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CAPTURING SPECIATION IN ACTION: RAPID POPULATION DIVERGENCE IN THE CARIBBEAN BIOLUMINESCENT OSTRACOD, *PHOTEROS ANNECOHENAE* (MYODOCOPIDA: CYPRIDINIDAE)

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By: Nicholas J Reda

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology -- Aquatic Science Concentration

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ABSTRACT

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Species that have complex courtship behaviors are some of the most evolutionarily diverse lineages observed in nature. Divergent, pre-mating calls are effective in both generating and/or maintaining reproductive isolation. Complex courtship displays provide numerous characters in which a small change can reinforce or lead to reproductive isolation. The multiple characters of these displays often evolve interactively, increasing potential variants of phenotypic display traits. Because many characters can be quantified and used to document variation among species, organisms that use complex courtship behaviors provide model systems for testing the influence of ecology on lineage diversification and trait evolution. Here, we quantify differences in the courtship behavior, morphology, and genetic trait change of male *Photeros annecohenae* over an intermediate range of geographic distances along reef habitats of the Mesoamerican barrier reef of Belize. Differences in bioluminescent ostracod behavior, morphology, and genetics have been documented across large geographic distances (500-1000 km) and at smaller geographic scales (~12km) *P.annecohenae* exhibits measurable population genetic structure but minimal behavioral and morphological differentiation. Our findings support the hypothesis that differences observed in behavioral, morphological, and genetic characters across isolated populations of *P. annecohenae* are occurring along this intermediate range at short geographic differences. The observed morphological, behavioral, and genetic isolation of male *P. annecohenae* offers novel insight toward our understanding of the speciation continuum.

ACKNOWLEDGEMENTS

Thank you to UW-La Crosse RSEL grant for financial support throughout this study. To Earl David Jr. for lodging and his insurmountable knowledge navigating the Mesoamerican barrier reef during time spent in the field. To Vanessa Gonzalez of the Global Genome Initiative for her expertise and guidance. Finally, to my committee members, Gretchen Gerrish, Todd Osmundson, and Alder Yu for guidance.

TABLE OF CONTENTS

PAGE

TABLE OF FIGURES

PAGE

INTRODUCTION

The complex process of speciation is affected by genetic, ecological, environmental and developmental factors that interact nonlinearly to drive divergence. The culmination of these multifaceted factors makes speciation a highly dynamic process that is difficult to capture in action. Based on the biological species concept, speciation is the evolution of reproductive isolating mechanisms that prevent the exchange of genes between nascently arising taxa (Bush, 1975; Turelli et al., 2001). Speciation is the initiation of reproductive barriers that reinforce the maintenance of both genetic and phenotypic identities of populations (Coyne & Orr, 2004; Price, 2008; Seehausen et al., 2014). The origin of reproductive barriers can be due to divergent selection that establishes either ecological or sexual reproductive isolation between populations or due to random evolutionary processes that cause genomic incompatibilities creating reproductive isolation. Barriers to gene flow that lead to pre-mating and/or post-mating reproductive isolation occur in allopatry and/or sympatry, and can be an indirect consequence of natural selection, or due to directional selection for pre-mating isolation (Schluter, 2001). Both pre-zygotic (e.g., behavior, habitat, ecology) and post-zygotic (e.g., hybrid inviability) isolation can interact and lead to multiple barriers of reproduction (Maan & Seehausen, 2011; Hendry et al., 2007; Abbott et al., 2013). True understanding of the causes and consequences of speciation requires comprehensive sampling, phylogenetic

construction, and precise distributional, morphological, and ecological data for diverging lineages at multiple temporal and geographic scales.

