Genetic spatial structure of an anchialine cave annelid indicates connectivity within -
but not between - islands of the Great Bahama Bank

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ABSTRACT

Land-locked anchialine blue holes are karstic sinkholes and caves with tidally influenced, vertically stratified water bodies that harbor endemic fauna exhibiting variable troglomorphic features. These habitats represent island-like systems, which can serve to elucidate evolutionary and biogeographic processes at local scales. We investigated whether the ‘continuous spelean corridor’ hypothesis may elucidate the biogeographical distributions of the stygobitic annelid *Pelagomacellicephala iliffei* (Polynoidae) collected from the Great Bahama and Caicos Banks of the Bahamas Archipelago. Phylogenetic reconstructions were performed using Bayesian Inference on individual and combined datasets of three molecular markers (16S rDNA, COI, 18S rDNA) and species delimitation employed three widely accepted methods in DNA taxonomy, namely GMYC, bPTP, and ABGD. Mantel tests were used to test the effect of geography on genetic structure. Using these analyses, we recovered five independently evolving entities of the focal species across four islands of the Great Bahama Bank including Cat, Eleuthera, Exumas and Long. Genetic data yielded strong correlations between islands and phylogenetic entities, signifying independent evolutionary histories within anchialine caves across the platform. The island of Eleuthera showed intra-island gene flow and dispersal capabilities between blue holes separated by 115 km, providing evidence of a crevicular spelean corridor within the island. However, no evidence of inter-island dispersal is present in the analyzed system. Consistent with previous biogeographic studies of cave crustaceans, the major barriers shaping the cave biota of the Bahamas Archipelago appears to be the deep trenches and channels separating the Bahamian banks.

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55 *iliffei*, Polynoidae, species delineation

1. Introduction

Anchialine habitats encompass land-locked water bodies of marine origin, partially isolated from the sea and harboring unique endemic fauna (Gonzalez et al., 2011; Holthuis, 1963; Iliffe and Bishop, 2007; Stock et al., 1986). They represent extremely isolated and discrete island-like ecosystems, serving to elucidate evolutionary and biogeographic processes (Gonzalez et al., 2011; Iliffe, 1986, 1990; Juan et al., 2010). Anchialine caves occur around the world, but are most abundant and extensive on islands and peninsulas along the northern Caribbean Sea and bordering the North Atlantic Ocean (Iliffe and Bishop, 2007).

Anchialine caves are characterized by total darkness, lack of photosynthesis, limited exposure to atmospheric oxygen, and reduced organic nutrient cycling (Iliffe, 2000). Typically, surface waters are fresh to brackish, but at depth, a well-defined halocline marks the transition to underlying seawater. In the Bahamas, they are referred to as inland blue holes, consisting of small pools or water-filled sinkholes, widening into deep circular shafts or long horizontal passages extending to the limits of current cave diving technology (Kornicker et al., 2002). Such blue holes are typically inhabited by eyeless, depigmented endemic fauna principally found in saline waters below the halocline. Investigations of anchialine caves have revealed diverse communities of seemingly isolated stygobitic fauna (*i.e.*, obligate, aquatic cave-dwellers) (Iliffe and Bishop, 2007). While several stygobitic genera inhabit anchialine caves on opposite sides of oceans or even across the globe (Wilkins et al., 2009), the majority of anchialine stygobitic species are recorded from a single cave or island (Gerovasileiou et al., 2016; Iliffe and Bishop, 2007). Disjunct occurrences of stygobitic relatives have long been attributed to relictualization of marine populations

inhabiting the Tethys Sea during the Mesozoic due to a combination of vicariant processes associated with plate tectonics or marine regressions and transgression (Bauzà-Ribot et al., 2012; Humphreys, 2000; Notenboom, 1991). Yet, similar stygobitic taxa are found in geologically young oceanic islands, and their occurrences have been attributed to deep-sea dispersal through a crevicular medium along oceanic spreading zones (Bauzà-Ribot et al., 2012; Martínez et al., 2013; Neiber et al., 2012).

On a more regional scale, Chace & Hobbs (1969) attempted to explain the occurrences of *Typhlatya monae* (Chace, 1954) (Pancrustacea, Atyidae) with apparent identical morphology from Puerto Rico and Honduras, suggesting a novel dispersal scenario through a 'continuous spelean corridor' which could be used by species to actively migrate within islands/landmasses, between islands/landmasses, or even across long distances using spaces among the strata. However, such an idea seemed preposterous for further investigations, and was abandoned.

Subsequently, Hart et al. (1985) introduced the term 'crevicular' for those habitats formed by crevices in and among rocks. Such a crevicular medium is equivalent to the 'continuous spelean corridor' that connects caves within and between islands, and may extend down submarine slopes of islands and continental margins via natural crevices into the deep-sea (Hart Jr et al., 1985; Iliffe, 1986, 1990).

Recent investigations tested the 'crevicular' or 'continuous spelean corridor' hypothesis using molecular methods on morphologically indistinguishable anchialine crustacean populations to separate long-term genetic isolation (thus denying the crevicular corridor) from continuous, even if weak, gene flow (potentially supporting a crevicular corridor). Strong isolation and restricted gene flow were found between

Hawaiian populations of anchialine crustaceans (Santos, 2006), while in Bermuda and the Caribbean active gene flow was shown to be present over larger spatial scales (Botello and Álvarez, 2010; Hunter et al., 2007). In most cases, gene flow is restricted and does not occur between islands, regardless of potential adult and larval dispersal capabilities of the analyzed crustaceans.

