to be a dry cave. There is no evidence that Jumbey Hole is or was an anchialine cave in recent history and island residents indicated that it has always been a dry cave.

FIELD OBSERVATIONS

The number of shrimps in anchialine pools are most abundant during high tide and absent during low tide. The presence or absence of shrimp in surface pools appears to be tidally driven. *Barbouria cubensis*, *P. sterreri*, and *M. lucifugum* began migrating into surface waters shortly after the start of flood tides and retreating during ebb tides. Further studies are necessary to understand the possible physiological mechanisms and environmental cues underlying this behavior, which may also further the understanding of the evolution of anchialine organisms. No remarkable agonistic behaviors were detected when *B. cubensis* and *P. sterreri* were observed in the same locality or housed in the same

aquarium. When housed together for an extended period of time, *B. cubensis* mirrored the coloration of *P. sterreri*. This was done by increasing the intensity of white coloration along the joints of pereopods 3 – 5 and expanding the white spots on the posterior surface of the uropodal exopods and the sixth abdominal somite (Fig. 2b). When *B. cubensis* and *M. lucifugum* were observed in the same locality, *B. cubensis* avoided *M. lucifugum*. *Barbouria cubensis* and *M. lucifugum* could not be housed in the same aquarium due to the aggressive behavior of *M. lucifugum*. *Barbouria cubensis* and *M. lucifugum* also exhibited no flight response to the presence of human observers, and often boldly foraged on the observers. *Parhippolyte sterreri* stayed deeper within the conduits and retreated in the presence of observers.

LABORATORY OBSERVATIONS

Barbouria cubensis was been found to exhibit phenotypic hypervariation (PhyV); defined as the presence of extensive morphological variation that far exceeds variation described in previous records and limits the usefulness of traditional taxonomic identification methods (Ditter *et al.*, 2019). Our initial observations showed the presence of PhyV in captive individuals of *B. cubensis* and exuvia from each specimen were examined monthly to determine if PhyV was retained after Ecdysis. If PhyV was lost after ecdysis it would possibly indicate physical trauma as a source of PhyV. This was not the case as all individuals retained identical variations over the course of two years in captivity, and no additional morphological variations were observed in any individuals. To our knowledge, the retention of extensive morphological variation has only been reported over the course of 130 d in *Palaemon longirostris* (Béguer *et al.*, 2010).

The color of *B. cubensis* has been described to range from white and nearly transparent to deep red or crimson (Hobbs *et al.*, 1977). Prior to light exposure, individuals appear nearly translucent (Fig. 3A), turning light to dark red within 4 min after exposure to full-spectrum light (Fig. 3B). Once returned to darkness, individuals lose color within 15 min. As ecdysis neared, the degree of color change in response to light decreased. When exposed to only blue light (455 nm) individuals of *B. cubensis* exhibited a flight response of erratic swimming seeking refuge. Individuals rapidly changed color from nearly translucent to deep red in the presence of light due to the presence of two types of chromatophores: smaller red and larger white (Fig. 4). Both types of chromatophores are distributed along the body and appendages. The white chromatophores are much larger and less abundant than the red chromatophores. The contrast in color change from pale to red decreased over a 30 d period. Once color change could no longer be detected, ecdysis occurred within 48 h. The increasingly red coloration of individuals appears to be a strong indicator for ecdysis.

Paired *B. cubensis* were highly active, either swimming or walking around the substrate, and cleaning themselves. Individuals were able to locate and consume food within 1 min. This species also exhibited agonistic behavior by means of antennal fencing (Dunham, 1972). Caridean shrimps have been found to behave aggressively to retain access to limited space, food, and potential mates (Ra'anana & Sagi, 1985; Karplus & Harpaz, 1990; Correa & Thiel, 2003). When not active, *B. cubensis* hid underneath the limestone shelters or the water filter. Isolated individuals exhibited behavior that was unlike their paired counterparts. They were less active, remaining underneath their shelters most of the time and taking 2–6 min to locate and consume their food. This may

be a possible explanation for the decreased activity and feeding response of isolated specimens.

Habitat destruction is a serious threat to the biodiversity of anchialine habitats (Culver & Sket, 2000; Iliffe & Kornicker, 2009). Many caves have already been altered or destroyed by pollution and development, and it is essential to document the distribution of anchialine organisms as their habitats are continually altered. The IUCN (2019) lists *B. cubensis* and *P. sterreri* as critically endangered, and *M. lucifugum* as least concern, but we believe reassessment of *M. lucifugum* would change its status to critically endangered (Iliffe, 1996a; Iliffe, 1996b; De Grave, 2013). Many unanswered questions about the dispersal, biology, and life history of anchialine organisms remain.

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FIGURE CAPTIONS

Figure 1. Map of Acklins, Bahamas adapted from Google Earth indicating the collection sites of *Barbouria cubensis*, *Parhippolyte sterreri*, and *Macrobrachium lucifugum* and the location of Jumbey Hole Cave. Magnified view of Harour Hill Cenote (**A**), Harbour Hill Cave (**B**), and Red Lantern Cave (**D**). Scale bar = 50 m. This figure is available in color at *Journal of Crustacean Biology* online. Reproduced with permission of Oxford University Press.

Figure 2. Dorsal views of *Barbouria cubensis* (**A**), *Parhippolyte sterreri* (**B**), and *Macrobrachium lucifugum* (**C**). Scale bar = 1 cm. This figure is available in color at *Journal of Crustacean Biology* online. Reproduced with permission of Oxford University Press.

Figure 3. Left lateral view of an individual of *Barbouria cubensis* exhibiting color change before and after light exposure, being nearly translucent pale when not exposed to light, (**A**) and deep red/crimson after five minutes of exposure to direct light (**B**). Scale bar = 1 cm. This figure is available in color at *Journal of Crustacean Biology* online. Reproduced with permission of Oxford University Press.

Figure 4. Dorsal view of the cephalothorax of *Barbouria cubensis* cephalothorax (40x magnification) with distinct small red and large white chromatophores visible. Scale bar = 1 mm. This figure is available in color at *Journal of Crustacean Biology* online.

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FIGURES

Figure 1.

Figure 2.

Figure 3.

Figure 4.

CHAPTER IV

ANCHIALINE ADJUSTMENTS: AN UPDATED PHYLOGENY AND
CLASSIFICATION FOR THE FAMILY BARBOURIIDAE CHRISTOFFERSEN, 1987
(DECAPODA: CARIDEA)

ABSTRACT

Barbouriidae Christoffersen, 1987 is a family comprised of 4 genera and 11 species of enigmatic shrimps restricted to anchialine or marine caves whose evolutionary history and relationships remain elusive. We investigated the evolutionary relationships among members of Barbouriidae with the inclusion of four genera and nine species, and newly collected material from Belize, the Bahamas, and the Yucatán Peninsula, Mexico.

Phylogenetic analyses based on seven mitochondrial and nuclear gene regions and genetic distances calculated using partial 16S gene regions have identified a need to revisit the relationships and classification within Barbouriidae. More specifically, we find evidence to suggest *Janicea* Manning & Hart, 1984 as a junior synonym of *Parhippolyte* Borradaile, 1900, *B. yanezi* Mejía, Zarza & López, 2008 as a synonym of *Barbouria cubensis* (von Martens, 1872), and define two new subfamilies, Calliasmatinae nov. and Barbouriinae Christoffersen, 1987. Included is a dichotomous key for the species of Barbouriidae that summarizes previous literature and includes new morphological characters. Our findings shed light on existing inaccuracies and gaps in molecular data from barbouriids. We also provide further clarity into evolutionary relationships among genera of Barbouriidae and their allies, suggesting phylogeographic divisions within the family. Our findings suggest an early Atlantic-Pacific divide among genera originating from a shallow-water reef ancestor.

INTRODUCTION

Anchialine pools are a common feature throughout the tropical western Atlantic, and are broadly defined as land-locked pools with limited tidal connections to adjacent marine

water bodies through a complex network of subterranean conduits ([Bishop *et al.*, 2015](#)).

Anchialine systems are considered to be isolated due to their unique species assemblages and endemism ([Iliffe & Kornicker, 2009](#); [Becking *et al.*, 2011](#); [Pérez-Moreno *et al.*, 2016](#)). Barbouriidae [Christoffersen, 1987](#) ([Fig. 1](#)) is a family of caridean shrimps that commonly inhabits anchialine or coastal caves along tropical latitudes ([Fig. 2](#)).

Barbouriidae is currently comprised of 4 genera and 11 species, but the genera within this family have undergone substantial revisions in recent years ([Chace, 1972, 1997](#); [Manning & Hart, 1984](#); [Christoffersen, 1987, 1990](#); [Fransen & Tomascik, 1996](#); [Wicksten, 1996](#); [De Grave *et al.*, 2014](#)).

The evolutionary history of Barbouriidae remains unclear ([De Grave *et al.*, 2014](#)). Members of this family had previously been classified as monotypic genera within Hippolytidae Spence Bate, 1888 until [Christoffersen \(1987, 1990\)](#) proposed recognizing Barbouriidae as a family with six genera (*Ligur* [Sarato, 1885](#); *Barbouria* [Rathbun, 1912](#); *Janicea* [Manning & Hart, 1984](#); *Parhippolyte* [Borradaile, 1900](#); *Somersiella* [Hart & Manning, 1981](#); and *Koror* [Clark, 1989](#)). [Kemp \(1914\)](#) synonymized *Parhippolyte* with *Ligur* without comment. Upon discovery of additional species with a multiarticulate carpus and propodus of the third to fifth pereopods, *Parhippolyte* was removed from the synonymy of *Ligur* ([Manning & Hart, 1984](#)). *Ligur* became a monospecific genus with the removal of *P. uveae* [Borradaile, 1900](#) and was transferred from Hippolytidae upon the resurrection of Lysmatidae Dana, 1852 ([Manning & Hart, 1984](#); [De Grave *et al.*, 2014](#)). The description of *Koror mysticus* [Clark, 1989](#) and *Somersiella sterreri* [Hart & Manning, 1981](#) placed them within Hippolytidae, but a lack of morphological evidence at the genus level for both genera led them to be synonymized with *Parhippolyte* ([Wicksten, 1996](#);

[Chace, 1997](#)). With these exclusions and reclassifications, recent phylogenetic analyses have supported the recognition of the family Barbouriidae ([Li et al., 2011](#); [De Grave et al., 2014](#); [Aznar-Cormano et al., 2015](#)). The current classification of Barbouriidae includes two species of *Barbouria* (*B. cubensis* (von Martens, 1872)) and *B. yanezi* ([Mejía, Zarza & López, 2008](#)), one species of *Janicea* (*J. antiguensis* ([Chace, 1972](#))), five species of *Parhippolyte* (*P. cavernicola* [Wicksten, 1996](#); *P. misticia* ([Clark, 1989](#)); *P. rukuensis* [Burukovsky, 2007](#); *P. sterreri* ([Hart & Manning, 1981](#)); and *P. uveae*), and the most recent addition of three species of *Calliasmata* [Holthuis, 1973](#) (*C. nohochi* [Escobar-Briones, Camacho & Alcocer, 1997](#); *C. pholidota* [Holthuis, 1973](#); and *C. rimolii* [Chace, 1975](#)). *Barbouria cubensis* and *P. sterreri* (listed as *Somersiella sterreri*) are listed as critically endangered on the IUCN Red List ([Iliffe, 1996](#)).

Although the genus *Calliasmata* is presently placed within Barbouriidae, [De Grave et al. \(2014\)](#) questioned the inclusion of this group within the family. *Calliasmata* lacks all of the defining characters shared by genera of Barbouriidae, including the unique subocular tooth posterodorsal to the orbital angle, which has been previously considered as a synapomorphy of the family ([Clark, 1989](#); [Chace, 1997](#); [De Grave et al., 2014](#)). Moreover, in spite of the troglodytic lifestyle of barbouriids, only *Calliasmata* exhibits characteristic adaptations of cave-dwelling animals, such as highly degenerate eyes ([Pérez-Moreno et al., 2016](#)). The conflicting morphology of *Calliasmata* with the other members of Barbouriidae further complicates the evolutionary history of this family, and Barbouriidae presently lacks an accurate formal description (see [De Grave et al., 2014](#)).

We also revisited the placement of the monospecific genus *Janicea* among other barbouriids. *Janicea* has the broadest distribution of the Atlantic species, ranging from the Canary Islands, Bermuda, and the Yucatán Peninsula, Mexico ([Manning & Hart, 1984](#); [Li et al., 2011](#)). *Janicea antiguensis* ([Chace, 1972](#)) has a gill compliment similar to that of *B. cubensis*, but multiarticulation of the carpus and propodus of the third to fifth pereopods and a cornea wider than the eyestalk suggest an affinity to *Parhippolyte* ([Manning & Hart, 1984](#)). The first description of *J. antiguensis* placed it within *Barbouria* with the belief that *Barbouria* would eventually be synonymized with *Ligur*, but *Janicea* was later recognized as a monotypic genus ([Chace, 1972](#); [Buden & Felder, 1977](#); [Manning & Hart, 1984](#)). The taxonomic uncertainty surrounding *J. antiguensis* justifies a closer examination of the phylogenetic placement of this species within Barbouriidae.

Barbouria yanezi [Mejía, Zarza & López, 2008](#) is the most recently described species of Barbouriidae and has only been reported from Cenote Tres Potrillos, Cozumel Island, Mexico ([Mejía et al. 2008](#)). *Barbouria yanezi* differs from *Barbouria cubensis* (von Martens, 1872) in the length and number of teeth of the rostrum, the length to width ratio of the scaphocerite, the proportions of the first and second pereopod, the number of articulations of the carpus and ischium of the second pereopod, and the length and terminal spines of the appendix masculina ([Mejía et al., 2008](#)). *Barbouria cubensis* has been more recently found to exhibit phenotypic hypervariation (PhyV), defined as the presence of extensive undescribed morphological variation at an uncommonly high frequency ([Ditter et al., 2019](#)). The differences that distinguish *B. yanezi* from *B. cubensis* fall within the range of morphological variations documented for *B. cubensis*

from the Bahamas ([Ditter](#)). In light of PhyV, the need to revisit the relationship between *B. yanezi* and *B. cubensis* using molecular tools is clear.

Although past molecular phylogenies have enhanced our understanding of barbouriid relationships, they are hindered by limited sampling across genera or incomplete genetic datasets ([Fiedler et al., 2010](#); [Li et al., 2011](#); [Baeza, 2013](#); [De Grave et al., 2014](#); [Aznar-Cormano et al., 2015](#)). Recent collections have allowed us to include nine species across all genera and provides further insight into the evolutionary relationships among these enigmatic crustaceans. We investigated the evolutionary relationships across current members of Barbouriidae with particular attention to the above-mentioned genera and species using multi-locus phylogenetic methods.