Species that have multiple axes of differentiation are prone to high divergence in the presence of ecological opportunity (Schluter, 2000; Losos and Mahler 2010; Yoder et al, 2010). In African cichlid fish, models of sexual selection interacting with ecological opportunity predict radiations in older lineages of deep lakes (Wagner et al, 2012; Seehausen, 2015). Lake Victoria cichlid fishes have multiple axes of differentiation: morphological traits, male coloration, distribution across depth, and feeding ecology (Keller et al, 2012). In river and lake ecotypes of cichlids, substantial prezygotic and postzygotic barriers contribute to reduced gene flow between ecotypes (Rajkov et al, 2018). Darwin's Galapagos finches exemplify evolutionary radiation through trait evolution of beak morphology in response to the ecological opportunity of their environment and seed preference (Grant and Grant, 2008; Losos and Ricklefs, 2009). In these case's allopatric distribution alone may cause radiations, but the high observed diversity is likely due to allopatric and sympatric mechanisms interacting to reinforce reproductive isolation (Losos and Ricklefs, 2009; Rundell and Price, 2009). In North American freshwater darter fish, male color patterns, a trait associated with sexual selection, plays a larger role than ecological adaptation traits, such as shape, in early lineage divergence (Martin and Mendelson, 2016). Identifying the relative impact of phenotypic and ecological drivers of reproductive isolation is useful for establishing the evolutionary mechanisms contributing to speciation.

While ecology provides opportunity for diversification, key innovations in behavior rapidly increase the rate of speciation within groups (Albert and Borgia, 2007;

Mendelson et al, 2014; Martin and Mendelson, 2016) and can either initiate and/or maintain reproductive isolation. Behavioral pre-mating reproductive isolation drives speciation through the divergent coevolution of male mating signals and female mate preference (Panhuis et al., 2001; Ritchie, 2007; Coyne and Orr, 2004). Sexual selection is distinct from natural selection as a mechanism of adaptive divergence because it is identifiable in phenotypic traits that lead to reproductive isolation (Safran et al, 2013). Acoustic mate signaling groups such as birds, frogs and crickets are strong examples of clades that exhibit rapid lineage diversification through key innovations in acoustic mate display (Kaiser et al, 2018; Graham et al, 2018, Blankers et al, 2018). The Hawaiian cricket *Laupala* has rapidly radiated across islands due to the tight coevolutionary bond of male signals and female preferences (Blankers et al, 2018). Geographic variation and directional sexual selection of multivariate male acoustic characters together play an important role in patterns of song divergence (Oh and Shaw, 2013). The Central American red-eyed tree frog has strong population level differentiation in male acoustic and visual communication, together enhancing local population mate preference (Kaiser et al, 2018). Lineages that use multivariate or complex communication signals are useful study systems for elucidating the role of reproductive behaviors as drivers of speciation.

Mapping lineage-specific traits onto phylogenies allows us to document speciation in action and evaluate evolutionarily important morphological, ecological, and genetic properties. When studying evolutionary radiations, it is imperative that biogeographic and phylogenetic analyses of a species evolutionary history (i.e. phenotypic behavioral and ecological traits) are considered (Losos, 2009; Bouchenak et al., 2015; Harmon et al, 2010; Lieberman, 2012; Moen and Morton, 2014). Phylogenies help assess rates of

speciation and extinction, while biogeography evaluates the evolution of groups across geographic space. Paired, these approaches allow accurate assessment of allopatric and sympatric divergence occurring throughout space and time (Simoes et al, 2016). Phylogenies used to assess Japanese and North American fireflies show the origin and loss of bioluminescent mate signaling (Suzuki et al, 1997; Stanger-Hall et al., 2007; Martin et al., 2017). The origin of ecological, morphological, behavioral, and genetic divergence involved in the rapidly evolving African cichlid fishes are identified through phylogenetic tools and analyses (Meyer 1993; Albertson and Markert, 1999; Salzburger et al., 2002; Salzburger and Meyer, 2004; Seehausen, 2006; Schwarzer et al., 2009; Schedel et al., 2019). Research that incorporates ecological factors, species interactions (competition and sympatric parameters), and comparative phylogenetic analyses are imperative for understanding how ecological and evolutionary forces interact to generate divergence at variable spatial scales.