The Bahamas consists of nearly 139,000 km² of aerial and submerged shallow water carbonate platforms, separated by channels of abyssal depths, which began forming ~200 Mya during the Early Jurassic (Buchan, 2000; Dietz et al., 1970; Meyerhoff and Hatten, 1974; Mylroie and Carew, 1990; Sealey, 1985). The 'Carbonate Island Karst Model' (CIKM) (Mylroie & Mylroie 2007) geomorphologically classifies the Bahamian islands as 'simple carbonate islands', where geology and hydrology is dependent solely on carbonates below the freshwater lens. The CIKM predicts that solutional processes would have formed crevicular spaces extending between and into the base of individual platforms, giving rise to potentially continuous spelean corridors within and between islands on individual banks.

Studies of anchialine lineages bring new insights into origin, dispersal, and colonization strategies, typically not leading to the panmixia, a common pattern usually described among their non-anchialine congeners in marine habitats (Kano and Kase, 2004; Russ et al., 2010). However, these investigations focused on crustacean taxa (*e.g.*, *Creaseria*, *Halocaridina*, *Typhlatya*), with scarce information existing for other phyla (Botello et al., 2013; Botello and Álvarez, 2010; Santos, 2006). Thus, the question remains if the crevicular spelean corridor hypothesis, as suggested for crustaceans, might explain distribution patterns in other anchialine lineages.

Following the description of the anchialine swimming polynoid *Pelagomacellicephala iliffei* Pettibone, 1985 from a cave on the Caicos Bank, scientific cave diving investigations over the last two decades has revealed its presence on several islands from the Great Bahama Bank (GBB) (Wollermann et al., 2007). We consider *P. iliffei* a suitable non-crustacean candidate to test the crevicular dispersal hypothesis given that it exclusively inhabits inland blue holes.

In this study, we describe and evaluate the DNA taxonomy and phylogeographic patterns of *P. iliffei* using three molecular markers from specimens sampled across four GBB islands. In particular, we explore the connectivity of the cave populations within and between islands to determine their potential geographic structure. We discuss our findings in relation to the crevicular spelean corridor theory as the underlying dispersal mechanism for the distribution of *P. iliffei*, and speculate on the estimated age of genetic divergences in relation to the fragmentation of the Bahamas platform.

2. Materials and methods

2.1. Sampling

Running since the 1980's, our survey of anchialine cave fauna in the Bahamas is the largest and longest on-going scientific cave diving project ever conducted. In this time, more than 130 inland and oceanic blue holes, extending over 13 islands from Great Bahama, Little Bahama, San Salvador, Acklins, Mayaguana, Cay Sal, and Caicos Banks have been scientifically explored (Fig. 1).

Since the majority of anchialine blue holes had never been previously surveyed for biological purposes, initial cave dives at each site (see Appendix A.1) targeted collections of individual specimens via sampling bottles and plankton tows. Subsequent collection dives were aimed at procuring additional individuals of the species, but in order to minimize potential population impacts, our collections were limited to one specimen from each cave for morphology/voucher purposes and three additional specimens for DNA sequencing.

2.2. Morphological analyses

Live animals were examined, photographed, and video-recorded (when possible) using digital cameras. Specimens were relaxed in the field using isotonic MgCl_2 solution and fixed in 4% formalin for morphology (see below) or directly preserved in 96-100% ethanol for molecular analysis.

Specimens from each locality were examined and photographed in detail using an Olympus IX70 inverted light microscope with a mounted Olympus DP73 camera and prepared for scanning electron microscopy (SEM) to provide additional information on detailed morphology and potential differences among populations. Specimens for morphological observation were post-fixed for 60 min in 1% osmium tetroxide buffered in 0.1 M cacodylate solution and rinsed in distilled water. Specimens were dehydrated using a graded ethanol series and transferred to 100% acetone, critical point dried, mounted on aluminum stubs, and sputter-coated with platinum/palladium using a high-resolution sputter coater (JFC-2300HR). Electron micrographs were obtained using a JEOL JSM-6335F field emission scanning

electron microscope at the Natural History Museum of Denmark, University of Copenhagen.

All morphological vouchers (Table 1), called hologenophores (the specimen vouchers from which the molecular samples are directly derived) and paragenophores (putatively conspecific specimen vouchers collected in the same sample of the hologenophore) (Pleijel et al., 2008), were deposited in the Natural History Museum of Denmark (Zoological Museum University of Copenhagen, ZMUC) under collection numbers ZMUC-POL-2389 – ZMUC-POL-2397.

2.3. *Distribution patterns of P. iliffei*

First, we analyzed the distribution of *P. iliffei* by testing if its occurrence was limited to caves near coastlines or also extended to more inland caves within the same island. We created a presence/absence matrix for all the biologically explored blue holes since the 1980's. Among 130 biologically investigated sites (see Appendix A.1), 77 localities were used from the geologically contiguous Bahamas Archipelago (including Turks and Caicos Islands), representing only inland blue holes which contained appropriate habitats (*i.e.*, stratified water bodies of marine origin) that could potentially support *P. iliffei*. Across the 77 suitable locations in GBB, 14 were found harboring *P. iliffei*. For each of the 77 caves, distances from the entrance pool to the nearest coastline were measured using Google Earth™, restricting our analysis to islands where *P. iliffei* was found. A logistic regression was used to test if occupied and non-occupied caves were different, with presence/absence data for the species as a response variable, and distance to the shoreline from the caves as an

explanatory variable. Distances were log-transformed in order to improve normality of residuals of the model.