MATERIAL AND METHODS

SAMPLING

We selected 7 genera and 16 species to be included in the phylogenetic analysis of Barbouriidae, including all four currently recognized genera of the family Barbouriidae (*Calliasmata*, *Barbouria*, *Parhippolyte* and *Janicea*). Species of the closely related genera *Latreutes* Stimpson, 1860, *Ligur*, and *Lysmata* [Risso, 1816](#) are included as outgroups. *Barbouria cubensis* and *P. sterreri* were collected on the San Salvador, Bahamas using baited minnow traps between June 2014 and July 2015. Tissue samples of *B. yanezi* were provided by LMMO collected from Cenote Tres Potrillos, Cozumel, Mexico. Specimens of *J. antiguensis* and *C. nohochi* were donated to the Florida International Crustacean Collection (FICC) by Dr. Thomas Iliffe. Additional sequences for *J. antiguensis* and *L. fucorum* were generated from specimens housed in the FICC

located on the Biscayne Bay Campus of Florida International University. Several specimens of *Parhippolyte misticia* were received as a loan from Dr. Tin-Yam Chan, National Taiwan Ocean University (NTOU) and several *Parhippolyte uveae* specimens were received as a loan from the Muséum national d'Histoire naturelle, Paris (MNHN). *Parhippolyte cavernicola* and additional specimens of *P. uveae* were received as loans from the Smithsonian Institution, United States National Museum of Natural History (NMNH). Attempts to generate additional sequences for *L. ensiferus* were unsuccessful. Loci were selected based on previous studies that confirm their utility in caridean phylogenetics ([Bracken-Grissom et al., 2009a](#); [Fiedler et al., 2010](#); [Li et al., 2011](#); [De Grave et al., 2014](#); [Aznar-Cormano et al., 2015](#)). To utilize taxa with sequences available in GenBank, loci were also selected so that individuals included in previous studies could be included. Loci included both protein coding and non-coding nuclear and mitochondrial gene regions. We generated 196 new sequences for seven partial gene regions from *Barbouria cubensis*, *B. yanezi*, *Calliasmata nohochi*, *Parhippolyte sterreri*, *P. cavernicola*, *P. misticia*, *P. uveae*, *Janicea antiguensis*, and *Latreutes fucorum* ([Fabricius, 1798](#)).

DNA EXTRACTION, PCR AND SEQUENCING

Total genomic DNA was extracted from muscle tissue of the abdomen, antennule or the third to fifth pleopod using DNeasy® Blood and Tissue Kits (Qiagen, Valencia, CA, USA). For incomplete tissue digestions, 10 µl of 10% DTT and 10 µl Proteinase K was added, and samples incubated until complete digestion was achieved. Total genomic DNA quality was visualized using 2% agarose gels and concentration was measured

using the Qubit dsDNA HS Assay kit on the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

We selected two partial mitochondrial genes and five partial nuclear genes for their utility in studies of phylogeny and genetic diversity among decapods ([Bracken *et al.*, 2009b](#); [Baeza, 2010](#); [De Grave *et al.*, 2014](#); [Aznar-Cormano *et al.*, 2015](#)). The mitochondrial genes included the 16S large ribosomal subunit (~550 basepairs (bps)) and protein-coding cytochrome oxidase I (~600 bps, COI). The nuclear genes included the 28S large ribosomal subunit (~750 bps) and protein coding genes: phosphoenolpyruvate carboxykinase (PEPCK, ~585 bps), enolase (~375 bps), histone H3 (~350 bps), and sodium-potassium ATPase alpha-subunit (NaK, ~565 bps). The large ribosomal subunit (16S) was amplified with primers 16S-1471/1472 ([Palumbi *et al.*, 1991](#); [Crandall & Fitzpatrick 1996](#)), COI was amplified with primers F/10, or LCO1490/HCO2198 ([Folmer *et al.*, 1994](#); [Bracken-Grissom *et al.*, 2014](#)), 28S was amplified with primers 28S01/28SR-02 ([Fiedler *et al.*, 2010](#)), PEPCK with primers -for/-rev ([Tsang *et al.*, 2008](#)), enolase with primers EA2/ES2 ([Li *et al.*, 2011](#)), H3 with primers AF/AR ([Li *et al.*, 2011](#)), and NaK with primers N79/N610 ([De Grave *et al.*, 2014](#)). Amplification was performed in 25µl volume reactions containing 12.5µl GoTaq DNA polymerase (Promega, Madison, WI, USA), 1µl forward and reverse primer for each gene, 9.5µl sterile H₂O, and 1µl template DNA. The thermal cycling profile conformed to the following parameters: Initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 sec at 94 °C, 45 sec at 48–56 °C, 45 sec at 72 °C, and a final extension of 5 min at 72 °C. PCR products were sent to Genewiz (South Plainfield, NJ, USA) for amplicon purification and subsequent sequencing.

All sequencing data was visually inspected, quality trimmed, manually cleaned, and assembled using Geneious 9.1.7 (Biomatters, Newark, NJ, USA). Once assembled, sequences were aligned using MAFFT v7.308 ([Katoh & Standley, 2013](#)). To identify potential pseudogenes, we translated protein-coding sequences and checked for insertions and deletions, stop codons, identified the open reading frames, and compared sequences among conspecifics following the protocol of [Song *et al.* \(2008\)](#).

PHYLOGENETIC ANALYSES

A dataset consisting of partial sequences of the 16S, 28S, COI, enolase, H3, NaK, and PEPCK gene regions ([Table 1](#)) was constructed to investigate generic and species level relationships across Barbouriidae. Missing data were designated as a “?” in our alignment. We constructed individual gene trees to compare topologies for both datasets. To improve resolution, multiple genes were concatenated into single alignments ([Ahyong & O’Meally, 2004](#); [Porter *et al.*, 2005](#); [Robles *et al.*, 2007](#); [Page *et al.*, 2008](#)). We conducted a partition test of heterogeneity and incongruence length difference test to determine if the gene regions were appropriate to combine for analyses, as implemented in PartitionFinder 2.7.1 and PAUP*, respectively ([Swofford, 2002](#); [Lanfear *et al.*, 2016](#)). The model of evolution that best fit the individual data sets was determined by Partitionfinder 2.7.1 ([Felsenstein & Churchill, 1996](#)). Independent models of evolution and parameters were partitioned in the Bayesian concatenated analysis.

The maximum likelihood (ML) analyses were conducted using RAxML (randomized accelerated maximum likelihood; [Stamatakis *et al.*, 2005](#)) with computations performed on the high-performance computing cluster at Florida

International University. Likelihood settings followed the general time reversible model with a gamma distribution and estimates of the proportion of invariable sites (GTR+I+G) and RAxML estimated all free parameters. Confidence in the resulting topologies was assessed using rapid bootstrapping and a search for the best scoring tree with 1,000 replicates ([Felsenstein, 1985](#)). We performed Bayesian inference (BI) analyses using parameters selected by PartitionFinder 2.7.1 and conducted in MrBayes v3.2.6 ([Huelsenbeck & Ronquist, 2001](#)). A MCMC algorithm ran for 10,000,000 generations, sampling one tree every 1,000 generations. Observation of likelihood scores allowed us to determine burnins and stationary distributions. Once split frequency in the Bayesian analysis reached < 0.01 , a 50% majority-rule consensus tree was obtained from the remaining trees. Posterior probabilities for clades were compared for congruence between analyses, bootstrap values > 70 for RAxML and > 0.90 for Bayesian are presented on the phylograms.

GENETIC DIVERSITY AND DISTANCE

We separately calculated nucleotide diversity (π) and Tajima's D (D) for each mitochondrial gene region in PopART v1.7 ([Leigh & Bryant, 2015](#)) to investigate genetic diversity and demographic history across the western Atlantic. To investigate genetic divergence between presently recognized genera of Barbouriidae, Nei's standard genetic distance (D_A) between partial 16S sequences were calculated using the *Dnadist* package of PHYLIP v3.695 ([Felsenstein, 1989](#); [Tuimala, 2004](#)). All calculations were conducted following standard parameters.

RESULTS

UPDATED PHYLOGENY OF BARBOURIIDAE

This combined analysis represents 39 terminals from the family Barbouriidae, representing all presently recognized genera, and seven outgroups, *Ligur ensiferus* (Risso, 1816), *Lysmata amboinensis* (De Man, 1888), *Lysmata debelius* Bruce, 1883, *Lysmata hochi* Baeza & Anker, 2009, *Lysmata intermedia* (Kingsley, 1878), *Lysmata wurdemanni* (Gibbes, 1850), and *Latreutes fucorum*. New sequences were generated for each gene region (16S, 28S, COI, enolase, H3, NaK, and PEPCK), for three *Barbouria cubensis*, three *B. yanezi*, four *Calliasmata nohochi*, three *Janicea antiguensis*, one *Parhippolyte cavernicola*, three *P. misticia*, three *P. sterreri*, five *P. uveae*, and one *Latreutes fucorum*. Individual gene phylogenies of 16S, 28S, enolase, H3, NaK, and PEPCK are without conflict (Supplementary material Figs. S1–S8). *Calliasmata* is recovered as sister to *Lysmata* in the COI phylogeny but has poor support at the conflicting nodes (bootstrap values < 50). All relationships within the concatenated phylogeny were recovered with significant support using ML and Bayesian analyses (Fig. 3). *Ligur* was the earliest branching lineage and a sister clade to Lysmatidae + Barbouriidae. *Calliasmata pholidota* and *C. nohochi* form a monophyletic group (clade 1) and is sister to the remaining barbouriids. Four individuals of *B. cubensis* and three of *B. yanezi* form a monophyletic group (clade 2) and is sister to a paraphyletic group of *Parhippolyte* + *Janicea antiguensis*. *Janicea antiguensis* is recovered as a sister species to *P. sterreri* (clade 3), which falls sister to a clade composed of remaining species of *Parhippolyte* (clades 4 and 5). Clade 4 represents *Parhippolyte uveae* (including

P. cf. uveae from GenBank). *Parhippolyte cavernicola* is recovered as a sister species to *P. misticia* (clade 5). Several misidentifications or unidentified GenBank individuals were revealed during this study (denoted as * in Figure 3, and these can be found denoted in Table 2).

GENETIC DIVERSITY AND DISTANCE

Tajima's D and nucleotide diversity (π) was calculated based on of the lack of geographic clustering between of *B. cubensis* and *B. yanezi* in the phylogeny (clade 2; [Fig. 3](#)). The highest values for Tajima's D were negative ($D = -2.34721$, $p_D = 0.998216$). Genetic diversity was also found to be very low ($\pi = 0.998216$). Mean and standard error values for Nei's D (D_A) for the 16S gene region are reported in [Table 3](#), a heat map with all 16S D_A values in [Table S9](#). Species within the same genus have a mean genetic distance of 0.010–0.113 ([Table 3](#)). Genera within the same subfamily have genetic distances of 0.113–0.260. Individuals within the same family have distances of 0.260–0.311 ([Table 3](#)). For individuals within the same superfamily we find genetics distances > 0.311 ([Table 3](#)). We find similar patterns for the genetic distances for the COI partial gene region amplified with the F/10 primers as we find for the 16S partial gene region ([Table 4](#)). We find individuals of the same species have genetic distances < 0.01 , and individuals within the same genus have genetic distances of ~ 0.01 –0.25 ([Table 4](#)). We find individuals within the same family to have genetic distances of ~ 0.25 –0.32 ([Table 4](#)). For individuals within the same superfamily we find genetic distances of > 0.33 ([Table 4](#)). Oddly, for this locus, we recover *Latreutes fucorum* to be more closely related to *Barbouria* and *Parhippolyte* than *Calliasmata nohochi* is to *Barbouria* and *Parhippolyte*.

RECLASSIFICATION OF BARBOURIIDAE

The following classification is proposed for Barbouriidae resulting from molecular evidence generated by this study and the accumulated morphological and molecular evidence in the literature.

Superfamily Alpheoidea [Rafinesque, 1815](#)

Family Barbouriidae [Christoffersen, 1987](#)

Subfamily Barbouriinae *nov.* Christoffersen, 1987

Genus *Parhippolyte* [Borradaile, 1900](#)

Parhippolyte antiguensis ([Chace, 1972](#))

Parhippolyte cavernicola [Wicksten, 1996](#)

Parhippolyte misticia ([Clark, 1989](#))

Parhippolyte rukuensis [Burukovsky, 2007](#)

Parhippolyte sterreri ([Hart & Manning, 1981](#))

Parhippolyte uveae [Borradaile, 1900](#)

Genus *Barbouria* [Rathbun, 1912](#)

Barbouria cubensis ([von Martens, 1872](#))

Subfamily Calliasmatinae *nov.*

Genus *Calliasmata* [Holthuis, 1973](#)

Calliasmata nohochi [Escobar-Briones, Camacho & Alcocer, 1997](#)

Calliasmata pholidota [Holthuis, 1973](#)

Calliasmata rimolii [Chace, 1975](#)

SUBFAMILY Calliasmatinae *nov.*

This is a new subfamily within the family Barbouriidae.

Diagnosis: Sensory dorsal organs of carapace, if present, highly reduced. Infraorbital angle of carapace depressed, inconspicuous below antennal tooth, subocular tooth absent. Rostrum, if present, highly reduced, unarmed. Carapace bearing minute scales or setules. Palp and incisor process of mandible, if present, highly reduced. Eyes highly degenerated, with eyestalks fused basally. First pereopod and third maxilliped notably robust in comparison to other pereopods.

Genera included: Calliasmata [Holthuis, 1973](#).

Material examined: see [Supplementary material Table S10](#).

Habitat: Exclusively found in anchialine caves.

Comments: Calliasmatinae represents a new subfamily. Further examination and molecular evidence of *C. nohochi* and *C. rimolii* may reveal that they represent a single species.

SUBFAMILY Barbouriinae *nov.* [Christoffersen, 1987](#)

Diagnosis: Carapace with 2 moderately large and 2 small sensory dorsal organs ([Fig. 4](#)); first large organ associated with and posterior to epigastric tooth, second organ medial along dorsal margin located medially within cardiac region, small posterolateral pair juxtaposed to second large organ ([Fig. 4](#)). Cornea darkly pigmented, may be broader or narrower than eyestalk. Mandible with 3-jointed palp, incisor process absent. Pereopods elongated, with or without arthrobranchs at bases of anterior 4 pairs of pereopods. First

pereiopods chelate; chelae slender, moderately small. Second pereiopods with carpus divided into articles, ischium and merus faintly subdivided. Third to fifth pereiopods with or without posterior segments subdivided into articles.

Genera included: *Parhippolyte* [Borradaile, 1900](#) and *Barbouria* [Rathbun, 1912](#).

Material examined: see [Supplementary material Table S10](#).

DISCUSSION

The classification of Barbouriidae has undergone numerous revisions and still lacks an accurate formal morphological definition ([De Grave et al., 2014](#)). Our tree, based on multi-locus phylogenetic methods, includes representatives of all barbouriid genera ([Fig. 3](#)). The tree excludes *Parhippolyte rukuensis* and *Calliasmata rimolii* as no sequence data is available for these species and attempts to locate molecular-grade material failed. All previous studies have at least one representative of *Parhippolyte* and found support for the continued recognition of Barbouriidae, with the inclusion of *Barbouria*, *Parhippolyte*, and *Janicea* ([Chace, 1997](#); [Fiedler et al., 2010](#); [Baeza, 2013](#); [De Grave et al., 2014](#); [Aznar-Cormano et al., 2015](#)). [De Grave et al. \(2014\)](#) found evidence for the inclusion of *Calliasmata* into Barbouriidae, based on their phylogenetic analysis of 16S, enolase and NaK, and because all species of *Calliasmata* are anchialine cave dwellers. While [De Grave et al. \(2014\)](#) advanced our understanding of hippolytid relationships, many generic- and species-level relationships within Barbouriidae remained unresolved due to limited sampling.

Anchialine species are often endemic and increasingly rare. It is important to examine evolutionary relationships between these species and their allies to improve

management efforts. Our study presents the most comprehensive treatment to date of barbouriid phylogeny. Our results recover *Ligur* as the earliest branching lineage and is markedly outside of *Lysmata*. [De Grave et al. \(2014\)](#) found *Ligur* to be included within Lysmatidae, but our finding shows *Ligur* to be a distinct lineage, using *Latreutus fucorum* as the outgroup taxon. It is possible *Ligur* may need to be considered outside of Lysmatidae; however, further analyses with the inclusion of additional specimens of *L. ensiferus*, and species of Lysmatidae and Hippolytidae is required to resolve this relationship.