Many terrestrial and freshwater taxa provide models for evaluating speciation mechanisms in time and space, but limited work in marine systems extensively apply these models to explain highly diverse groups. Marine systems are vastly connected at large and small spatial scales and defining connectivity depends on understanding life history and dispersal of target taxa. The geographic scale at which phylogenetic studies are evaluated offers the ability to make certain inferences on the eco-evolutionary forces that promote local adaptation. Large-scale genetic analyses reflect species history and contemporary evolutionary processes linked to population connectivity and environmental conditions (e.g., temperature, oceanic currents). Many marine flora and fauna rely on thermal ocean currents for dispersal and migration. In marine coastal

regions, where physical barriers to dispersal are absent, temperature initiates ecological speciation (Teske et al., 2019). In the Indonesian Spermonde archipelagos, vertebrate (*Amphiprion ocellaris*) and invertebrate (*Polycarpa aurata*) species had panmictic population structure on the northern end where there is stronger ocean currents and had significantly restricted gene flow at small geographic scales on the southern end where there is less connectivity (e.g., weaker current, shallow reef shelf) (Timm et al., 2017). However, strong currents, upwelling and geographic distance may act to restrict gene flow in the New Zealand sea urchin, *Evechinus chloroticus,* where there is strong fine-scale and broad-scale population differentiation (Nagel et al., 2015). Small-scale, population genetic analyses are fundamental to understanding the complex, interwoven biological mechanisms (larval duration, larval behavior, isolation by distance, limited dispersal capabilities) that drive genetic divergence (Pascual et al., 2017). The complex, interconnected relationship between abiotic and biotic factors results in a level of spatial genetic differentiation greater than expected (Charrier et al., 2006; Calderon et al., 2007; Palero et al., 2008). Benthic marine organisms in their larval stage can be transported substantial distances and are affected by many of these factors which play a significant ecological and evolutionary role in adult marine populations and their connectivity. Life history characteristics include adult mobility, breeding or broadcast spawning modes of reproduction, and larval development, motility and behavior. Abiotic (e.g., geographical and physical) factors include oceanic currents, tidal currents, eddies, and geographic location and history. These abiotic factors, especially geographic barriers, significantly affect dispersal, gene flow, and population connectivity (Treml et al. 2008; Rasmussen 2009).

The Meso-American barrier reef off the coast of Belize has a suite of small island-like cays, and large cuts (e.g., spur and grooves) in the forereef that provide an intriguing study environment to assess regional population dynamics of marine coral reef organisms. These geographic barriers are capable of restricting gene flow between populations and initiating reproductive isolation. For example, a group of Caribbean reef hamlets are speciating in full sympatry with extensive gene flow across macro- and micro geographic scales, where genes responsible for mate recognition, in vision and pigmentation, are maintained via a long scale linkage disequilibrium and driven by both assortative mating and natural selection (Hench et al, 2019). At a regional or intermediate spatial scale, however, there is a lack of information on the connectivity between local and global populations and the eco-evolutionary forces and that may be responsible for morphological, ecological, and/or genetic character state divergence (Edgar et al., 2017). The non-uniform ecological fragmentation of the Meso-American barrier reef may be driving high/pre-zygotic isolation in populations at variable spatial scales.

Bioluminescent ostracods (Myodicopida:Cypridinidae) that use luminescence for courtship have a wide geographic distribution in the Caribbean Sea (Morin, 2019) and produce one of the most complex courtship displays observed in marine systems (Morin, 1986; Rivers and Morin, 2008; Rivers and Morin, 2009). Multiple species of ostracods can be observed displaying in the same geographic region partitioning their mating displays in space and time (Gerrish and Morin, 2016). When geographically isolated, sister taxa maintain some display characteristics but the direction, timing of displays and habitat in which displays take place change (Gerrish et al., 2016; Gerrish, 2018: 18th International Symposium of Ostracoda, oral presentation). The dynamic multivariate

characters of their courtship display traits and spatio-temporal habitat use for mating creates a landscape for divergence on top of the already heterogenous ecological opportunity of the reef habitat. At the large scale, surveys throughout the Caribbean Sea (Panama, Belize, Puerto Rico, Roatan, Jamaica) indicate that each collection location has a unique assortment of species that differ in behavioral, morphological, and genetic characters (Gerrish et al., in prep). On the small scale, *Photeros annecohenae* (a grass-bed dwelling species), (Torres and Morin, 2007) shows strong population genetic structure across a 12 km range (Gerrish, 2008). Species are clearly delineated at 500-1000 km scales and population genetic structure can be identified at 0-12 km scales. However, population divergence resulting in speciation is likely happening somewhere between small and large spatial scales. The research presented here aims to (1) identify the geographic range at which morphological, behavior, and genetic trait change is occurring, (2) assess whether trait change is associated with barriers to gene flow or dispersal limitation, and (3) to evaluate how sympatric (behavioral isolation) and allopatric (geographic isolation) barriers of diversification apply to populations of *P. annecohenae*.