2.4. Molecular laboratory methods

To evaluate the evolutionary relationships of *P. iliffei*, mitochondrial 16S rDNA and cytochrome c oxidase subunit I (COI), and nuclear 18S rDNA data were selected as putatively reliable markers following previously published work for resolving intra-familial relationships (Bleidorn et al., 2003, Canales-Aguirre et al., 2011, Martinez et al., 2015). Since 16S rDNA and COI are fast evolving markers, they were chosen to resolve inter- and intraspecific relationships of the ingroup, whereas 18S rDNA is a conserved marker, selected to investigate deeper relationships.

Total genomic DNA was obtained from tissue samples consisting of 1-2 posterior segments using the Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. DNA was eluted in 160 µL of AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0). DNA extraction was performed for three separate individuals from each cave (when possible) in order to provide provisional estimates of variation within populations (see section 2.5 for details on sequences included from each specimen replicate). Although desirable, larger sampling size was not possible due to low occurrences of *P. iliffei* in all localities and for ethical conservation concerns.

Polymerase chain reactions (PCR) (see Appendix A.2) were prepared to final volumes of 25 µL with 12.5 µL of GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 1 µL of each primer (10 µM concentration), 10-8.5 µL of Milli-Q water (adjusted to DNA extract), and 0.5-2 µL of non-diluted DNA template. Reaction

mixtures were heated in a Bio-Rad S1000 Thermal Cycler at 94°C for 3 min, followed by 35-40 cycles (primer specific) of 94°C for 30 s, specific primer pair annealing temperatures for 30 s, and 68°C for 45 s (unless indicated otherwise), and final extension of 5 min at 72°C. The 16S rDNA primer set and the COI primer set each have two cycling steps varying in temperature and time. Annealing temperatures for 16S rDNA were 45°C and 49°C respectively, while COI were 45°C and 51°C for 45 s respectively. Sequences of 18S were obtained using three overlapping fragments. 18S rDNA fragments (1) and (3) had annealing temperatures at 49°C, with fragment (2) at 52°C.

PCR reactions were visualized on 1% agarose gels stained with GelRed™ (Hayward, CA, USA) and purified using E.Z.N.A.® Cycle-Pure Kit (Norcross, GA, USA) following the manufacturers protocol. Bound DNA was eluted in 40 µL of elution buffer. Purified products were sequenced on an ABI DNA Analyzer (Applied Biosystems) in the Marine Genomics Lab of Texas A&M University at Galveston or by MacroGen Europe (Amsterdam, the Netherlands).

Chromatogram reading and contig assembly were performed in Sequencher 4.10.1 (GeneCodes Corporation, Ann Arbor, MI, USA). All contigs were verified using NCBI BLAST to check for contamination. Sequences were visualized and trimmed using BioEdit (Hall, 1999).

Newly generated sequences were deposited in GenBank® with the following accession numbers BG##### - BG##### (Table 1).

2.5. Taxa selection and alignment

Outgroup selection was based on ongoing phylogenetic analyses of the scale worm Suborder Aphroditiformia (Gonzalez et al. 2015) and selected based on available GenBank sequences, including two Polynoidae species from the deep-sea subfamily Macellicephalinae, *Austropolaria magnicirrata* Neal, Barnich, Wiklund & Glover, 2012 and *Macellicephala violacea* (Levinsen, 1887); other deep-sea polynoids, *Branchipolynoe symmytilida* Pettibone, 1984; *Bathykurila guaymasensis* Pettibone, 1989; *Branchinotogluma sandersi* Pettibone, 1985; as well as *Gesiella jameensis* (Hartmann-Schröder, 1974), the only other known stygobitic swimming polynoid inhabiting an anchialine lava tube cave on Lanzarote, Canary Islands.

All sequences were aligned using an iterative refinement method implemented in MAFFT (Katoh and Standley, 2013). The data set on 16S rDNA was aligned under the Q-INS-I iterative refinement method, suitable for highly variable ribosomal marker (Katoh and Toh, 2008), while the dataset on 18S rDNA was aligned under the E-INS-I iterative refinement method suited for sequences of close relatedness (Katoh et al., 2005). Alignments of COI sequences were trivial as they were constant in length. Additional alignment strategies were also performed and compared, exhibiting nearly identical results.

Individual gene fragments and those of the two concatenated alignments (16S+COI and 16S+COI+18S) were independently analyzed and examined for the best partition scheme using PartitionFinder (Lanfear et al., 2012). Concatenated alignments were assembled using Sequence Matrix (Vaidya et al., 2011). Optimal partitioning treated 16S rDNA and 18S rDNA independently, without further partitioning, while COI was found to be optimally divided into two partitions (partition 1 = codons 1+2 and partition 2 = codon 3) for all datasets. Prior to phylogenetic

reconstructions, identical sequences in both individual and combined datasets were identified using pairwise distances, and subsequently removed to avoid inclusion of redundant information.