Calliasmata is the earliest branching lineage of Barbouriidae, which is in congruence with previous molecular findings ([De Grave et al., 2014](#)). More surprising, the genetic distance separating *Calliasmata* from the remaining barbouriids is comparable to the family-level distances between Lysmatidae and Barbouriidae, suggesting that *Calliasmata* represents a separate taxonomic group ([Tables 3, 4](#)). From a morphological standpoint, *Calliasmata* lacks any significant synapomorphy previously identified for Barbouriidae as stated by [De Grave et al. \(2014\)](#). The genus differs from genera of Barbouriidae in having the rostrum formed by a simple spine, degenerate and immovable eyes that may or may not be fused, the scaphocerite not reaching beyond the distal margin of the third antennular peduncle and lacking a 3-jointed palp, and the presence of a single podobranch on the second maxilliped and a single arthrobranchs on the third maxilliped ([Holthuis, 1973](#); [Chace, 1975](#); [Escobar-Briones et al., 1997](#)). The relationships formed within the multi-locus phylogenetic tree ([Fig. 3](#)), genetic distances and morphological evidence, provide strong support that the sub-families Barbouriinae *nov.* and Calliasmatinae *nov.* be erected as a monogeneric subfamilies within

Barbouriidae. Erecting these subfamilies resolves the disparity between morphological and molecular evidence as to the relationship between *Calliasmata* and other barbouriids ([Fig. 3](#); [Tables 3–5](#)), but Barbouriidae still lacks a complete definition ([De Grave et al., 2014](#)).

Another surprising result is the phylogenetic position of *Janicea antiquensis*, significantly supported within *Parhippolyte* and as the sister taxon to *P. sterreri* (clade 3; [Fig. 3](#)). In all phylogenetic trees, including single gene phylogenies, *J. antiquensis* was found nested within the genus *Parhippolyte*. *Janicea antiquensis* is similar to *Parhippolyte* in the subdivision of the three posterior pereopods and having a cornea that is wider than the eyestalk, and is superficially most similar to *P. misticia* ([Chace, 1972](#); [Clark, 1989](#)). *Janicea antiquensis* is similar to *Barbouria* in its gill complement ([Chace, 1972](#); [Manning & Hart, 1984](#)). [Clark \(1989\)](#) drew attention to the appendix masculina (AM) being longer than the appendix interna (AI) as a diagnostic character for *Janicea*. In the likely case that barbouriid genera are protandric simultaneous hermaphrodites similar to *P. misticia*, however, the length of the AM versus the AI will change as individuals transition from the male to female phase ([Onaga et al., 2012](#)). This character and other similar sexually dimorphic characters are thus not viable characters to delineate among species. Morphologically, *Janicea* seems to represent an intermediate between *Parhippolyte* and *Barbouria*, but our molecular results suggest a clear affinity between species of *Parhippolyte* and *J. antiquensis* (clade 3; [Fig. 3](#)).

Our phylogeny ([Fig. 3](#)) finds *Parhippolyte* to be paraphyletic. The paraphyly can be resolved by either considering *Janicea* as a junior synonym of *Parhippolyte*, or by

resurrecting the genus *Somersiella*. In our tree, *P. sterreri* is found to form a group with *J. antiquensis* (clade 3; [Fig. 3](#)) and is sister to a clade composed of *P. misticia* + *P. uveae* + *P. cavernicola* (clades 4 and 5; [Fig. 3](#)). It is noteworthy that *Parhippolyte sterreri* was first described as *Somersiella sterreri* ([Hart & Manning, 1981](#)) and *Somersiella* was considered distinct from *Parhippolyte* due to the lack of a podobranch on the second maxilliped ([Manning & Hart, 1984](#)), and whether or not the telson terminated in a sharp point ([Christoffersen, 1987](#)). The examination of material of *P. sterreri* revealed a podobranch on the second maxilliped previously diagnosed as being absent, and that characters that are distinct among genera are likely species-specific ([Wicksten, 1996](#)). There is no morphological evidence supporting the resurrection of *Somersiella* ([Wicksten, 1996](#)). The inclusion of more material, including *P. cavernicola*, has helped resolve the relationships among species of *Parhippolyte*. Based on a lack of morphological evidence and our multi-locus phylogeny, we find the best solution is to consider *Janicea* as a junior synonym of *Parhippolyte* and retain *P. sterreri* within the genus *Parhippolyte*.

The relationship between *P. misticia* and *P. uveae* could not be resolved using only the available sequences from GenBank. The inclusion of additional material of *P. misticia*, *P. cavernicola*, and *P. uveae* has revealed the evolutionary relationships within this genus and mistaken or unresolved identification of species within *Parhippolyte* ([Table 2](#)). Updating the GenBank and museum records for the corresponding sequences for these *Parhippolyte* species resolves the conflicts in the phylogenies created by the inclusion of these sequences ([Table 2](#)).

Our phylogeny finds *Barbouria cubensis* and *Barbouria yanezi* form a single species, represented by a polytomy in our tree ([Fig. 3](#)). *Barbouria yanezi* was described as a distinct species within *Barbouria* based on the length of the rostrum, number and positioning of rostral teeth, length to width ratio of the scaphocerite, length ratios of the articles of the first and second pereopod, length of the appendix masculina *versus* the appendix interna, number of terminal spines, and maximum recorded carapace length ([Mejía *et al.*, 2008](#)). All of the differences between *B. yanezi* and *B. cubensis* fall within the range of PhyV documented in *B. cubensis* ([Ditter *et al.*, 2019](#)). Based on the lack of genetic morphological differences we consider *Barbouria yanezi* to be a synonym of *B. cubensis*. This reverts *Barbouria* to a monotypic genus.

Some noteworthy phylogeographic patterns emerge in our barbouriid tree ([Figs. 2, 3](#)). *Barbouria cubensis* (Atlantic species) is the earliest branching lineage of Barbouriinae (clade 2; [Fig. 3](#)), sister to a clade comprised of *P. sterreri* + *J. antiguensis* (clade 3; [Fig. 3](#)) and *P. misticia* + *P. cavernicola* + *P. uveae* (clades 4 and 5; [Fig. 3](#)). This would suggest that Barbouriinae originated in the Atlantic after the invasion of caves. Pacific barbouriids also appear superficially similar to Atlantic counterparts, with similar a morphological appearance between *P. uveae* and *B. cubensis*, *P. cavernicola* and *P. sterreri*, and *P. misticia* and *J. antiguensis* ([Figs. 1, 5](#)). Our phylogeny also suggests that Barbouriidae likely originated from shallow-water reef species prior to invading caves, instead of deep-sea species, a hypothesis based on the close relationship of Barbouriidae with Lysmatidae. A cornea that is narrower than the eyestalk is a predominant troglodytic adaptation found in Barbouriinae but only present in *B. cubensis*. Only *B. cubensis* and *P. sterreri* have the propodus and carpus of the third to fifth pereopods not articulated.

Barbouria cubensis and *J. antiquensis* lack arthrobranches on the first and second maxillipeds, whereas all species of Barbouriinae have two arthrobranches on the first and second maxillipeds. These characters suggest that the ancestor of Barbouriinae likely possessed a greater number of troglodytic adaptations that have been lost over time. It is peculiar, however, that *J. antiquensis* shares characters with both Atlantic and Pacific species of Barbouriidae. Such distribution suggests that after *Barbouria* further diverged from its troglodytic ancestor barbouriids before invading the Pacific. It would be of particular interest to investigate the results of an ancestral state reconstruction to gain a better understanding when these invasions and divergences occurred.

Our results are not expected to fully resolve the classification of all barbouriids but represents progress towards unraveling the relationships among these species, which have remained enigmatic to caridean systematists. This study is intended to provide clarity into the evolutionary history of Barbouriidae and provide a framework for future studies.

KEY FOR SUBFAMILIES BARBOURIINAE AND CALLIASMATINAE

Carapace armed with unique subocular tooth posterodorsal to the orbital angle; mandible with 3-jointed palp; carapace with sensory dorsal organs, the first associated and posterodorsal to the epigastric tooth and the second along the dorsal median margin within cardiac region, with smaller pair posterolateral juxtaposed to second sensory dorsal organ Barbouriinae

Infraorbital angle of the carapace depressed and inconspicuous below small antennal tooth, subocular tooth absent; eye highly reduced with eyestalks fused basally;

rostrum, if present, unarmed, not reaching past eyestalk; palp and incisor process
of mandible, if present, highly reduced; third maxilliped and first pereopod
notably robust; sensory dorsal organs, if present, highly reduced
..... Calliasmatinae

KEY TO SPECIES OF *CALLIASMATA*, SUBFAMILY CALLIASMATINAE

(Modified from Escobar-Briones *et al.*, 1997)

1. Rostrum overreaching eyes, antennal spine overreaching distal margin of eyes, third to
fifth pleura of abdominal somites with strong ventral tooth, telson margin bearing
3 spines *C. pholidota*
- Rostrum not overreaching eyes, antennal spine almost reaching distal margin of eyes,
third to fifth pleura of abdominal somites without ventral tooth, telson margin
without spines 2
2. Carapace and integument with setules, third to fifth pleura posteroventral angle acute
produced into tooth, propodus of fourth pereopod 5 times longer than dactylus
..... *C. nohochi*
- Setules of carapace and integument, if present, highly reduced, posteroventral angle of
third to fifth pleura acute without tooth, propodus of fourth pereopod 4 times
longer than dactylus *C. rimolii*

KEY TO GENERA OF SUBFAMILY BARBOURIINAE

1. Eyes large, cornea darkly pigmented, broader than eyestalk 2
Eyes reduced, cornea darkly pigmented, narrower than or subequal to the eyestalk
..... *B. cubensis* ([Fig. 5B](#))
2. Carpus and propodus of third to fifth pereopods multiarticulate 3
Carpus and propodus of third to fifth pereopods not multiarticulate
..... *P. sterreri* ([Fig. 5D](#))
3. Anterior 4 pereopods with arthrobranchs 4
Anterior 4 pereopods without arthrobranchs *P. antiguensis* ([Fig. 5F](#))
4. Pleuron of fourth abdominal somite rounded or subacute without terminal point 5
Pleuron of fourth abdominal somite acute or subacute with terminal point, fifth and sixth
pleuron produced into tooth, appendix masculina of second male pleopod not
reaching as far as distal end of appendix interna, terminal margin of telson formed
into point, terminating in a strong tooth *P. uveae* ([Fig. 5A](#))
5. Pleuron 5 of abdominal somite unarmed, appendix masculina of second male pleopod
shorter than appendix interna *P. cavernicola* ([Fig. 5C](#))
Pleuron 5 of abdominal somite produced into tooth, appendix masculina of second male
pleopod longer than appendix interna 6
6. Rostrum armed with more than 1 dorsal and ventral teeth, extending almost to the
distal end of basal segment of antennular peduncle, terminal margin of telson
formed into point, terminating in a weak tooth *P. misticia* ([Fig. 5E](#))
Rostrum armed with a single dorsal and ventral tooth, not reaching to the distal end of the
basal segment of the antennular peduncle *P. rukuensis*

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TABLES

Table 1. Species used in phylogenetic reconstruction for the family Barbouriidae [see Material and Methods for the museum abbreviations]. Accession numbers for partial sequences of COI we generated using the LCO1490/HCO2198 and F/10 primers respectively. Individuals with successful amplification for both regions will have two accession numbers for the COI gene. Reproduced with permission from Oxford University Press.

Taxon	Collection locality	Museum catalog no.	GenBank accession no.							
			16S	28S	Enolas	H3	NaK	PEPCK	COI	
<i>Barbouria cubensis</i>	San Salvador, Bahamas	HBG1937	MT505235	MT505191	MT527421	MT527448	MT527503	MT527477	MT524340	MT540990
<i>B. cubensis</i>	San Salvador, Bahamas	HBG2151	MT505236	MT505192	MT527422	MT527449	MT527504	MT527478	MT524341	MT540991
<i>B. cubensis</i>	San Salvador, Bahamas	HBG2198	MT505237	MT505193	MT527423	MT527450	MT527505	MT527479	MT524342	MT540992
<i>B. cubensis</i>	San Salvador, Bahamas	OUMNH.ZC.2010-05-003	KF023098	—	KF023142	—	KF023173	—	—	—
<i>B. yanezi</i>	Cozumel, Mexico	HBG9168	MT505238	—	—	MT527451	—	—	—	MT540993
<i>B. yanezi</i>	Cozumel, Mexico	HBG9180	MT505239	MT505194	MT527424	MT527452	MT527506	MT527480	—	MT540994
<i>B. yanezi</i>	Cozumel, Mexico	HBG9181	MT505240	MT505195	—	MT527453	—	MT527481	—	MT540995
<i>Calliasmata pholidota</i>	Hawaii, USA	OUMNH.ZC.2010-04-003	KF023119	—	KF023151	—	KF023182	—	—	—
<i>C. nohochi</i>	Mexico	A. Baeza, pers. coll.	—	—	KF178861	KF178838	—	—	—	—
<i>C. nohochi</i>	Mayaguana, Bahamas	HBG10007	MT505231	MT505187	MT527417	MT527444	MT527499	MT527473	MT524336	MT549845
<i>C. nohochi</i>	Mayaguana, Bahamas	HBG10008	MT505232	MT505188	MT527418	MT527445	MT527500	MT527474	MT524337	MT549846
<i>C. nohochi</i>	Mayaguana, Bahamas	HBG10009	MT505233	MT505189	MT527419	MT527446	MT527501	MT527475	MT524338	MT549847
<i>C. nohochi</i>	Mayaguana, Bahamas	HBG10010	MT505234	MT505190	MT527420	MT527447	MT527502	MT527476	MT524339	MT549848
<i>Janicea antiguensis</i>	Cape Verde, Africa	OUMNH.ZC.2004-15-002	MT505241	MT505196	MT527425	MT527454	MT527507	MT527482	MT524343	—
<i>J. antiguensis</i>	Giant Cave, Belize	HBG9999	MT505242	MT505197	MT527426	MT527455	MT527508	MT527483	—	MT542150
<i>J. antiguensis</i>	Giant Cave, Belize	HBG10000	MT505243	MT505198	MT527427	MT527456	MT527509	MT527484	MT524344	MT542151
<i>Parhippolyte cavernicola</i>	Baja California Sur, Mexico	HBG10136/USNM-273315	MT505253	MT505208	MT527431	MT527470	MT527524	—	MT534287	—
<i>P. misticia</i>	Odo Point, Okinawa, Japan	Fiedler et al., 2010	HQ315615	HQ315560	—	—	—	—	—	—
<i>P. misticia</i>	Shagakko-mae, Japan	HBG10126/NTOU-M01157-E	—	MT505204	—	MT527465	MT527518	MT527491	MT524349	MT542196
<i>P. misticia</i>	Shagakko-mae, Japan	HBG10127/NTOU-M01157-F	—	MT505205	MT527436	MT527466	MT527519	MT527492	MT524350	MT542197
<i>P. misticia</i>	Shagakko-mae, Japan	HBG10128/NTOU-M01157-G	—	MT505206	MT527437	MT527467	MT527520	MT527493	—	MT542198
<i>P. sterreri</i>	San Salvador, Bahamas	HBG1941	MT505244	MT505199	MT527428	MT527457	MT527510	MT527485	MT524345	MT542201
<i>P. sterreri</i>	San Salvador, Bahamas	HBG1943	MT505245	MT505200	MT527429	MT527458	MT527511	MT527486	MT524346	MT542200
<i>P. sterreri</i>	San Salvador, Bahamas	HBG2149	MT505246	MT505201	MT527430	MT527459	MT527512	MT527487	MT524347	MT542199
<i>P. sterreri</i>	Iguana Cay, Exumas, Bahamas	MNHN-IU-2012-1057	KP725619	KP726001	—	KP726178	—	—	KP759480	—
<i>Parahippolyte</i> sp.	Japan NTOU	M01675/TWH-2014	KF023096	—	KF023140	—	KF023171	—	—	—
<i>P. cf. uveae</i>	Okinawa, Japan	JAB-2013	KF178886	—	KF178877	KF178855	—	—	—	—
<i>P. uveae</i>	Society Islands, French Polynesia	HBG10114/MNHN-IU-2012-1001	MT505247	MT505202	MT527432	MT527460	MT527513	MT527488	—	MT542192
<i>P. uveae</i>	Society Islands, French Polynesia	HBG10115/MNHN-IU-2012-1002	MT505248	MT505203	MT527433	MT527461	MT527514	MT527489	—	MT542193
<i>P. uveae</i>	Coral Sea	HBG10117/MNHN-IU-2018-3568-B	MT505249	—	MT527434	MT527462	MT527515	MT527490	—	MT542194
<i>P. uveae</i>	Aldabra, Seychelles	HBG10130/USNM-280216-85(1)	MT505251	—	MT527438	MT527468	MT527521	MT527494	MT524351	—
<i>P. uveae</i>	Aldabra, Seychelles	HBG10134/USNM-280216-85(2)	—	—	—	MT527469	MT527522	MT527495	MT524352	MT542202
<i>P. uveae</i>	Aldabra, Seychelles	HBG10135/USNM-280216-86(3)	MT505252	MT505207	MT527432	MT527460	MT527513	MT527488	MT524353	MT542203
<i>Lysmata amboinensis</i>	Not available	HBG395/KC9045	MT505229	MT527439	—	—	MT527472	—	MT542204	—
<i>L. debellus</i>	Not Available	MLP121	DQ079718	DQ079793	—	DQ079681	—	—	—	—
<i>L. hochi</i>	Long Key, FL, USA	UMML32.9460	EU861507	—	KF178810	KF178848	—	—	KC962174	—
<i>L. intermedia</i>	Bocas Del Toro, Panama	UMML32.9461	EU861484	—	KF178871	KF178849	KF023169	—	KC962203	—
<i>L. wurdemannii</i>	Gulf of Mexico	HBG546/KC4529/ULLZ17433	MT505230	MT505186	MT527440	MT527443	MT527498	—	—	—
<i>Ligur ensiferus</i>	Guadeloupe	MNHN-IU-2012-1000	KP725542	KP725926	KF023141	KP726104	KF023172	—	—	—
<i>Latreutes fucorum</i>	Gulf of Mexico	HBG2764	MT505228	MT505184	MT527441	MT527442	MT527497	MT527471	—	MT563442

Table 2. Original and corrected identifications of taxa in Barbouriidae. See Material and Methods for museum abbreviations.