2.6. Phylogenetic reconstruction

Phylogenetic reconstructions were performed with Bayesian Inference using BEAST v1.8.2 (Drummond et al., 2012) as implemented on the CIPRES Science Gateway (Miller et al., 2010). BEAUti (Bayesian Evolutionary Analysis Utility version) v1.8.2 generated xml files for all BEAST runs. Independent BEAST runs were performed for both individual molecular markers, as well as combined and partitioned datasets (16S+COI-p1/p2 and 16S+COI-p1/p2+18S). Two separate analyses were run for each dataset with varying tree priors since population size is unknown and intra-specific sequences are being employed. Tree priors were thus selected both under a Yule Process (Nee, 2006) and under a Coalescent Process with constant population size. Nucleotide substitution models for all BEAST runs were estimated by the corrected Akaike information criterion (AICc) implemented within PartitionFinder and were as follows: generalized time reversible (GTR + G) for 16S rDNA; Hasegawa, Kishino and Yano (HKY) for COI codon 1+2; Symmetrical (SYM + G) for COI codon 3; and Tamura-Nei (TrN + I) for 18S rDNA. All datasets had independent MCMC analyses with 10^8 generations, sampled every 10,000 generations. All parameters were unlinked between markers for reconstructions using alignments of concatenated markers. TRACER v1.6.0 (Rambaut and Drummond, 2007) assessed convergence of MCMC runs. A maximum clade credibility (MCC) consensus tree was

obtained after annotating the remaining 7000 trees after burn-in in TreeAnnotator v1.8.2.

2.7. Testing for evidence of crevicular dispersal through a continuous spelean corridor

Analyses and simulations described below were performed in R v3.2.1 (R Core Team, 2014):

2.7.1. Species delimitation

To determine if *P. iliffei* represented a single species throughout the sampled localities, or at the opposite extreme, if each cave population could be considered a separate and independently evolving entity, an integrative taxonomical approach was used. This included morphological studies (SEM) and three widely accepted methods in DNA taxonomy (Fontaneto et al., 2015), namely the generalized mixed Yule-coalescent (GMYC) model (Fujisawa and Barraclough, 2013), the Bayesian implementation of the Poisson Tree Process (bPTP) (Zhang et al., 2013), and the Automated Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). Outgroups were removed prior to implementation under all three methods. The GMYC model was implemented on ultrametric trees obtained from BEAST, currently considered the most reliable method to obtain ultrametric trees for GMYC (Tang et al., 2014). The model was performed in the R package SPLITS v1.0-19 (Ezard et al., 2009) on phylogenies obtained for each individual molecular marker and combined datasets. The PTP model for species delineation, based on the number of mutations between taxa, was carried out on the bPTP online server (<http://species.h-its.org/>) (Zhang et

al., 2013). Putative entities were identified using 10^4 MCMC generations with a burnin of 0.1 using ultrametric BEAST trees of individual and combined marker datasets. Individual 16S and COI datasets were uploaded on the ABGD online platform (<http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>) and analyzed using pre-set parameters (Puillandre et al., 2012).

The most conservative estimates in common among the employed methods and genetic markers were used to identify phylogenetic entities, providing evidence of independently evolving entities as units of diversity in subsequent analyses where their occurrence in separate caves and islands were assessed. The term evolutionary, phylogenetic, or independently evolving entity rather than species is used throughout the manuscript, as not to place any emphasis on the taxonomic consequences of our results. Such consequences could be explicitly addressed only when material from the type locality of *P. iliffei* in Turks and Caicos (Pettibone, 1985) becomes available for further morphological and molecular comparisons.

2.7.2. Cave- and island-specificity in independently evolving entities of *P. iliffei*

We evaluated whether cave-specificity and/or island-specificity was present for the identified independently evolving entities. We then tested whether such specificity was significant or could be considered just a consequence of small sample size, providing misleading specificity by chance alone. We performed an association test by resampling our results of occurrence of individuals in caves (or islands) from the phylogeny with all individuals included. For each phylogenetic entity, we counted the number of caves (or islands), identified where each lineage was found (see Appendix A.1), and tested whether the observed numbers of caves and islands were

significantly lower than those obtained by a random distribution of caves (or islands) as attributes of the terminals in the phylogeny. We repeated the simulation 1000 times and calculated the distribution of the estimated number of caves (or islands) per lineage with the observed numbers. Results were compared to the distribution of the simulation, checking how many times observed numbers were lower than ones expected under a random distribution of individuals across the GBB. Alternative scenarios were also tested: 1) a random distribution of individuals lacking association between islands and caves suggests frequent dispersal across different caves and islands; 2) island but not cave specificity indicates that a crevicular spelean corridor facilitates dispersal within islands, but not between islands; and 3) island and/or cave specificity suggests that there is no dispersal among caves within or among islands. We acknowledge that the low within-cave sample size to minimize the number of suppressed animals may cause a lack of significance in some of our tests, and thus we place emphasis only on the significant correlations, and not on the lack of significant correlations.

2.7.3. Effect of geography on genetic structure

We assessed whether patristic distances (*i.e.*, sum of branch lengths) between phylogenetic entities were correlated to geographic distances between collection sites, by implementing Mantel tests under the R package FOSSIL v0.3.7 (Vavrek, 2011). Since no georeferencing was possible inside submerged caves, we calculated geographical distances between sampled populations based on coordinates of each entrance pool. Patristic distances were calculated in the R package VEGAN v2.3-0 (Oksanen et al., 2015), as average distances between entities. Strong correlations

between geography and genetics would suggest gene flow is highly limited in space, whereas a lack of correlation could have several causes, difficult to disentangle.