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Museum catalog no.	Taxon	
	Original	Corrected
HBG9168	<i>Barbouria yanezi</i>	<i>Barbouria cubensis</i>
HBG9180	<i>Barbouria yanezi</i>	<i>Barbouria cubensis</i>
HBG9181	<i>Barbouria yanezi</i>	<i>Barbouria cubensis</i>
HBG66/OUMNH.ZC.2004-15-002	<i>Janicea antiguensis</i>	<i>Parhippolyte antiguensis</i>
HBG9999	<i>Janicea antiguensis</i>	<i>Parhippolyte antiguensis</i>
HBG10000	<i>Janicea antiguensis</i>	<i>Parhippolyte antiguensis</i>
HBG10114/MNHN-IU-2012-1001	<i>Parhippolyte uveae</i>	<i>Parhippolyte misticia</i>
HBG10115/MNHN-IU-2012-1002	<i>Parhippolyte uveae</i>	<i>Parhippolyte misticia</i>
HBG10117/MNHN-20018–3568	<i>Parhippolyte uveae</i>	<i>Parhippolyte misticia</i>
NTOU M01675/TWH-2014	<i>Parhippolyte sp.</i>	<i>Parhippolyte misticia</i>
JAB-2013	<i>Parhippolyte cf. uveae</i>	<i>Parhippolyte uveae</i>

Table 3. Mean (and standard error) of genetic distances between genera of Barbouriidae for the 16S partial gene regions.

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	<i>Ligur</i>	<i>Lysmata</i>	<i>Calliasmata</i>	<i>Barbouria</i>	<i>Janicea</i>	<i>Parhippolyte</i>
<i>Ligur</i>	0.022 (0.025)	0.309 (0.024)	0.321 (0.006)	0.329 (0.000)	0.344 (0.010)	0.304 (0.027)
<i>Lysmata</i>	0.309 (0.024)	0.112 (0.104)	0.329 (0.015)	0.334 (0.021)	0.352 (0.018)	0.330 (0.029)
<i>Calliasmata</i>	0.331 (0.005)	0.344 (0.015)	0.099 (0.118)	0.311 (0.006)	0.303 (0.022)	0.300 (0.021)
<i>Barbouria</i>	0.329 (0.000)	0.334 (0.021)	0.311 (0.006)	0.000 (0.000)	0.260 (0.007)	0.238 (0.014)
<i>Janicea</i>	0.344 (0.010)	0.352 (0.018)	0.303 (0.022)	0.260 (0.007)	0.007 (0.009)	0.113 (0.018)
<i>Parhippolyte</i>	0.304 (0.027)	0.330 (0.029)	0.300 (0.021)	0.238 (0.014)	0.113 (0.018)	0.089 (0.054)

Table 4. Genetic distances for the CO1 partial gene region in species of Barbouriidae amplified with the F/10 primers. GenBank accession numbers and alternative museum catalog numbers are listed in Table 1. Individuals with asterisk (*) represent misidentified species in GenBank. See Table 2 for the correct identifications. Reproduced with permission of Oxford University Press.

	<i>Latreutes fucorum</i>	<i>Calliasmata nohochi</i>	<i>Barbouria cubensis</i>	<i>Barbouria yanezi</i>	<i>Parhippolyte sterreri</i>	<i>Parhippolyte uveae*</i>	<i>Parhippolyte misticia</i>	<i>Parhippolyte uveae</i>	<i>Parhippolyte cavernicola</i>	<i>Janicea antiguensis</i>
Museum cat. no.	HBG2764	HBG10010	HBG1937	HBG9181	HBG2149	HBG10114	HBG10125	HBG10135	HBG10136	HBG10000
<i>Latreutes fucorum</i>	0	0.6878	0.4425	0.4425	0.4875	0.4338	0.4257	0.4302	0.4782	0.4031
<i>Calliasmata nohochi</i>	0.6878	0	0.5072	0.5072	0.5343	0.5007	0.5030	0.5074	0.6459	0.5014
<i>Barbouria cubensis</i>	0.4425	0.5072	0	0	0.2717	0.3173	0.3184	0.2760	0.2887	0.2904
<i>Barbouria yanezi</i>	0.4425	0.5072	0	0	0.2717	0.3173	0.3184	0.2760	0.2887	0.2904
<i>Parhippolyte sterreri</i>	0.4875	0.5343	0.2717	0.2717	0	0.2241	0.2297	0.2180	0.2509	0.2041
<i>Parhippolyte uveae*</i>	0.4338	0.5007	0.3173	0.3173	0.2241	0	0.0073	0.2119	0.2556	0.2478
<i>Parhippolyte misticia</i>	0.4257	0.5030	0.3184	0.3184	0.2297	0.0073	0	0.2126	0.2595	0.2437
<i>Parhippolyte uveae</i>	0.4302	0.5074	0.2760	0.2760	0.2180	0.2119	0.2126	0	0.0300	0.2345
<i>Parhippolyte cavernicola</i>	0.4782	0.6459	0.2887	0.2887	0.2509	0.2556	0.2595	0.0300	0	0.2674
<i>Janicea antiguensis</i>	0.4031	0.5014	0.2904	0.2904	0.2041	0.2478	0.2437	0.2345	0.2674	0

Table 5. Comparison between diagnostic characters of Barbouriinae and Calliasmatinae. Reproduced with permission of Oxford University Press.

	Barbouriinae <i>nov.</i>	Calliasmatinae <i>nov.</i>
1	Rostrum with dorsal and ventral margin armed, usually reaching beyond the eyestalk.	Rostrum, if present, highly reduced and unarmed, not reaching beyond eyestalk.
2	Carapace with subocular tooth.	Carapace without subocular tooth.
3	Carapace smooth, without setules.	Carapace and integument bearing setules.
4	Sensory dorsal organs of carapace well developed; associated with epigastric tooth and within cardiac region (Fig. 4).	Sensory dorsal organs of carapace, if present, highly reduced.
5	Mandible with 3-jointed palp.	Palp of mandible, if present, much reduced.
6	Cornea darkly pigmented and well developed.	Cornea, if present, highly degenerated.
7	Eyestalk not fused basally.	Eyestalk fused basally.
8	Third maxilliped and first pereopod narrow and elongated, slightly more robust <i>vs.</i> remaining pereopods.	Third maxilliped and first pereopod notably more robust <i>versus</i> remaining pereopods.

FIGURE CAPTIONS

Figure 1. Examples of Barbouriidae taxa. *Parhippolyte uveae* (Photo by Meerwasser-Aquaristik-Studio-Korallenkiste) (**A**). *Barbouria cubensis* (Photo by R.E. Ditter) (**B**). *Parhippolyte cavernicola* (Photo by A. Kerstitch) (**C**). *Parhippolyte sterreri* (Photo by R.E. Ditter) (**D**). *Parhippolyte misticia* (Photo by J. Starmer in Legall & Poupin, 2018) (**E**). *Janicea antiguensis* (Photo by T. Iliffe) (**F**). Reproduced with permission of Oxford University Press.

Figure 2. Distribution of the genera *Barbouria*, *Calliasmata*, *Janicea* and *Parhippolyte*. *P. rukuensis* excluded because it was described from a single female collected from Yeh-Him Island, Ryukyu Archipelago, Japan. Reproduced with permission of Oxford University Press.

Figure 3. Bayesian (BI) phylogram for Barbouriidae ($N = 8$) based on a 16S, 28S, COI, Enolase, H3, NaK and PEPCK concatenated data set. BI posterior probabilities and Maximum Likelihood (ML) bootstrap values noted above branches. Values > 0.7 for ML and $> 90\%$ for BI are shown and represented by percentages. Vertical black bars represent the native range for each species. Catalog numbers represent voucher specimens housed in the Florida International Crustacean Collection (FICC). * Indicates a clade likely comprised of a single species. ** Indicates chimeric individuals. Reproduced with permission of Oxford University Press.

Figure 4. Sensory Dorsal Organ (SDO) of Barbouriidae on *Barbouria cubensis*

(Illustrated by R.E. Ditter). SDO associated with and posterior to the epigastric tooth (A).

Single large SDO within cardiac region and a small pair juxtaposed to the larger SDO

(B). Scale bar = 1mm. Reproduced with permission of Oxford University Press.

Figure 5. Illustrated examples of Barbouriidae taxa. *Parhippolyte uveae* (from Fransen &

Tomascik, 1996, fig. 1) (A). *Barbouria cubensis* (from Hobbs, Hobbs & Daniel 1977, fig.

33) (B). *Parhippolyte cavernicola* (from Wicksten, 1996, fig. 1) (C). *Parhippolyte*

sterreri formerly *Somersiella sterreri* (from Hart & Manning, 1981, fig. 1) (D).

Parhippolyte misticia (from Clark, 1989, fig. 1) (E). *Janicea antiguensis* (from Chace,

1972, fig. 40b) (F). Reproduced with permission of Oxford University Press.

FIGURES

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

SUPPLEMENTARY MATERIAL (CAPTIONS)

Supplementary material is available at Journal of Crustacean Biology online.

S1 Figure. 16S loci phylogeny with support values calculated using RAxML.

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S2 Figure. 28S loci phylogeny with support values calculated using RAxML.

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S3 Figure. Enolase loci phylogeny with support values calculated using RAxML.

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S4 Figure. H3 loci phylogeny with support values calculated using RAxML. Reproduced with permission of Oxford University Press.

S5 Figure. NaK loci phylogeny with support values calculated using RAxML.

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S6 Figure. PEPCK loci phylogeny with support values calculated using RAxML.

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S7 Figure. COI loci (amplified with F/10 primers) phylogeny with support values calculated using RAxML. Reproduced with permission of Oxford University Press.

S8 Figure. COI loci (amplified with LCO1490/HCO2198 primers) phylogeny with support values calculated using RAxML. Reproduced with permission of Oxford University Press.

S9 Table. Heat map and genetic distances (DA) for the 16S partial gene region.

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S10 Table. Material of Barbouriidae examined. Reproduced with permission of Oxford University Press.

Figure S1.

Figure S2.

Figure S3.

Figure S4.

Figure S5.

Figure S6.

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Figure S7.

Figure S8.

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Table S9.