2.8. Molecular clock

For a rough divergence time estimation of *P. iliffei*, a time-calibrated phylogeny was reconstructed in BEAST v1.8.2 using the concatenated dataset 16S+COI+18S. Given the absence of fossils for the Macellicephalinae for time calibration or published group specific rates, we employed strict molecular clock for COI and 18S rDNA using previously estimated rates (Tang et al., 2014). A strict clock was chosen as it has been shown to be superior for low- to moderate-rate variation between branches, especially within intra-species (Brown and Yang, 2011). The substitution rate calibration used for COI was 1.19% Myr (deep-sea annelid specific; see Wilke et al., 2009) and for 18S rDNA was 0.02% Myr (Bargues et al., 2000; Ochman and Wilson, 1987). We acknowledge the uncertainties associated with clock estimations, especially regarding models lacking calibration points, where implementation of external clock rates may lead to the underestimation of intraspecific rates (Bauzà-Ribot et al., 2012). However, considering various clock rates employed for stygobitic crustaceans (*i.e.*, 2.3-20% Ma; see Bauzà-Ribot et al., 2012; Craft et al., 2008), their estimated ages would be even older than the rates we could obtain. Thus, we chose the most conservative method, providing bias towards more recent estimates while providing a more convincing argument favoring ancient phylogenetic subdivisions within the GBB. Nucleotide substitution models used for each partition were as previously mentioned. Tree priors were set to speciation birth-death process with a uniform distribution, suggested as an appropriate null model for phylogenetic

diversification (Mooers and Heard, 1997; Nee, 2001). MCMC chain length was 600k, with tree sampling every 20,000 generations and a final burn-in of 33%. Convergence of the MCMC run was assessed using TRACER v1.6.0. MCC consensus tree was obtained after burn-in; annotating the remaining trees using TreeAnnotator v1.8.2.

3. Results

3.1. Distribution patterns of *P. iliffei*

Of the 77 anchialine inland blue holes explored throughout the Bahamas, only 14 (18.18%) harbored *P. iliffei* (Fig. 1) from Cat, Eleuthera, Exumas, and Long Islands; in addition, the species was already known from Middle Caicos Island, Turks and Caicos Islands on the Caicos Bank, which we were not able to sample. Our results showed that caves with *P. iliffei* were not closer to the coastline than caves lacking *P. iliffei* (GLM: $z = -0.058$, $p = 0.953$).

3.1. Morphology and phylogeny

Morphological evaluation of collected specimens using light and SEM confirmed that GBB specimens conform to the original description of *P. iliffei* by Pettibone in 1985. (Fig. 2). Slight variations, including the presence of papillae, were observed along elytral surfaces and margins (Fig. 2 f-g). Additionally, ciliated areas along the body, prostomium, and parapodia (Fig. 2 b, h) were discovered based on SEM micrographs not employed at the time of the original description. All examined animals exhibited

these features and no differences were observed among them, or among populations in different caves.

In all molecular analyses, *P. iliffei* was recovered as monophyletic and sister to *G. jameensis*, together forming a highly supported clade with the two other deep-sea Macellicephalinae terminals. Within the *P. iliffei* clade, terminals from each island formed individual subclades. However, the two collection localities of *P. iliffei* from Cat Island formed separate and independent subclades, including a deep branching subclade and another sister to the terminals from Eleuthera (Fig. 3).

3.2. Potential crevicular dispersal through a continuous spelean corridor

The distribution of *P. iliffei* was tested from various perspectives in order to investigate the patterns of connectivity within and between islands, and to determine the potential for geographical structure.

3.2.1 Species delimitation

PTP and ABGD consistently estimated five phylogenetic entities (Table 2), corresponding to the clades referred to as A-E (Fig. 3). GMYC revealed the same five entities as the minimum estimate when combining 16S+COI markers, as well as when combining all three markers (16S+COI+18S). On the other hand, no statistical support was found when using phylogenies from the individual mitochondrial molecular markers (16S rDNA and COI). No differences in the results of DNA taxonomy were observed between Yule or Coalescent BEAST tree priors (Table 2). Uncorrected genetic distances among entities for COI ranged from 5.4% to 20.7%, whereas the largest uncorrected COI distance within an entity was 3.1% (See

Appendix A.3).

3.2.2. Cave- and island-specificity in independently evolving entities of *P. iliffei*

Entities B, C, D, and E were each associated with one island and one cave, whereas entity A from Eleuthera was present in three caves (Fig. 3; see Appendix A.1). Such association between phylogenetic entities and islands cannot be obtained by chance alone given our dataset (resampling test: $p < 0.0001$). Conversely, islands could be inhabited by more than one entity, as entities B and E were found in caves only 1.2 km apart on Cat Island.

Multiple caves within Eleuthera were inhabited by a single phylogenetic entity (A). However, the overall association between phylogenetic entities and caves is equally as strict as the one with islands, and cannot be obtained by chance alone (resampling test: $p < 0.0001$): each cave hosted only one phylogenetic lineage.

3.2.3. Effect of geography on genetic structure

Genetic distances among entities were not correlated with geographic distances (Mantel test: $p = 0.269$). Such lack of correlation could be due to the presence of the distantly related entities E and B on Cat Island. Yet, when removing entity E from the analyses and dealing only with the sister clade formed by all other entities, correlation between genetic and geographic distances for the other four entities was not significant (Mantel test: $p = 0.821$), hereby not providing any support for the hypothesis that the genetic structure of *P. iliffei* is related to isolation by distance, even if we cannot rule out the effect of a low sample size.