		<i>Barbouria yanezi</i>		<i>Janicea antiquensis</i>			<i>Parhippolyte sterrei</i>			<i>Parhippolyte uveae</i>		<i>Parhippolyte cf. uveae*</i>	<i>Parhippolyte misticia*</i>	<i>Parhippolyte misticia</i>			<i>Parhippolyte sp.*</i>	<i>Parhippolyte cavernicola</i>
	Accession #	HBG9180	HBG9181	HBG9999	HBG10000	HBG66	HBG1941	HBG1943	HBG2149	HBG10130	HBG10135	KF178886	HBG10114	HBG10115	HBG10117	HQ315615	KF023096	HBG10136
<i>Latreutes fucorum</i>	HBG2764	0.365824	0.365824	0.415082	0.415082	0.406113	0.389588	0.385521	0.385521	0.423012	0.414966	0.411927	0.397846	0.38596	0.393763	0.394733	0.388535	0.410047
<i>Ligur ensiferus</i>	KP725541	0.332399	0.332399	0.358349	0.358155	0.334909	0.317445	0.316608	0.316608	0.313373	0.313373	0.313373	0.317145	0.306951	0.310718	0.308855	0.305622	0.238938
	KF023097	0.332399	0.332399	0.358349	0.358155	0.334909	0.317445	0.316608	0.316608	0.313373	0.313373	0.313373	0.317145	0.306951	0.310718	0.308855	0.305622	0.238938
	KP725542	0.327993	0.327993	0.361689	0.358456	0.337797	0.305497	0.304704	0.304704	0.313156	0.313156	0.313156	0.320181	0.310042	0.313738	0.308801	0.308728	0.239931
<i>Lysmata debelius</i>	DQ079718	0.331156	0.331156	0.343521	0.343521	0.326453	0.330152	0.329315	0.329315	0.341839	0.329377	0.329377	0.313571	0.286012	0.309195	0.317019	0.298723	0.268663
<i>Lysmata intermedia</i>	EU861484	0.328078	0.328078	0.386011	0.386011	0.374252	0.362533	0.361599	0.361599	0.369931	0.359669	0.356797	0.349322	0.33861	0.356843	0.377834	0.356507	0.284403
<i>Lysmata amboinensis</i>	KF023091	0.345003	0.345003	0.374373	0.374373	0.355774	0.356814	0.355848	0.355848	0.350279	0.345276	0.342299	0.328287	0.296102	0.327823	0.337267	0.301749	0.278659
<i>Lysmata wurdemanni</i>	HBG546	0.3976	0.3976	0.427344	0.430433	0.411942	0.405539	0.404449	0.404449	0.40648	0.39762	0.39762	0.375339	0.378808	0.380218	0.378586	0.361908	0.367499
<i>Lysmata hochi</i>	EU861507	0.295098	0.295098	0.340632	0.340632	0.329497	0.329002	0.328159	0.328159	0.341886	0.3321	0.329249	0.317237	0.299363	0.310218	0.328814	0.306692	0.254464
<i>Calliasmata pholidota</i>	KF023119	0.306875	0.306875	0.286379	0.283618	0.277285	0.2656	0.264977	0.264977	0.269345	0.265729	0.268661	0.272932	0.301442	0.274814	0.303705	0.295264	0.294686
<i>Calliasmata nohochi</i>	HBG10007	0.314351	0.314351	0.34753	0.350585	0.328733	0.323746	0.322957	0.322957	0.330455	0.324862	0.328048	0.315543	0.299374	0.318562	0.299324	0.304212	0.280738
	HBG10009	0.314351	0.314351	0.34753	0.350585	0.328733	0.323746	0.322957	0.322957	0.330455	0.324862	0.328048	0.315543	0.299374	0.318562	0.299324	0.304212	0.280738
	HBG10010	0.314351	0.314351	0.34753	0.350585	0.328733	0.323746	0.322957	0.322957	0.330455	0.324862	0.328048	0.315543	0.299374	0.318562	0.299324	0.304212	0.280738
<i>Barbouria cubensis</i>	HBG1937	0	0	0.278128	0.280927	0.266742	0.265531	0.264913	0.264913	0.250927	0.242562	0.242562	0.237913	0.236037	0.234676	0.252026	0.241521	0.225807
	HBG2151	0	0	0.278128	0.280927	0.266742	0.265531	0.264913	0.264913	0.250927	0.242562	0.242562	0.237913	0.236037	0.234676	0.252026	0.241521	0.225807
	HBG2198	0.001923	0.001923	0.275406	0.278198	0.264054	0.262804	0.262194	0.262194	0.253729	0.245256	0.245256	0.235208	0.238729	0.231986	0.252026	0.238743	0.231825
<i>Barbouria yanezi</i>	HBG9168	0	0	0.278128	0.280927	0.266742	0.265531	0.264913	0.264913	0.250927	0.242562	0.242562	0.237913	0.236037	0.234676	0.252026	0.241521	0.225807
	HBG9180	0	0	0.278128	0.280927	0.266742	0.265531	0.264913	0.264913	0.250927	0.242562	0.242562	0.237913	0.236037	0.234676	0.252026	0.241521	0.225807
	HBG9181	0	0	0.278128	0.280927	0.266742	0.265531	0.264913	0.264913	0.250927	0.242562	0.242562	0.237913	0.236037	0.234676	0.252026	0.241521	0.225807
<i>Janicea antiquensis</i>	HBG9999	0.278128	0.278128	0	0.003833	0.021425	0.128802	0.128543	0.128543	0.116051	0.112616	0.114902	0.118787	0.143228	0.123344	0.165212	0.141693	0.165967
	HBG10000	0.280927	0.280927	0.003833	0	0.021425	0.128748	0.12849	0.12849	0.116002	0.11257	0.114855	0.118736	0.143168	0.123292	0.159654	0.14163	0.16068
	HBG66	0.266742	0.266742	0.021425	0.021425	0	0.119649	0.119411	0.119411	0.095669	0.094996	0.097229	0.098274	0.120323	0.10274	0.14074	0.122382	0.118873
<i>Parhippolyte sterrei</i>	HBG1941	0.265531	0.265531	0.128802	0.128748	0.119649	0	0.001952	0.001952	0.157872	0.153034	0.150772	0.134507	0.152092	0.136819	0.166888	0.14106	0.119993
	HBG1943	0.264913	0.264913	0.128543	0.12849	0.119411	0.001952	0	0	0.157537	0.152719	0.150463	0.134224	0.151777	0.136531	0.166485	0.140755	0.124726
	HBG2149	0.264913	0.264913	0.128543	0.12849	0.119411	0.001952	0	0	0.157537	0.152719	0.150463	0.134224	0.151777	0.136531	0.166485	0.140755	0.124726
<i>Parhippolyte uveae</i>	HBG10130	0.250927	0.250927	0.116051	0.116002	0.095669	0.157872	0.157537	0.157537	0	0	0.00198	0.079744	0.103226	0.093262	0.134882	0.130844	0.093228
<i>Parhippolyte cf. uveae*</i>	HBG10135	0.242562	0.242562	0.112616	0.11257	0.094996	0.153034	0.152719	0.152719	0	0	0.001926	0.079744	0.102435	0.093262	0.134882	0.129317	0.093228
<i>Parhippolyte misticia*</i>	KF178886	0.242562	0.242562	0.114902	0.114855	0.097229	0.150772	0.150463	0.150463	0.00198	0.001926	0	0.081986	0.104709	0.09555	0.137621	0.131795	0.093228
<i>Parhippolyte misticia*</i>	HBG10114	0.237913	0.237913	0.118787	0.118736	0.098274	0.134507	0.134224	0.134224	0.079744	0.079744	0.081986	0	0.066771	0.01201	0.070296	0.05801	0.098945
<i>Parhippolyte misticia</i>	HBG10115	0.236037	0.236037	0.143228	0.143168	0.120323	0.152092	0.151777	0.151777	0.103226	0.102435	0.104709	0.066771	0	0.053533	0.043516	0.022626	0.088661
	HBG10117	0.234676	0.234676	0.123344	0.123292	0.10274	0.136819	0.136531	0.136531	0.093262	0.093262	0.09555	0.01201	0.053533	0	0.0554	0.044503	0.093239
	HQ315615	0.252026	0.252026	0.165212	0.159654	0.14074	0.166888	0.166485	0.166485	0.134882	0.134882	0.137621	0.070296	0.043516	0.0554	0	0.036637	0.092258
<i>Parhippolyte sp.*</i>	KF023096	0.241521	0.241521	0.141693	0.14163	0.122382	0.14106	0.140755	0.140755	0.130844	0.129317	0.131795	0.05801	0.022626	0.044503	0.036637	0	0.090394
<i>Parhippolyte cavernicola</i>	HBG10136	0.225807	0.225807	0.165967	0.16068	0.118873	0.119993	0.124726	0.124726	0.093228	0.093228	0.093228	0.098945	0.088661	0.093239	0.092258	0.090394	0

Table S10.

Taxon		FICC catalog no.	Alt. museum catalog no.	Collection date	Locality	No.
original	corrected					
<i>Calliasmata nohochi</i>		HBG 10006	TI-07-001	Jul 2007	Mayaguana, Bahamas	8
<i>Barbouria cubensis</i>		HBG3188	ULLZ-11739	Nov 2009	Abaco, Bahamas	1
<i>B. cubensis</i>		HBG3355–5600		Mar 2015–Mar 2016	Abaco, Bahamas	42
<i>B. cubensis</i>		HBG3390, HBG8028–8151		Jul 2017	Acklins Island, Bahamas	74
<i>B. cubensis</i>		HBG10001–10005		Jan 2005	Eleuthera, Bahamas	18
<i>B. cubensis</i>		HBG441/HBG829	KC-7621/ULLZ-11771	2007	Eleuthera, Bahamas	2
<i>B. cubensis</i>		HBG5623–5695		Feb–Mar 2016	Eleuthera, Bahamas	95
<i>B. cubensis</i>		HBG3187, HBG7091	ULLZ-11738	Jan 2007	Mayaguana, Bahamas	3
<i>B. cubensis</i>		HBG2772–2775	BS1, BS12, BS19, BS22	Jan 2003	Quintana Roo, Mexico	4
<i>B. cubensis</i>		HBG1296–1390, HBG1762–1906		Jun 2012	San Salvador, Bahamas	158
<i>B. cubensis</i>		HBG1391–1694, HBG1907–1972		Jun 2013	San Salvador, Bahamas	70
<i>B. cubensis</i>		HBG1977–2124		Jun 2014		109
<i>B. cubensis</i>		HBG2137–3422, HBG8151–8160		Jun 2015	San Salvador, Bahamas	115
<i>Barbouria yanezi</i>	<i>Barbouria cubensis</i>	HBG9158–9181		Oct 2011	Cozumel, Q. Roo, Mexico	24
<i>Janicea antiguensis</i>	<i>Parhippolyte antiguensis</i>	HBG 66	OUNH.ZC.2009-15-002	Apr 2004	Mayaguana, Bahamas	1
<i>J. antiguensis</i>	<i>P. antiguensis</i>	HBG 9999, HBG10000	TI-19-002	Mar 2019	Belize	2
<i>Parhippolyte cavernicola</i>		HBG 10136	USNM-IC-273315	May 1994	Baja California Sur, Mexico	1
<i>Parhippolyte misticia</i>		HBG 10121	NTOU-M01675	Nov 2009	Japan	1
<i>P. misticia</i>		HBG10122–10128	NTOU-M01157	Nov 2009	Dive 22 Shogakko-mae, Japan	7
<i>Parhippolyte sterreri</i>		HBG1761–3389		Jun 2011	San Salvador, Bahamas	53
<i>P. sterreri</i>		HBG1927–1944	USNM-IC-1234891 – USNM- IC-1234894	Jun 2013	San Salvador, Bahamas	4
<i>P. sterreri</i>		HBG8046–8162		Jul 2017	Acklins Island, Bahamas	29
<i>Parhippolyte uveae</i>	<i>Parhippolyte misticia</i>	HBG10119	MNHN-IU-2019-2058	1967	Coral Sea	1
<i>P. uveae</i>	<i>Parhippolyte misticia</i>	HBG10120	MNHN-IU-2019-2059	1967	Coral Sea	1
<i>P. uveae</i>	<i>Parhippolyte misticia</i>	HBG10114	MNHN-IU-2012-1001	Mar 1990	Coral Sea	1
<i>P. uveae</i>	<i>Parhippolyte misticia</i>	HBG10115	MNHN-IU-2012-1002	Mar 1990	Coral Sea	1
<i>P. uveae</i>	<i>Parhippolyte misticia</i>	HBG10116–10118	MNHN 2018-3568	Mar 1990	Coral Sea	3
<i>P. uveae</i>		HBG10130, HBG10134	USNM-IC-280216-85	Mar 1996	Aldabra, Seychelles	2
<i>P. uveae</i>		HBG10131–10135	USNM-IC-280216-86	Mar 1996	Aldabra, Seychelles	4

CHAPTER V

ENDANGERED CAVE SHRIMP REVEAL HIGH CONNECTIVITY DESPITE AN
ANCHIALINE ISOLATION PARADIGM ACROSS THE TROPICAL WESTERN
ATLANTIC (DECAPODA: CARIDEA: *BARBOURIA CUBENSIS*)

ABSTRACT

Barbouria cubensis is a critically endangered anchialine shrimp distributed across the tropical western Atlantic. Anchialine systems are generally considered to be disjointed, with little to no connectivity between anchialine pools or between islands. The present study analyzes the population structure and genetic diversity of *B. cubensis* from Abaco, Acklins and San Salvador, Bahamas and the Yucatán Peninsula, Mexico to test the isolation paradigm of anchialine systems. Single nucleotide polymorphisms from reduced representation genome-sequencing were examined in 47 individuals from 24 distinct localities and a hybrid *de novo* partial draft genome assembly was constructed using Illumina short reads and Oxford Nanopore long reads. The results indicated a single population of *B. cubensis* distributed throughout the western Atlantic with low genetic diversity ($F_{ST} = 0.0033$) and a small effective population size ($N_e = 198$). Such a low F_{ST} value despite their wide distribution suggests that *B. cubensis* has a high level of connectivity between anchialine systems, likely resulting from strong dispersal capabilities. The results also suggest that a partial draft genome can greatly improve downstream analyses in next-generation molecular studies. The ability to use a partial draft genome is particularly valuable in research involving vulnerable or threatened species, or species with large genomes. Our findings also suggest that a disturbance to a single anchialine pool could affect an entire species as a whole, which indicates the need for improved conservation of seemingly disjointed anchialine systems and increased protection of the highly connected and endangered species that call them “home”.

INTRODUCTION

Isolation has long been recognized as a driving force behind evolution since Charles Darwin first described natural selection in his work on the origin of species (Darwin, 1859). Islands provide a natural laboratory for the study of evolutionary process and patterns across spatially disjunct populations (Edwards, 1996). Akin to the islands on which they are typically found, anchialine systems can offer a similar opportunity to study seemingly isolated populations with unique and sometimes endemic species (Edwards, 1996, Rose *et al.*, 2016; Pérez-Moreno *et al.*, 2016).

Anchialine systems are globally distributed subterranean estuaries with landlocked surficial bodies of water and tidal influence where crustaceans constitute the greatest biodiversity (Culver & Sket, 2001; Bishop *et al.*, 2015). In spite of tidal influences, anchialine systems have been described as “islands within islands,” which fit within an isolation paradigm as a consequence of their lack of biological connectivity with the surrounding environment (Edwards, 1996; Santos, 2006; Porter, 2007; Humpreys *et al.*, 2009; Iliffe & Kornicker, 2009; Russ, Santos & Muir, 2010; Becking *et al.*, 2011; Bishop *et al.*, 2015; Dawson, 2016; Gonzalez *et al.*, 2017; Pérez-Moreno *et al.*, 2017). The isolation of anchialine systems has been found to be closely linked with speciation as a result of niche partitioning (Turner *et al.*, 2008; Martin & Wainwright, 2013; Herman *et al.*, 2018) and geographical barriers to gene flow (Neighbor *et al.*, 2012; Becking *et al.*, 2013; Pérez-Moreno *et al.*, 2016). Isolation caused by geographic barriers (e.g., cave system) has also been demonstrated to cause population and phenotypic differentiation in hypogean systems, which may be an early indicator of evolution processes taking place (Rose *et al.*, 2016; Pérez-Moreno *et al.*, 2017; Herman *et al.*,

2018). Evidence from the Scaly Pearl Oyster (*Pinctada longisquamosa*) further suggests that anchialine systems remain relatively isolated except during extreme weather events where geographic barriers may be disrupted (Cole *et al.*, 2007). In contrast to the “islands within islands” hypothesis, a “continuous spelean corridor” hypothesis has been proposed, suggesting anchialine systems are connected within islands on the basis of intra-island gene flow, and limited genetic exchange over small spatial scales (Edwards, 1996; Chace & Hobbs, 1969; Gonzalez *et al.*, 2017).

Gene flow detected among populations of crustaceans inhabiting similarly isolated environments was found to exist between islands but is highly dependent on marine dispersal capabilities (Kano & Kase, 2004; Buhay & Crandall, 2005; Santos, 2006; Zakšek *et al.*, 2009, Cook *et al.*, 2009; Russ *et al.*, 2010; Cutter, 2013). Anchialine species with strong dispersal mechanisms may exhibit genetic exchange between populations up to 200–600 km (Kano & Kase, 2004; Weese *et al.*, 2013). The tropical western Atlantic is an ideal testing ground for the proposed isolation of anchialine habitats because of their separation by geographic distance, and deep trenches and channels (Culver & Sket, 2000; Gonzalez *et al.*, 2017).

Barbouria cubensis is critically endangered cave shrimp that has colonized an expansive variety of anchialine localities throughout the western Atlantic (Bishop & Iliffe 2013). *Barbouria cubensis* is also relatively unique among anchialine species, because they are believed to have strong dispersal capabilities (Hobbs, 1978; Bauer, 2005; Onaga *et al.*, 2012). The widespread distribution and likely dispersal capabilities of *B. cubensis* nominates them as an ideal candidate for investigating connectivity patterns among vulnerable anchialine communities and as potential indicator species for the health of

anchialine habitats (Bohonak, 1999; Kano & Kase, 2004; Porter, 2007; Becking *et al.*, 2011; Bishop & Iliffe, 2012; Ditter *et al.*, 2015; Pérez-Moreno *et al.*, 2017; Ditter *et al.*, 2019a). *Barbouria cubensis* has also been found to exhibit phenotypic hypervariation (PhyV), defined as the presence of extensive morphological variations at rate far beyond the prescribed norm (Ditter *et al.*, 2019b). Phenotypic hypervariation has been hypothesized to be a result of, in part, low genetic diversity, a possible result of severe inbreeding. *Barbouria cubensis* was found to lack genetic diversity between populations from the Yucatan Peninsula and the Bahamas in the 16S and COI mitochondrial gene regions (Ditter *et al.*, 2019; Ditter *et al.*, 2020). Morphological abnormalities have been correlated to low genetic diversity leading to the accumulation of deleterious mutations (O'Grady *et al.*, 2006; Duarte *et al.*, 2008; Fernandes *et al.*, 2011; Lacy & Alaks, 2012; McMillan *et al.*, 2016). Highly fragmented populations can be susceptible to an increased risk from infectious diseases and loss of genetic diversity, fitness and ability to respond to environmental changes stemming from inbreeding, drift, competition and habitat loss (Wilson, 1985; Brian *et al.*, 2006; Perez-Enriquez *et al.*, 2009; Bazin *et al.*, 2006; Airoidi *et al.*, 2008; Lacy & Alaks, 2012). Phenotypic hypervariation may be an indicator of low population health and resilience, and a need for immediate conservation efforts.