3.3. Molecular clock and age of divergence

The node supporting anchialine (*P. iliffei* – *G. jameensis*) and deep-sea clades yielded a divergence time of ~63.71 Ma (52.6-71.81 Ma; 95% HPD) and *P. iliffei* and *G. jameensis* diverged at ~55.55 Ma (44.48-67.55 Ma; 95% HPD). The *P. iliffei* clade shows a split between entity E and entities D-A at ~25.51 Ma (20.31-31.3 Ma; 95% HPD). The youngest split in the *P. iliffei* clade is between entities A and B, estimated at ~5.07 Ma (3.76- 6.62 Ma; 95% HPD).

4. Discussion

This study represents the first molecular and ecological examination of the enigmatic stygobitic swimming polynoid *Pelagomacellicephala iliffei* from one of the largest anchialine environments in the Caribbean and North Atlantic. Using an integrative taxonomical approach, we offer support against a spelean corridor between islands, but show that such corridors may be effective for dispersal within islands.

4.1. Systematic significance

The type locality for *P. iliffei* is Conch Bar Cave, Middle Caicos Island, Caicos Bank, with the Turks and Caicos Islands being a geological and geographical extension of the Bahamas Archipelago. This study extends the known geographical range of *P. iliffei* from the type locality on Caicos Bank, more than 300 km northwest to four islands within the GBB. Morphologically, the specimens of *P. iliffei* obtained in the Bahamas are nearly identical for overall size, length of dorsal cirri, and chaetal characters. Only slight differences were observed in elytral morphology when

compared to the original description. Our conclusion after examining the Bahamian specimens is that they appear to be in a state of morphological stasis, notwithstanding deep genetic diversity and ancient origin.

Our phylogenetic findings support a sister relationship between *P. iliffei* and *G. jameensis*, the only other known anchialine polynoid. A deep-sea origin was originally proposed for these two taxa based on morphology, and now as a result of concurrent phylogenetic analyses, *P. iliffei* and *G. jameensis* have been shown to be strongly supported as members of the deep-sea family Macellicephalinae (Gonzalez et al., 2015). While our study validates that of Gonzalez et al. (2015), it cannot further contribute to the original hypotheses on our understanding of their distribution, as we may be biased due to sampling limitations and the complexity of subterranean labyrinths.

4.2. Crevicular dispersal throughout the Great Bahama Bank

Our phylogenetic investigation recovered five separate entities of *P. iliffei* (A-E; Fig. 3) with clear geographic structure relative to the islands of GBB. Given the results of the association tests, each entity (A-E) was island specific (Fig. 3). van Hengstum and Scott (2012) illustrated that anchialine water bodies are capable of migrating vertically based on coastal circulation. This suggests that lineage diversification would be independent of geologic parameters such as isolation by uplift or eustatic changes, as animals would migrate vertically along with the water bodies. Therefore, we interpret our results as signifying that each entity may represent geographically independent diversification events throughout the Bahamas.

Evidence for dispersal through a crevicular spelean corridor was only found within, but not between islands. Entity A was comprised of specimens from three inland blue holes on the island of Eleuthera (Table 1). Distances between entrance pools range from 0.73 to 115 km, illustrating the potential for intra-island movement of individuals via crevicular subterranean corridors. Similar patterns of connectivity within a single landmass have been shown for crustaceans in cenotes of the Yucatán Peninsula, Mexico. Evidence for genetic exchange between cenotes 200-235 km apart was found in the anchialine shrimp *Typhlatya mitchelli* Hobbs & Hobbs, 1976, *Typhlatya pearsei* Creaser, 1936, and *Creaseria morleyi* (Creaser, 1936), suggesting hypogean connectivity within continental landmasses (Botello and Álvarez, 2010; Hunter et al., 2007). Similar results were also obtained with *Halocaridina rubra* Holthuis, 1963 in Hawaii, but across much shorter distances (30-45 km; Santos, 2006).

Given the genetic differences and habitat specificity of *P. iliffei*, exclusive to inland blue holes having no above ground connection to the ocean and up to 2.2 km from the coast (Appendix A.1), it is unlikely that oceanic dispersal occurs. *Pelagomacellicephala* larvae are unknown and annelid larvae were absent from plankton tows during sampling. Furthermore, no oceanic dispersing larvae are known for anchialine stygobites. While reproductive strategies and lifecycle stages are unknown for *P. iliffei*, the possibility of long distance planktonic dispersal for both colonization and gene flow should not be completely ruled out. However, it is highly doubtful and this pathway was likewise dismissed for *H. rubra* (Santos, 2006).

On Cat Island, two divergent entities (E and B) occur at separate inland blue holes. It is unknown if this divergence could be due to barriers to dispersal, given that

the 2.2 km distance is well within the actual potential of dispersal of entity A of *P. iliffei* (>100 km), but the two cave systems appear geologically and hydrologically compartmentalized from one another, being present in different karstic features separated by a hill line (25 m elevation). Gene flow in stygobitic lineages is only permitted if they are within the same geologic basin permitting water flow. Populations of *H. rubra* were also identified as having geologic barriers, where connectivity and gene flow was lacking (Santos, 2006). Additionally, Mantel tests found no correlation (Mantel test: $p = 0.269$) between geographic distance and genetic distances between entities, and thus it is difficult, if not impossible to track a colonization history for the five entities of *P. iliffei*. Unfortunately, Caicos specimens were not available for morphological or genetic comparison and integration into our hypothesis testing regarding the colonization and speciation among anchialine environments of the Caribbean Sea, but we may reliably assume that these animals will be even more genetically diverse from the ones we analyzed in the Bahama Bank. Thus, the following questions persist: Do similar patterns of a single island entity exist within the Turks and Caicos? Is there evidence of a founder effect into the Bahamas with subsequent allopatry? Where is the origin of colonization and can a pattern of directionality be extrapolated based on the breakup of the Bahamas megabank?