The advent of next-generation molecular techniques, such as reduced representation genome-sequencing (RADseq) permits unprecedented resolution of a variety of long-standing questions regarding phylogeography and population structure (Davey & Blaxter, 2010; Juan *et al.*, 2010; Metzker, 2010; Lemmon *et al.*, 2012; Peterson *et al.*, 2012; McCormack *et al.*, 2013). However, the effectiveness of population level analyses using RADseq data is limited in non-model organisms that lack full genome

sequences, because effective population size (N_e) and linkage distances between markers cannot be calculated accurately (Eklom & Galindo, 2011; Hoban *et al.*, 2016). The RADseq datasets that lack a reference genome also generally call fewer SNPs than genome referenced datasets, reducing the calculated inbreeding coefficient (F_{IS}) and the expected transition-to-transversion ratio (T_S/T_V) (Shafer *et al.*, 2017). The lack of a reference genome may also result in underestimates of genetic diversity and introduce genealogical biases from nonrandom haplotype sampling (Arnold *et al.*, 2013).

The current study used a hybrid *de novo* assembly of Illumina® short read sequences and Oxford Nanopore generated long read sequences to generate a draft genome for *Barbouria cubensis*. The use of a hybrid *de novo* genome assembly greatly decreases the number of scaffolds and base call errors, while it increases the accuracy and coverage depth (Austin *et al.*, 2017; Istace *et al.*, 2017; Janser *et al.*, 2017; Tan *et al.*, 2017). Using a full or partial genome when conducting population analyses provides increased resolution by allowing the calculation of linkage disequilibrium, estimating effective population size (N_e) and allows researchers to analyze structural variations within sequences (Slatkin, 2008; Calafell *et al.*, 2001; Li & Durbin, 2009; Istace *et al.*, 2017).

The objective of the present study is to use next generation molecular techniques to test the isolation paradigms of anchialine systems. By evaluating population dynamics and dispersal abilities of the critically endangered *B. cubensis* we aim to test the “island within islands” and “spelean corridor” hypothesis. We hope to demonstrate the usefulness of partial genome sequencing by leveraging a hybrid *de novo* assembled draft genome to evaluate the health of ecosystems and vulnerable species, and to determine if PhyV in *B.*

cubensis is due to inbreeding and low genetic diversity (Fernandes *et al.*, 2006; Oha & Paal, 2004; Timm *et al.*, 2020).

METHODS

SAMPLE SELECTION

A single freshly preserved specimen of *Barbouria cubensis* (FICC-HBG-3393) from San Salvador, Bahamas was selected for hybrid *de novo* assembly of the draft genome. To explore population genomics of *B. cubensis*, 47 individuals representing the broadest geographical distribution of *B. cubensis* housed within the Florida International Crustacean Collection (FICC) were selected from three islands in the Bahamas, Abaco (n=12), Acklins (12), and San Salvador (n=12), and from the Yucatán Peninsula (n=11) (Appendix Table 1). An approximate genome size estimate was generated by averaging C-values for available species on the Animal Genome Size Database that were returned as highly similar to *B. cubensis* from BLASTn queries of 16S (rRNA) and H3 (nDNA) gene regions (Altschul *et al.*, 1990; Gregory, 2020). Using the approximate genome size of ~8 Gbp and DNA fragment proportions of enzyme digestions from Agilent BioAnalyzer 2100 (Agilent Technologies) trace results, we determined that a single lane of HiSeq® 4000 (Illumina, San Diego, CA, USA) would produce a sufficient number of reads with a least 30x coverage for 48 individuals.

DNA EXTRACTION

Genomic DNA was extracted from muscle tissue of the antennule, third to fifth pleopod, or abdomen using DNeasy® Blood and Tissue Kits (Qiagen, Valencia, CA,

USA), following the manufacturer's protocol. For incomplete tissue digestions, 10 µl of Proteinase K and 10 µl of 10% DTT was added, and samples incubated until digestion was complete. Quality of total genomic DNA was visualized using 2% agarose gels stained with GelRed® (Biotium, Fremont, CA, USA), and concentration was measured using the Qubit dsDNA HS Assay kit on the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

LONG READ SEQUENCING

To generate genomic long reads, 4.8 µg of high molecular weight DNA from FICC-HBG-3393 (see appendix for metadata; Table A1) was cleaned with AMPure XP beads to remove small DNA fragments for sequencing using Oxford Nanopore Technologies Minion device. Long read libraries were prepared using the Rapid Sequencing (SQK-RAD004) kit (Oxford Nanopore). Long read sequencing was performed using two R9.4 flow cells on a MinION Mk1B device with MinKNOW 3.1.3 software, and with base calling performed using Guppy 4.0.11 (Oxford Nanopore). Each flow cell was run four times consecutively for 14 hours using 400–1000 ng of DNA. Flow cells were flushed between each run using a Flow Cell Wash Kit (WSH-002, Oxford Nanopore).

SHORT READ SEQUENCING

An additional paired end (PE) library was prepared from a single individual (FICC-HBG-3393) using an NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® and NEBNext® Multiplex Oligos for Illumina® (E6609S) for use in hybrid *de*

novo genome assembly. Samples used for reduced representation library preparation had a minimum of 3000 ng of high molecular weight DNA (metadata list in appendix, Table A1). Library preparation was conducted following a modified protocol for NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (E7805L) and NEBNext® Multiplex Oligos for Illumina® (E6440S) kits (New England Biolabs, Ipswich, MA, USA).

AMPure XP Beads (Beckman Coulter, Brea, CA, USA) were used to clean DNA samples. Size selection of 250 bp conducted on a PippinPrep (Sage Science, Beverly, MA, USA) was conducted after PCR enrichment and pooling, with a final AMPure XP bead. Final PE library quality was accessed again using an Agilent BioAnalyzer 2100 before sending the library for 2x150 paired end sequencing with 5% Phi-X Spike-in on an Illumina HiSeq 4000 by Genewiz (South Plainfield, NJ, USA).

HYBRID DE NOVO ASSEMBLY

An initial genome size was estimated using PE and Nanopore reads separately using k-mer counting with Jellyfish 2.2.7 (Marçais & Kingsford, 2011) and GenomeScope 1.0 (Vurture *et al.*, 2017) to visualize k-mer count distribution. The average estimated genome size (1.8 Gbp) was used to assemble raw nanopore reads twice using Canu 1.7.1 (Koren *et al.*, 2017) with corrected error rates of 10.5% and 14.4%, respectively. Genome assembly quality evaluation was conducted using QUAST 5.0.2 (Gurevich *et al.*, 2013). The most contiguous assembly was used to re-estimate the genome size (4.5 Gbp). Raw nanopore reads were then corrected using 10 iterations of Canu -correct with a corrected output coverage of 500, a corrected minimum coverage of 1, corrected Mhap sensitivity at “high”, and a genome size of 4.5 Gbp (Koren *et al.*,

2017). The 10th iteration of read correction was trimmed and assembled using Canu flags -trim and -assemble with utg graph deviation set to 50 and the corrected error rate set to 30% (Koren *et al.*, 2017). The final genome assembly was indexed and aligned against the corresponding corrected PE using Bowtie2 2.4.1 and polished using Nanopolish 0.13.2 (Altschul *et al.*, 1990; Loman *et al.*, 2015).

SHORT READ FILTERING AND DATA ASSEMBLY

Raw sequences were processed with the STACKS 2.3d (Catchen *et al.*, 2011; Catchen *et al.*, 2013) pipeline on the FIU High Performance Computing Cluster (HPC). Any PCR clones were removed using the clone_filter package of Stacks 2.3d while retaining oligos (Catchen *et al.*, 2011). Using the process_shortreads package of Stacks 2.3d, PE reads were demultiplexed, cleaned (-c), quality filtered (-q), chastity/purity filtered (--filter-illumina), and truncated/trimmed to 35 bp. The denovo_map.pl pipeline was used to execute the Stacks pipeline (ustacks, cstacks, sstacks, tsv2bam, gstacks, and populations) with allowed number of mismatches allowed between stacks within individuals set to -M 3 and between individuals set to -n 4 (Catchen *et al.*, 2011; Catchen *et al.*, 2013). Additional options of specific pipeline components were passed to the deno_map.pl program using the -X option (-X ustacks:"-M 3 -m 5"). Putative paralogs were excluded by setting the maximum Hamming distance between reads in a stack to 3 and setting the minimum depth of coverage required to create a stack to 5 (Timm *et al.*, 2020). The *populations* tool was rerun to reduce the total number of loci to between 500 and 1000 (Catchen *et al.*, 2011). Only one random SNP was called per locus to generate a final alignment of SNPs (FSD). All FSD loci were retained by allowing 0% missing data

at each site and within each individual. No F_{ST} outliers within the final SNP dataset (FSD) were detected by plotting results of BayeScan v2.1 in R (Foll & Gaggiotti, 2008).

DRAFT GENOME CHARACTERIZATION

To assemble the draft genome, a combination of Illumina-sequenced short reads and MinION-generated long reads were mapped together (Austin *et al.*, 2017). For the purposes of the present manuscript, we refer to the *de novo* assembled SNP dataset(s) as the “BcubSNP”, the *Barbouria cubensis* (FICC-HBG-3393) draft genome (PENDING) referenced SNP assembly dataset(s) as “BcubGNM”, and the *Palaemon carinicauda* (GCA_004011675.1) referenced SNP assembly dataset(s) as “PcarGNM”. Paired end reads were aligned to the BcubGNM and PcarGNM assemblies using Bowtie2 2.4.1. Read assembly was conducted *de novo*, BcubGNM and PcarGNM were reference-aligned using the STACKS *denovo_map.pl* and *ref_map.pl* pipelines respectively of Stacks 2.5.3 (Fig. 1). To compare assembly methods, 30 BcubSNP, BcubGNM and PcarGNM test datasets each were generated, one random SNP was retained with a maximum of 15% missing at each locus using *populations* 2.5.3 (Fig. 1). For each test dataset (BcubSNP, BcubGNM & PcarGNM) divergence from Hardy-Weinberg equilibrium for each locus and SNP-based F statistics were calculated as part of the Populations 2.5.3. No F_{ST} outliers within the BcubSNP, BcubGNM and PcarGNM test datasets were detected by plotting results of BayeScan v2.1 in R (Foll & Gaggiotti, 2008). Mean F_{ST} values were tested for normality using the Shapiro-Wilk statistics and compared using a one-way ANOVA in R version 3.3.1 (R Core Team, 2016).

POPULATION GENOMIC ANALYSES

The analysis suite implemented in GENODIVE 3.04 (Meirmans & Van Tienderen, 2004) was utilized to calculate population allele frequencies, which determined the distribution that alleles were randomly drawn from to replace missing data of the BcubSNP, BcubGNM and PcarGNM datasets (Eaton *et al.*, 2017). Measures of genetic diversity were calculated within GENODIVE as well as, the inbreeding coefficient (G_{IS}), Observed, expected and total heterozygosity (H_O , H_S and H_T , respectively). Each locus was tested for departures from Hardy-Weinberg equilibrium (HWE), using jackknifing to calculate to standard deviation. Hierarchical Analyses of Molecular Variance (AMOVA) and pairwise populations differentiation (F_{ST}) were calculated in GENODIVE as well. The AMOVA was conducted under the Infinite Alleles Model and significance was assessed over 1000 permutations (Felsenstein, 1985). A single set of SNPs that was closest to the means of the genetic diversity measures from the BcubSNP, BcubGNM and PcarGNM datasets was used to conduct a discriminant analysis of principal components using FastSTRUCTURE (Raj *et al.*, 2014) to assess the impact of each assembly method on the population structure results.

POPULATION STRUCTURE: FINAL SNP DATASET

The final dataset (FSD) of random snps that was generated with 0% missing data from the BcubGNM was used to analyze population structure. Using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) used to analyze k-means clustering. STRUCTURE 2.3.4 was run for 10 iterations for each number of clusters (K) between 2 and 10 for 100,000 generations, with a burn-in of 25,000 generations, and with/without admixture using prior

population information. Evanno's ΔK (Evanno, Regnaut, & Goudet, 2005) was calculated with STRUCTURE HARVESTER (Earl & vonHoldt, 2012) and used to determine the most likely number of genetic populations. Cluster Matching and Permutation Program (CLUMPP) files generated by STRUCTURE HARVESTER (Earl & vonHoldt, 2012) were used to average membership probabilities among runs with CLUMPP 1.1 (Jakobson & Rosenberg, 2007) and the results were visualized with DISTRUCT 1.1 (Rosenberg, 2004) using Clustering Markov Packager Across K with CLUMPAK 1.1 (Kopelman *et al.*, 2015).

The selection coefficient (Tajima's D) was calculated for the SNPs using PopART 1.7.4 (Leigh & Bryant, 2015). PopART 1.7.4 was also used to generate TCS networks for phased haplotypes and recovered mitochondrial loci (Bandelt *et al.*, 1999; Clement *et al.*, 2002). Effective population size (N_e) using the molecular co-ancestry method of Nomura (2008) was calculated with NeEstimator V2.1 for the draft genome aligned SNPs (Do *et al.* 2014). Relative migration networks were constructed using the divMigrate function of the R package diveRsity (Jost, 2008; Keenan *et al.*, 2013).

RESULTS

DRAFT GENOME: LONG READ SEQUENCING AND ASSEMBLY

We obtained 5,974,504 Oxford Nanopore long reads (1,383,044,009 base pairs) from two R9.4 flow cells on a MinION Mk1B device. The R9.4 flow cells produced reads up to 172,000 bp in length and the longest read with >10x coverage of was ~95,000 bp. Ten rounds of Canu -correct resulted in 386,827 reads, representing 1,164,309,982 bases with 0.64x coverage at an estimated genome size of 1.8 Gbp.

After quality filtering and correcting long reads, multiple rounds of CANU - assemble was conducted to generate the partial BcubGNM. Our first round of CANU long read genome assembly produced 519 contiguous sequences (contigs) from 149,512 long read sequences with a total length of 408,198,075 bp and an estimated genome size of between 1.5 Gbp and 1.8 Gbp. The second round of CANU long read genome assembly produced 795 contigs with a total length of 5,851,284 bp (N50 13,055; L50 134; Fig. 2).

DRAFT GENOME AND FSD: SHORT READ SEQUENCING

Two lanes of Illumina® HiSeq® 4000 run for the BcubGNM and FSD produced over 250 gigabytes of short read sequencing data. We obtained 673,442,914 filtered short reads for FICC-HBG-3393 and 1,529,981,090 filtered short reads for 47 of 48 individuals (excluding FICC-HBG-2773 because of low coverage) analyzed in this study (average 16,276,395, standard deviation 5,974,504; NCBI Bio-project PENDING; NCBI SRA Accessions PENDING).

DRAFT GENOME EVALUATION

Thirty rounds of *de novo* (BcubSNP), BcubGNM aligned, and PcarGNM aligned short read assemblies have an average of 358, 6897 and 40 remaining variant sites, respectively (Fig: 1). The proportion of total genetic variance contained within subpopulations (F_{ST}) exhibit normal distribution and significantly different between each test dataset (p-value = 3.36968×10^{-12} , $df = 29$, $\alpha = 0.05$; Fig. 3). We find F_{ST} to be more similar between BcubGNM and PcarGNM datasets. A similar pattern is present among

other diversity measures except for the number of random SNPs retained (Table 1 & Fig. 3). Populations structure of the BcubSNP, BcubGNM and PcarGNM test datasets examined in fastSTRUCTURE (Pritchard *et al.*, 2010) (Fig. 4A) yield the same results, and suggests an optimal $K = 2 - 29$, with contribution from one to two groups. For analysis of the 47 individuals using PCA, the first principal component explained 18.1% of variance and the second principal component explained 12.8% of variance (Fig. 4B). The PCA clustered individuals from the Bahamas together, whereas some individuals from the Yucatán showed some differentiation.