4.3. Divergence estimates of *P. iliffei*

Divergence times between *P. iliffei* and *G. jameensis* inferred with our molecular clock (Fig. 4) shows a very early split, 55 Ma (44.48-67.55 Ma; 95% HPD). This age

may fit with late opening stages and formation of the Atlantic, and therefore does not rule out vicariant events for the origin of either anchialine taxon.

Based on its phylogenetic position, the split between entity E and entities A-D appears to represent the oldest diversification event within the GBB. The split was estimated to be approximately 25 Mya (Fig. 4). During this time, it has been proposed that the Bahamas banks were still partially fused as a megabank, being altered to their current states by seawater scouring (Meyerhoff and Hatten, 1974). Younger splits between entities A-D exhibit divergence times ~8.36 Ma to ~5.07 Ma, possibly reflecting the progression when island-to-island connectivity ceased to exist among the varying islands of the GBB, although this description is highly speculative. While existing anchialine habitats occur in significantly younger geology than the original bank, the nature of the platform suggests that analogous anchialine habitats would have been present.

4.4. Support for a 'continuous spelean corridor'

While nearly all anchialine studies have been limited to depths of < 50 m with the occasional excursion to > 70 m, it is human physiology, not geological or biological constraints restricting our knowledge of how deep crevicular and cave habitats extend (Iliffe 1990). Evidence now suggests that submarine caves or crevices may exist at virtually any depth of the ocean. This is supported by submersible dives observing hydrologically active caves with speleothems at depths of > 350 m on Johnston Atoll (Keating, 1985) and submarine lava tubes and crevicular fissures across volcanic seamounts and oceanic ridges (Fornari et al., 1985). Chace and Hobbs (1969) introduced the proposal of the existence of 'continuous spelean

corridor' to explain disjunct distribution of anchialine stygobites with little morphological differences. Given the results presented here with *P. iliffei* and those of *Typhlatya* (Hunter *et al.*, 2007) and *Creaseria* (Botello and Álvarez, 2010), the potential for a 'continuous spelean corridor' appears to be only evident at small spatial scales within islands, contiguous platforms, or shallow water banks, but not between islands. While it is possible that ancient subterranean corridors once existed between larger geographic areas (*i.e.*, before the scouring and isolation of islands of the GBB, the oceanic barriers present today cannot be easily overcome, with each anchialine cave potentially representing a unique isolated habitat for independent evolution of scale worms and other marine life forms.

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Data Accessibility

All sequences used and obtained in this study have been deposited in the GenBank[®] database with accession numbers BG##### - BG#####. All morphological paragenophore and halogenophore vouchers were deposited at the Natural History Museum of Denmark (ZMUC) under collection vouchers ZMUC-POL-2389-ZMUC-POL-2397.

Author Contributions

BCG and TMI collected *P. iliffei* used in this study from inland anchialine blue holes. BCG, DF, and AM jointly conceived the testing of the study. BCG, EB, and AM contributed to obtaining and post-processing of molecular sequences. Supervised laboratory research was performed under the direction of EB and KW. Funding was provided through KW. BCG wrote the manuscript with input from all authors.

Tables

815 **Table 1** Details of specimens used in this study including vouchers, collection localities, and sequences. Bold specie names,
816 vouchers, and accession numbers were used throughout the analyses reported in this study.
817

Family	Subfamily	Taxon	Voucher	Cave/Blue hole	Rock type	Origin	18S rDNA	16S rDNA	COI
Polynoidae	Macellicephalinae	<i>Austropolaria magnicirrata</i>	NHM2012.92	-	-	Pine Island Bay, Amundsen Sea	JX863895	JX863896	-
	-	<i>Bathyrurila guaymasensis</i>	-	-	-	Southern California Margin	DQ074765	-	DQ074766
	Branchinotogluminae	<i>Branchinotogluma sandersi</i>	SMNH118960	-	-	Juan de Fuca Ridge	JN852821	JN852889	JN852923
	Branchipolynoinae	<i>Branchipolynoe symmytilida</i>	-	-	-	East Pacific Rise	-	AF315055	AY646021
	Macellicephalinae	<i>Macellicephala violacea</i>	NHMUK:2012.12	-	-	Mid-Atlantic Ridge, N. Atlantic	-	-	JX119016
	Gesiellinae	<i>Gesiella jameensis</i>	ZMUC-POL-2389	Túnel de la Átlantida	Volcanic	Lanzarote, Canary Islands	□	□	□
	Gesiellinae	<i>Gesiella jameensis</i>	ZMUC-POL-2390	Túnel de la Átlantida	Volcanic	Lanzarote, Canary Islands	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2391	White Lake Blue Hole	Limestone	Cat Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	White Lake Blue Hole	Limestone	Cat Island, Bahamas	-	□	-
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	White Lake Blue Hole	Limestone	Cat Island, Bahamas		□	
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2392	Big Fountain Blue Hole	Limestone	Cat Island, Bahamas	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Big Fountain Blue Hole	Limestone	Cat Island, Bahamas	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Big Fountain Blue Hole	Limestone	Cat Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2393	Valentine's Cave	Limestone	Eleuthera Island, Bahamas	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Valentine's Cave	Limestone	Eleuthera Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Preachers Blue Hole	Limestone	Eleuthera Island, Bahamas	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2394	Preachers Blue Hole	Limestone	Eleuthera Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2395	Mermaids Hole	Limestone	Eleuthera Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Mermaids Hole	Limestone	Eleuthera Island, Bahamas	□	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Mermaids Hole	Limestone	Eleuthera Island, Bahamas		□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Windermere Abyss	Limestone	Eleuthera Island, Bahamas	□		□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2396	Oven Rock Cave	Limestone	Exuma Islands, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	Basil Minns Blue Hole	Limestone	Exuma Islands, Bahamas		□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	The Grotto Hole	Limestone	Long Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	-	The Grotto Hole	Limestone	Long Island, Bahamas	-	□	□
	Macellicephalinae	<i>Pelagomacellicephala iliffei</i>	ZMUC-POL-2397	The Grotto Hole	Limestone	Long Island, Bahamas	□	□	□