FINALIZED SNP DATASET (FSD)

After finding improved SNP calling with the BcubGNM referenced loci, we reran diversity measures using the FSD. The BcubGNM aligned short reads produced the FSD with 824 of the 927 variant sites retained and unlinked and with 0% missing data between individuals or within populations.

FSD: POPULATION STRUCTURE ANALYSIS

Population structure of the 47 *B. cubensis* in the FSD examined with the program STRUCTURE and 827 SNPs with the program ADMIXTURE, found $K = 3$ to be optimal using the ΔK method of Evanno and the optimal number of populations ($Q = 2$) respectively (Fig. 5). Individuals all share a high degree of ancestry from groups one and two with some ancestry from group three, except for two individuals from the Yucatán population that show a high level of ancestry from groups one and three (Fig. 5; top). High levels of admixture are present among all populations (Abaco, Acklins, San

Salvador, Bahamas and the Yucatán), with some separation between the Bahamas and the Yucatán populations (Fig. 5; bottom). For analysis of the 47 individuals using PCA, the first principal component explained 24.1% of variance and the second principal component explained 18.2% of variance (Fig. 5; right). The PCA clustered individuals from the Bahamas and the Yucatán together.

Both the recovered mitochondrial loci and phased haplotype networks reveals a high degree of interrelatedness between populations (Fig. 6; top), with little distinction based on geographic location (Fig. 6; bottom) The overall nucleotide diversity (π) is 0.117955, with 607 segregating sites and 440 parsimony-informative sites, and a Tajima's D of -1.2585 (calculated in PopArt 1.7.4).

FSD: GENETIC DIVERSITY

We find indications of very low genetic diversity among populations of *B. cubensis* ($\pi = 0.0538 \pm 0.0049$) with an average pairwise F_{ST} of 0.0214 ± 0.0017 , and evidence of outbreeding ($G_{IS} = -0.3540 \pm 0.0638$) and very little divergence ($\Delta_{XY} = 0.0006 \pm 4.05 \times 10^{-5}$) among Abaco, Acklins and San Salvador, Bahamas, and the Yucatán populations (Table 3 and Fig. 7). This suggests that *B. cubensis* in the tropical western Atlantic represents a single population, possibly with subpopulations in the Bahamas and the Yucatán Peninsula. Genetic diversity values across populations are very similar (H_O : $0.0482 - 0.0626$; H_S : $0.0333 - 0.0475$; G_{IS} : $-0.3093 - -0.4474$), indicating outbreeding between populations or recent population growth (Table 2). Recalculation of the inbreeding coefficient, treating all sampling localities as single population, resulted in an overall G_{IS} of 0.0033 (Table 3). An analysis of Genetic Membership Probability also

provides evidence of two mixed subpopulations (Bahamas and Yucatán) within the western Atlantic, with membership probabilities similar to geographic distances (Fig. 8).

The overall effective population size ($N_e = 198$) calculated using linkage disequilibrium (LD) locus pairing across chromosomes for treating all 47 individuals as single population is particularly low considering the wide geographic sampling range and the high densities *B. cubensis* are reported in (Charlesworth, 2009). When treating the individuals as two subpopulations, the Bahamas and Yucatán we find the effective population sizes of 346.2 and 3.2 respectively. The relative migration networks indicate high magnitudes of migrations from Acklins Island to Abaco, San Salvador and the Yucatán (>0.96). We also find moderate to high magnitudes of migrations between all localities (0.45 – 0.96).

DISCUSSION

This research represents the first genomic study to examine diversity and connectivity of enigmatic anchialine crustaceans in the western Atlantic. Using a hybrid of Illumina short reads and Nanopore long reads, we test the anchialine isolation paradigm using *Barbouria cubensis* (Edwards, 1996; Gonzalez et al., 2017). The idea that inland bodies of water within islands completely lack connectivity is largely based on their differences in colonization patterns, associated species assemblages, salinity regimes and their departure from island biogeographic theory (Edwards, 1996). This complete lack of connectivity is most often supported in studies of organisms that lack planktonic dispersal mechanisms or have undergone sympatric speciation as a result of niche partitioning (Neighbor et al., 2012 Becking et al., 2013; Rose, Masonjones &

Jones, 2016). However, when study organisms exhibit some form of weak dispersal or limited planktotrophic larvae, dispersal through crevicular spelean corridors is found, but only within a single land mass (Santos, 2006; Botello & Álvarez, 2010; Russ *et al.*, 2010; Hunter *et al.*, 2007; Gonzalez *et al.*, 2017). Organisms with intra-island connectivity better fit within the rules of island biogeography theory as island populations are isolated from one another, and gene flow is restricted over a small spatial scale, but their ability to use spelean corridors does not offer insight into how these organisms are able to colonize island-to-island. Further investigation of anchialine crustaceans has found some species are able to exchange genetic material between islands at distances around 200 km, and in some cases up to 600 km (Russ *et al.*, 2010; Weese *et al.*, 2016)

GENETIC CONNECTIVITY AND MIGRATION PATTERNS OF BARBOURIA CUBENSIS ACROSS THE WESTERN ATLANTIC

In spite of a variety of potential barriers to gene flow among anchialine populations in the tropical western Atlantic, we find little evidence of isolation among populations of *B. cubensis*. Across the Bahamian islands of Abaco, Acklins and San Savlador, and the Yucatán Peninsula, we found evidence of migration, outbreeding and admixture between populations, indicating gene flow exceeding 1200 km. (Table 3). These distances might be somewhat shorter if we consider a Cuban population acting as an intermediate for genetic exchange between the Yucatán and the Bahamas. Despite this possibility, we find evidence of a significant high magnitude of migration between the Bahamas and the Yucatán (Fig. 8). Alternatively, a population to the South of Cuba in Cayman Brac, Cayman Islands or another Caribbean population could be acting as an

intermediary. The distance between the Yucatán and Cayman Brac is ~760 km, which still exceeds the 600 km limit previously reported for anchialine crustaceans (Weese *et al.*, 2016). The direction of water flow in the Atlantic resulting from the Caribbean Current and Florida Current is a potentially important factor to consider in connectivity of species with planktotrophic larvae. Any individuals or larvae emigrating from the Yucatán or the Bahamas could be swept away by the currents and become entrained in the North Atlantic Gyre, where individuals would mix before immigrating to another population (Fig. 8). Our relative migration networks suggest that *B. cubensis* are exchanging between the Bahamas and the Yucatán, with the shortest possible distances of over 1200 km (Table 3). This is at least twice the distance of the farthest recorded migration among anchialine species (Weese *et al.*, 2016; Gonzalez *et al.*, 2017). Our findings suggest Acklins is acting as an intermediary for genetic exchange, based on the magnitudes of gene flow between populations. It makes sense that Acklins would be a central hub for individuals after completing a cycle in the North Atlantic Gyre, as it is the first location individuals would reach in the Bahamas.

DISPERSAL ABILITY OF BARBOURIA CUBENSIS

Observations of numerous small oocytes in an ovigerous female *B. cubensis* (Hobbs, 1978), the lack of a large yolk on newly hatched zoeae of the *Parhippolyte sterreri* (Hart & Manning, 1981) (unpublished data), and the sexual system of *Parhippolyte misticia* (Clark, 1989) (Onaga, *et al.*, 2012) are indicators that shrimps of the family Barbouriidae Christoffersen, 1987 are likely to exhibit extended planktotrophic larval stages (Bohonak, 1999; Bauer, 2005). The distance *B. cubensis* is

traveling between islands within our study and their likely extended planktotrophic larvae suggests that they have strong dispersal mechanisms. Compared to other anchialine shrimps, such as *Creaseria morleyi* (Botello *et al.*, 2010), *Metabeteaeus lohena* (Russ *et al.*, 2010) *Halocardia rubra* (Santos, 2006), and species of *Typhlatya* (Hunter *et al.*, 2007), the potentially strong dispersal capabilities of barbouriid shrimps appears to be unique. This raises the question of how is *B. cubensis* able to locate and settle in a suitable habitat after traveling such a long distance across what are thought to be typical barrier to gene flow.

POPULATION STRUCTURE OF BARBOURIA CUBENSIS

Analysis of genetic structure of *B. cubensis* identified one population in the western Atlantic, with very little genetic differentiation between the Bahamas and the Yucatán. We find equal values of nucleotide diversity and inbreeding coefficients between Abaco, San Salvador and the Yucatán ($G_{IS} = -0.309 - -0.341$ and $\pi = 0.0557 - 0.05$ with slightly lower genetic diversity and inbreeding coefficient for Acklins ($G_{IS} = -0.447$, $\pi = 0.0466$). This result indicates the presence of outbreeding among populations. When G_{IS} was recalculated treating *B. cubensis* within the western Atlantic as a single population we found an inbreeding coefficient of 0.003, and the AMOVA identified 99% of molecular variance was explained by variance within individuals and only 1% was explained by variance among regions. Discriminant analysis of principal components revealed high levels of admixture between populations. The results of these analyses all suggest that there is a single highly connected population of *B. cubensis* in the western Atlantic. Similarly, we see highly interconnected branching patterns in both our nuclear

and mitochondrial based haplotype networks and our analysis of genetic membership found two mixed subpopulations. Our results suggest that there is presently only one population of *B. cubensis* in the western Atlantic, which may have historically been divided into two to three separate lineages, and that individuals appear to be primarily mixing within Acklins Island, Bahamas.

HOW LOW CAN THEY GO? GENETIC DIVERSITY

According to our finding genetic diversity ($F_{ST} = 0.0033$). Our finding suggests that *B. cubensis* has undergone a recent population expansion, which typically occurs following a bottleneck (Provan *et al.*, 2005; Maggs *et al.*, 2008; Campo *et al.*, 2009). This is not that surprising when considering the increased access to existing habitat, and the formation or opening of new anchialine habitats as sea-levels rose following last glacial maxima occurring around 20,000 years ago (Myroie *et al.*, 1995, Myroie & Myroie, 2011). We also found very low effective population size ($N_e = 198$). Anchialine habitats often occur in areas of high demand for tourism, and are being rapidly altered by development, pollution, and climate change (Iliffe, Jeckells & Brewer, 1984; Sket, 1999; Iliffe, 2002; Schawbe, Cathcart & Carew, 2010; Pérez-Moreno *et al.*, 2016). Due to their endemism or restricted distributions anchialine organisms are more easily impacted by disturbances (Humphreys *et al.*, 2009; Bishop & Iliffe, 2012, Pérez-Moreno *et al.*, 2016). The combination of low genetic diversity and a small effective population size suggests that *B. cubensis* may be vulnerable to future disturbances as a result of their high connectivity resulting from inbreeding depression (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). This is particularly concerning, because of the rampant

harm anthropogenic effects are having on anchialine systems. While *B. cubensis* is widespread throughout the tropical western Atlantic, our findings suggest that *B. cubensis* may be in need of conservation efforts.

VALUE ADDED: PARTIAL DRAFT GENOME

The use of a hybrid *de novo* draft genome assembly of Illumina short reads and Nanopore long reads provided more utility for our short read data and higher resolution with greater accuracy to results. Results of Downstream analyses from more effectively assembled loci better reflect the actual population as a result of reduced nonrandom haplotype sampling bias (Arnold *et al.*, 2013). A comparison of genetic diversity indices for *de novo* (BcubSNP), *Barbouria cubensis* draft genome referenced (BcubGNM) and *Palaemon carinicauda* genome referenced (PcarGNM) assembled datasets suggests that the BcubGNM more closely resembles the PcarGNM and that the BcubGNM likely more closely represents the actual genetic diversity of *B. cubensis* than the BcubSNP. The genetic diversity measures of the BcubGNM are nearly 3x higher than those of the BcubSNP, which is in line with *de novo* assembled SNPs underestimating diversity (Arnold *et al.*, 2013). While we found lower genetic diversity and nucleotide diversity in the BcubGNM versus the BcubSNP, we found higher values for all other measures of genetic diversity and significantly more unlinked loci. This also indicates the BcubGNM is more accurate and of greater utility as these loci more closely resemble the actual population of *B. cubensis*. The BcubSNP implicates the Yucatán as the intermediary for dispersal, while the BcubGNM implicates Acklins, Bahamas as the intermediary for dispersal. This may be the result of bias from higher similarity in sequences between the

Bahamian populations and the draft genome, as the draft genome was constructed from an individual from San Salvador, Bahamas. However, this is likely not the case, as the PcarGNM implicates both Acklins and San Salvador as intermediaries for dispersal and bias caused by differences in sample size between the Bahamas and the Yucatán. The similarity in which populations are implicated as intermediaries for gene flow lends further support that the use of a partial draft genome improves SNP assembly and downstream analyses. Overall, our results suggest that even the use of a partial draft genome greatly reduces the biases associated *de novo* assembled short read datasets, and yields results comparable to complete reference genome assembled dataset. For little extra cost, the use of a draft genome also offers greater value and utility in downstream analyses of genomic datasets by providing the ability to calculate linkage distances.

CONCLUSION

We propose a new “anchialine isolation by dispersal” hypothesis to explain how different species inhabiting the same anchialine systems can exhibit a range connectivity patterns, from complete isolation to intra-island gene flow to high levels of genetic exchange between geographically distant populations. Simply stated, the more time that the larvae of a species spends in the water column before settling out the greater distance the larvae will travel, ultimately leading to a greater likelihood of genetic connectivity among populations. Larval dispersal mechanisms of a given organisms is the primary determining factor if populations among anchialine systems will exhibit an isolated population structure or genetic connectivity over a range of geographic distances, initially described by Weese, Fuijita and Santos (2016). In the case of a species suspected of

having strong dispersal capabilities, like *B. cubensis*, we would expect and found high inter-island connectivity across vast geographic distances.

When it comes to population genomics and the current technology available, having a complete reference genome is the best option. Regrettably, when working with non-model species or species with large genomes a complete high coverage genome sequence is not typically available. Fortunately, low cost sequencing platforms are available to generate long read data that can be combined with short reads to assemble a draft genome. Even a partial draft genome can vastly improve the resolution of downstream analyses, increase the utility of short read sequencing data and has little extra cost. This is especially valuable in studies that include vulnerable species or habitats that may be in need of conservation efforts.

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TABLES

Table 1. Mean measures of genetic diversity for 47 *Barbouria cubensis* from four geographical populations calculated from random unlinked single nucleotide polymorphic loci or the BcubSNP, BcubGNM, and PcarGNM datasets (generated with identical “populations” 2.53 parameters).

Assembly type	Population	H_o	H_s	F_{IS}	π	N_e	A_m	A_p
de novo	Abaco	0.5670	0.3246	-0.7470	0.3304	1.6140	273	22
	San Salvador	0.5603	0.3187	-0.7580	0.3364	1.6090	237	11
	Acklins	0.5615	0.3276	-0.7140	0.3346	1.6140	280	42
	Yucatan	0.4678	0.3182	-0.4700	0.2941	1.5950	156	9
draft genome aligned	Abaco	0.0884	0.0700	-0.2633	0.1034	1.1000	2858	1129
	San Salvador	0.0891	0.0709	-0.2563	0.1051	1.1010	2805	1144
	Acklins	0.0872	0.0653	-0.3364	0.0986	1.0950	2893	1069
	Yucatan	0.0933	0.0747	-0.2495	0.1098	1.1060	2596	940
Palaemon genome aligned	Abaco	0.0495	0.0530	0.0656	0.0604	1.2120	17	9
	San Salvador	0.0338	0.0377	0.1033	0.0314	1.2190	13	6
	Acklins	0.0383	0.0381	-0.0054	0.0332	1.2250	14	6
	Yucatan	0.0590	0.0575	-0.0256	0.0628	1.2230	13	5

For each column, colors indicate similar in values for each column. H_o , observed heterozygosity; H_s , heterozygosity within populations (“genetic diversity”); F_{IS} , inbreeding coefficient; N_e , effective population size bias; π , nucleotide diversity (calculated with invariant loci); A_m , polymorphic sites; A_p , private alleles.