Table 2 Species delimiting results for *Pelagomacellicephala iliffei* Pettibone, 1985 using GMYC, PTP, and ABGD methods for individual and combined gene datasets (* = $p \leq 0.05$).

YULE MODEL

	GMYC			PTP		ABGD
	ML entities (C.I.)	likelihood ratio	<i>P</i>	est. ent.	mean	est. ent.
16S rDNA	1	5.5248	0.0631	5-10	6.22	5
COI	1	4.3552	0.1133	5-9	6.27	5
16S rDNA + COI	7 (6-8)	7.8771	0.0195*	5-8	6.13	<i>na</i>
16S rDNA + COI + 18S rDNA	6 (5-7)	6.8285	0.0329*	5-9	6.02	<i>na</i>

COALESCENT MODEL: CONSTANT POP

	GMYC			PTP		ABGD
	ML entities (C.I.)	likelihood ratio	<i>P</i>	est. ent.	mean	est. ent.
16S rDNA	1	5.5131	0.0635	5-10	6.18	5
COI	1	4.3693	0.1125	5-9	6.28	5
16S rDNA + COI	7 (6-8)	7.8771	0.0195*	5-8	6.13	<i>na</i>
16S rDNA + COI + 18S rDNA	6 (5-7)	6.7275	0.0346*	5-9	5.98	<i>na</i>

Figures

Fig. 1 Known localities of *Pelagomacellicephala iliffei* Pettibone, 1985. (a) Map showing the location of blue holes yielding *P. iliffei* across the Great Bahama Bank. Locations indicated by a solid circle (•). Type locality of *P. iliffei* is indicated by a solid star (★). (b) Example of inland anchialine blue hole entrance pool from the Bahamas Archipelago harboring *P. iliffei*. Photo by Tamara Thomsen.

Fig. 2 Morphological comparison of geographically diverse specimens of *Pelagomacellicephala iliffei* Pettibone, 1985 using scanning electron microscopy. (a) Dorsal overview of entire animal including elytra and elongated dorsal cirri. Pharynx everted. (b) Close up of prostomium and median antenna attachment. White arrow indicates a ciliary band. (c) Close detail of prostomium, everted pharynx, and first three segments. (d) Mouth detail including jaws and papillae. Position of palps shown. (e) Parapodial detail illustrating chaetal position and acicular lobes. (f) Elytra (whole) with areas of previously undocumented papillae illustrated by white line. (g) Close-up detail of elytral papillae and margin crenulations. (h) Detail of parapodia showing ciliary tufts along the margin. (i) Notochaetae with spinous pockets along margins. (j) Neurochaetae with distinct terminal widening and spinous pockets along margin. (k) Posterior segments showing elytral attachment. Definition of abbreviations: I-III, segment numbers; aC, anal cirri; acL, acicular lobe; dC, dorsal cirrophore of dorsal cirri; dTc, dorsal tentacular cirri; dTu, dorsal tubercles; el, elytra; elph, elytraphore; ja, jaw; m, mouth; mAn, median antenna; ne, neuropodia; neC, neurochaetae; no,

notopodia; noC, notochaetae; pa, palps; pap, papillae; pr, prostomium; prob, proboscis; st, style of dorsal cirri; vTc, ventral tentacular cirri.

Fig. 3 Phylogenetic reconstruction of *Pelagomacellicephala iliffei* Pettibone, 1985 using Bayesian Inference. Node values represent Bayesian posterior probabilities generated in BEAST. (a) Islands associated with the Great Bahama Bank (GBB) and their relative position within the Bahamas Archipelago. Dashed lines around islands represent the 100-fathom depth profile. Phylogenetic entities (Ent) recovered by the delineation are labeled A-E. Tree tips are color-coded corresponding to island. Dashed red line corresponds to the most conservative estimates of entities based on the delineation results of GMYC and PTP. A solid grey box on a branch corresponds to strictly deep-sea lineages, while a solid black box represents lineages of anchialine taxa. (b-c) Live photographs of collected *P. iliffei* from Cat Island (b) and from Exumas (c). Photographs by TMI.

Fig. 4 BEAST ultrametric tree inferred using a concatenated alignment of 16S rDNA + COI + 18S rDNA. Node ages were calculated using a strict molecular clock with separate substitution rates for COI (1.19% Ma) and 18S rDNA (0.02% Ma). Solid horizontal red bars represent 95% HPD.