Table 2. Measures of genomic diversity for 47 *Barbouria cubensis* from for geographical populations calculated using the FSD from random unlinked single nucleotide polymorphic loci and 0% missing loci between individuals or within.

Population	H_o	H_s	G_{IS}	π	A_v	A_m	A_p
Abaco	0.0618	0.0472	-0.30932	0.056755	927	360	215
San Salvador	0.0626	0.0475	-0.31789	0.056361	927	361	214
Acklins	0.0482	0.0333	-0.44745	0.046586	927	261	141
Yucatán	0.0621	0.0463	-0.34125	0.055665	927	280	156

H_o , observed heterozygosity; H_s , mean expected heterozygosity; G_{IS} , inbreeding coefficient; π , nucleotide diversity (calculated with invariant loci); A_v , variant sites; A_m , polymorphic sites; A_p , private alleles.

Table 3. Measures of genomic diversity between populations of *Barbouria cubensis* from for geographical populations within the Bahamas and the Yucatán Peninsula, Mexico, calculated using the FSD dataset from random unlinked single nucleotide polymorphic loci and 0% missing loci between individuals or within populations. Also, with marine geographic distances between nearest sampling sites among populations of *Barbouria cubensis*. Distances reported are the shortest possible direct path through water among populations sampled in this study.

Population-Pair	F_{ST}	G_{ST}	Δ_{xy}	Distance (km)
Abaco - San Salvador	0.01958	-0.00328	0.00053	350
Abaco - Acklins	0.01994	-0.00363	0.00056	500
San Salvador - Acklins	0.02318	-0.00363	0.00055	150
Abaco - Yucatán	0.02003	-0.00321	0.00058	1200
San Salvador - Yucatán	0.02232	-0.00345	0.00059	1400
Acklins - Yucatán	0.02334	-0.00392	0.00064	1450

F_{ST} , biallelic genetic distance (Wright, 1969); G_{ST} , multiallelic genetic diversity (Nei, 1973); Δ_{XY} , genetic divergence between populations as a function of π (Hedrick, 2005).

FIGURE CAPTIONS

Figure 1. Workflow for generating and comparing *de novo*, *Barbouria cubensis* partial draft genome referenced and *Palaemon carinicauda* genome referenced assembly test datasets.

Figure 2. An example of the histograms of k-mer counting used as part of estimating genome size and scaffold contiguity; (A) before short read polishing and (B) after short read polishing.

Figure 3. (A) fastSTRUCTURE analysis without admixture using total number of sample sites as the maximum number of clusters ($K = 1-29$). fastSTRUCTURE results are the same for the *de novo* and aligned test SNP data sets; (B) PCA of *Barbouria cubensis de novo* assembled SNPs with 95% confidence ellipse.

Figure 4. Proportions of total genetic variance contained within subpopulations; Abaco, Acklins, San Salvador (SanSal), Bahamas and The Yucatán Peninsula.

Figure 5. Inferred population structure for 47 *Barbouria cubensis* as estimated by the software STRUCTURE using the ancestry linkage model (**bottom**); inferred number of populations and populations admixture for 827 SNPs as estimated by the software ADMIXTURE (**bottom**); PCA of *Barbouria cubensis* using the final dataset of 827 SNPs (**right**).

Figure 6. Haplotype networks constructed using phased haplotypes from finalized SNP dataset (**top**), and a corresponding haplotype network map of the western Atlantic (**bottom**).

Figure 7. Diversity metrics (observed heterozygosity H_o , expected heterozygosity H_e , and inbreeding coefficient G_{IS}) are compared between collection localities in the Bahamas and Mexico for *Barbouria cubensis* (note H_o and H_e are on the primary y-axis and G_{IS} is on the secondary y-axis).

Figure 8. Relative migration levels between populations of *Barbouria cubensis* in the western Atlantic generated with the *divMigrate* function of diversity package in R. Manually depicted populations with N_m values (**A**), Jost's D (**B**) and G_{ST} models. The direction of arrows indicates the direction and thickness represents the magnitude of gene flow. (1) Abaco, Bahamas (2) San Salvador, Bahamas, (3) Acklins, Bahamas and (4) the Yucatán Peninsula, Mexico.

FIGURES

Figure 1.

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Figure 6.

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Figure 8.

APPENDICES CAPTIONS

Appendix Table 1. Metadata for all samples included in this study, including Florida International Crustacean Collection (FICC) museum catalog number, collection locality, collection site, collection date and GPS coordinates for the collection site. *, individual used to generate partial draft genome assembly.

APPENDICES

Appendix Table 1.

FICC museum catalog no.	Collection		Coordinates		
	Locality	Site	Date	Latitude	Longitude
HBG3188	Abaco, Bahamas	Dean's Cave	11-Dec-09	26.35°N	-77.11°W
HBG3356	Abaco, Bahamas	Lora's Pond	31-Mar-15	26.31°N	-77.00°W
HBG3362	Abaco, Bahamas	Lora's Pond	31-Mar-15	26.32°N	-77.00°W
HBG3363	Abaco, Bahamas	Dripping Stone Cave	31-Mar-15	26.32°N	-77.00°W
HBG3364	Abaco, Bahamas	Dripping Stone Cave	31-Mar-15	26.32°N	-77.00°W
HBG3372	Abaco, Bahamas	Runge's Sinkhole	31-Mar-15	26.31°N	-77.01°W
HBG3374	Abaco, Bahamas	Runge's Sinkhole	31-Mar-15	26.31°N	-77.01°W
HBG3375	Abaco, Bahamas	Runge's Sinkhole	31-Mar-15	26.31°N	-77.01°W
HBG3376	Abaco, Bahamas	Runge's Sinkhole	31-Mar-15	26.31°N	-77.01°W
HBG3377	Abaco, Bahamas	Runge's Sinkhole	31-Mar-15	26.31°N	-77.01°W
HBG5591	Abaco, Bahamas	Lora's Pond	17-Mar-16	26.31°N	-77.00°W
HBG5594	Abaco, Bahamas	Lora's Pond	17-Mar-16	26.31°N	-77.00°W
HBG1806	San Salvador, Bahamas	Oyster Pond	29-Jun-12	24.11°N	-74.46°W
HBG1832	San Salvador, Bahamas	Watling's Blue Hole	29-Jun-12	23.95°N	-74.55°W
HBG1841	San Salvador, Bahamas	Plantation Pond	29-Jun-12	24.04°N	-74.45°W
HBG1849	San Salvador, Bahamas	Mermaid Pond	29-Jun-12	23.97°N	-74.52°W
HBG1907	San Salvador, Bahamas	Pain Pond	17-Jun-13	24.11°N	-74.46°W
HBG1954	San Salvador, Bahamas	Little Lake	29-Jun-13	24.05°N	-74.51°W
HBG1980	San Salvador, Bahamas	Dunk City Pond	03-Jun-14	23.96°N	-74.53°W
HBG2018	San Salvador, Bahamas	Lighthouse Cave	08-Jun-14	24.10°N	-74.45°W
HBG2021	San Salvador, Bahamas	Major's Cave	11-Jun-14	24.07°N	-74.51°W
HBG2067	San Salvador, Bahamas	Blue Hole #2	16-Jun-14	23.96°N	-74.55°W
HBG2101	San Salvador, Bahamas	Big Rob Pond	18-Jun-14	24.03°N	-74.46°W
HBG2133	San Salvador, Bahamas	William's Pond	15-Jun-14	24.02°N	-74.52°W
HBG8032	Acklins, Bahamas	Nibbler's Cave	19-Jul-17	22.22°N	-74.20°W
HBG8066	Acklins, Bahamas	Flamingo Pond	20-Jul-17	22.21°N	-74.22°W

Appendix Table 1. Con't.

FICC museum		Collection		Coordinates	
catalog no.	Locality	Site	Date	Latitude	Longitude
HBG8069	Acklins, Bahamas	Nibbler's Cave	19-Jul-17	22.22°N	-74.20°W
HBG8071	Acklins, Bahamas	Nibbler's Cave	19-Jul-17	22.22°N	-74.20°W
HBG8090	Acklins, Bahamas	Pinefield Pond	23-Jul-17	22.65°N	-73.89°W
HBG8091	Acklins, Bahamas	Pinefield Pond	23-Jul-17	22.65°N	-73.89°W
HBG8104	Acklins, Bahamas	Red Lantern Cave	23-Jul-17	22.72°N	-73.89°W
HBG8115	Acklins, Bahamas	Red Lantern Cave	23-Jul-17	22.72°N	-73.89°W
HBG8118	Acklins, Bahamas	Big Pond	26-Jul-17	22.55°N	-73.86°W
HBG8119	Acklins, Bahamas	Big Pond	26-Jul-17	22.55°N	-73.86°W
HBG8121	Acklins, Bahamas	Big Pond	26-Jul-17	22.55°N	-73.86°W
HBG8124	Acklins, Bahamas	Big Pond	26-Jul-17	22.55°N	-73.86°W
HBG2772	Akumal, Q. Roo, Mexico	Cenote 21 Pasos	01-Jan-03	20.42°N	-87.36°W
HBG2774	Akumal, Q. Roo, Mexico	Cenote 21 Pasos	01-Jan-03	20.42°N	-87.36°W
HBG2775	Akumal, Q. Roo, Mexico	Cenote 21 Pasos	01-Jan-03	20.42°N	-87.36°W
HBG9162	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9164	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9165	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9169	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9172	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9178	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9179	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
HBG9180	Cozumel, Mexico	Cenote Tres Potrillos	29-Oct-11	20.46°N	-86.97°W
*HBG3393	San Salvador, Bahamas	William's Pond	26-Jun-15	22.22°N	-74.20°W

CHAPTER VI

CONCLUSTIONS AND FUTURE DIRECTIONS

The series of works that is presented here were completed with the goal of increasing our understanding of crustacean evolution and ecology from multiple directions. Starting with a taxonomic study of the presence of extensive morphological variation, referred to as phenotypic hypervariation (PhyV), these results far exceed all previous records for any anchialine crustacean. Molecular barcoding was used to confirm the identity of *Barbouria cubensis* exhibiting high PhyV and to determine if PhyV is the result of cryptic speciation. This is followed with records of geographic range extensions for three anchialine crustaceans (*Barbouria cubensis*, *Parhippolyte sterreri* and *Macrobrachium lucifugum*), and reporting on observations of their behavior during field collection and the behavior of *B. cubensis* in a laboratory setting over course of two years. During the process of genetic identification of *B. cubensis* inaccuracies in the molecular record for species from the family Barbouriidae were uncovered. To remedy the errors in barbouriid genetics an in-depth phylogenetic study of Barbouriidae was conducted using traditional molecular methods. In chapter V a population genomic approach is used to test the isolation paradigm of anchialine systems, shining further light on the source of PhyV and demonstrating the value a draft genome can add to genomic studies. These studies emphasize the importance of using variety approaches when studying evolutionary processes and echo a theme of needing improved conservation of anchialine systems.

In documenting phenotypic hypervariation (PhyV) and molecular barcoding of *Barbouria cubensis* from Abaco, Eleuthera and San Salvador, Bahamas, no geographic distribution pattern or cryptic diversity is detected. Phenotypic hypervariation was found to be present in at least one morphologically informative character in approximately 90% of specimens examined (n = 463). Phylogenetic analysis of the 16S and COI

mitochondrial gene regions identified all specimens ($n = 70$ included in publication; $n \approx 130$ not included publication) as *B. cubensis*. Genetic diversity is found to be very low among *B. cubensis* ($n = 70$), suggesting high PhyV may be the result of inbreeding. The lack of geographic distribution pattern in PhyV and the lack of genetic diversity across Abaco, Eleuthera and San Salvador, Bahamas suggests that populations of *B. cubensis* could be highly connected. *Janicea antiguensis* was also found to be nested within *Parhippolyte* in the phylogenetic analyses of 16S and COI, indicating a possible mistake in the species identities of GenBank sequences or a need to revisit the classification of the family Barbouriidae.

As part field work on Acklins Island, Bahamas collecting material to revisit the classification of Barbouriidae a geographic range extension was discovered for *Barbouria cubensis*, *Parhippolyte sterreri* and *Macrobrachium lucifugum*. Observations on the Behavior of the species during collection was reported. Observations on the behavior of *B. cubensis* was also reported from over two years in a laboratory setting as part of an attempted life history study. *Barbouria cubensis* was found to have large white and small red chromatophores that provide the capability of rapidly changing color in response to light and mimicking the color pattern of *Parhippolyte sterreri*. We also found individuals of *B. cubensis* housed separately exhibit different behavior than those housed in pairs. We conclude that it is important to report even minor range extensions, especially in threatened anchialine habitats and for critically endangered species.

In chapter IV, classification of the family Barbouriidae was examined using both morphological and molecular evidence from two mitochondrial and five nuclear gene regions. Several mistakes in the species identity of GenBank sequences were uncovered

and corrected. Phylogenetic analysis revealed that *Barbouria yanezi* is a synonym for *Barbouria cubensis*. Based on molecular and morphological evidence we found that *Janicea* is junior synonym for *Parhippolyte* and erected two new subfamilies (Barbouriinae and Calliasmatinae). An updated identification key was provided and provide a synapomorphy for the subfamily Barbouriinae. We found that Barbouriidae originated from a shallow water reef dwelling species from the Atlantic and gained, then lost troglomorphic adaptations. Our results also suggest that Calliasmatinae may need to be elevated to the family level and that *Ligur* likely does not belong within *Lysmata*. We concluded that the study was intended to provide insight into the evolutionary history of Barbouriidae and not fully resolve the classification of Barbouriidae or *Ligur*.

A population genomic study was conducted testing the isolation paradigm of anchialine systems to better understand how anchialine communities are connected and to further investigate the source of high PhyV in *B. cubensis*. As part of the population study we found that a partial draft genome assembled from short and long genomic reads greatly improves the accuracy and utility of next generation sequencing for little extra cost ($\leq \$1000$). These findings are especially relevant for studies of critically endangered species or species with large genomes, and in studies with limited funding. Testing the “islands within islands” and “spelean corridor” hypotheses lead the proposal of a new “anchialine isolation by dispersal” hypothesis. We found that *B. cubensis* represents a single highly connected population with very low genetic diversity in the tropical western Atlantic and likely has strong reproductive dispersal capabilities. This suggests that PhyV is the result of an increased susceptibility environmental disturbances and a possible accumulation of deleterious alleles as a consequence of their ability to exchange genetic

material of large geographic distances. We also discussed the need for improved conservation of anchialine systems, because of their rapid alteration or destruction by anthropogenic activity, they are home to numerous endemic species, and effects on a single seemingly disjointed pond may have broad ranging consequences.

The sixth mass extinction that is a blight of the Anthropocene stands to claim numerous anchialine species. The study of a broad range of evolutionary and ecological questions using traditional and next-generation tools stands to aid in the fight to stem the global biodiversity crisis. We find across all life, species have an astonishing ability to recover following disturbances, and establishing a baseline helps to identify when additional protection is necessary to aid in their recovery. By investigating the population dynamics and structure of species with strong dispersal mechanisms we can more easily identify threats across seemingly disjointed systems. Protecting biodiversity should be our highest priority as new health threats emerge and we become more and more reliant on the environment for sources to combat these threats.

VITA

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