MATERIALS AND METHODS

Site Selection

Sample sites were selected within a 196 km range off the Meso-American barrier reef of Belize. Approximate locations were selected based on nautical depth contour maps in locations where seagrass habitats were visible on Google Earth (Moutainview, California) at distances 15-20 km apart. Upon arrival at locations, sites were selected to allow consistent depth (5-20 ft) and prominent seagrass, *Thallasia testudinum,* the preferred habitat conditions of *P. annecohenae*. Distance between all sites ranged from 9-184 km (Fig 1) and neighboring sites ranges from 9 – 17 km apart. Sampling locations between Middle Long Caye and Caye Caulker (3N and 4N) were assessed while diving at night but no courtship displays were observed in the appropriate habitats of two independent locations. Artificial lights from Belize City were highly visible at these locations and could have excluded luminescent ostracods from these seemingly

appropriate habitats. Video and specimens were collected from all sites during a single lunar cycle from May 1st to May 10th, 2018 (Table 1).

Figure 1. Sampling locations of *P. annecohenae* along the Meso-American barrier reef.

Table 1. Detailed sampling site descriptions of locations where collection of *P. annecohenae* took place on the Meso-American barrier reef of Belize, CA.

Site	Approximate latitude and longitude coordinates	Estimated *T. testudinum* cover (categorical density) and length (grass height in cm)	Depth (m)	Sampling date
Caye Caulker (4N)	17.731535, -88.017272	Medium high; 25-30	3	5/7/2018
Middle Long Caye (3N)	17.250760, -88.056294	Medium high; 25	3	5/5/2018
Gator Caye (2.5N)	17.190426, -88.061955	Medium high; 20 shallow, 25 deep	2-4	5/8/2018
Columbus Caye (1.5N)	17.101775, -88.026714	Medium low; 20	2	5/12/2018
Tobacco Caye (1N)	16.918507, -88.058218	High; 20	4	5/1/2018
South Water Caye (0SWC)	16.812549, -88.082698	Medium; 30	2	5/1/2018
South Cut (1S)	16.698827, -88.087278	Low; 25-30	4	5/2/2018
Gladdens Caye (2S)	16.480513, -88.030192	Low; 25-30	4	5/2/2018
Three Queens Caye (3S)	16.454876, -88.041352	Medium high; 30	7	5/3/2018
Ranguana Caye (4S)	16.327939, -88.153994	High; 30	6-7	5/3/2018

Tom Owens Caye (5S)	16.180289, -88.237580	Medium; 25	5	5/10/2018
Ragged Caye (6S)	16.100007, -88.286045	Medium; 25	4	5/10/2018

Animal Collection and Identification

We collected signaling male *P. annecohenae* during their bioluminescent signals and courtship displays by sweeping a hand net (125 µm) in the direction of the light display (Torres and Morin, 2007). At each site we swept 60 *P. annecohenae* displays following the protocol of Gerrish and Morin (2016). Live *P. annecohenae* individuals were sorted by their relative length: height ratio and keel shape (N =30) (Morin and Cohen, 2017). Individuals from each population were measured on a Nikon SMZ-800 microscope with an eyepiece micrometer at 50x magnification. Length and height measurements were taken from 30 individuals with keel and eye measurements taken from 15 of the same individuals (Gerrish and Morin, 2016; Morin and Cohen, 2017). The 30 measured and recorded individuals were then placed in 1.5 mL centrifuge tubes and preserved with 100% ethanol to serve as additional morphological diagnoses as needed. An additional 30 individuals were also preserved in 1.5 mL centrifuge tubes with 100% ethanol for genetic analyses.

Behavioral Analysis

Courtship displays of multiple individuals from each population were recorded using a Sony A7S low light camera with 25 mm Canon lens, recording in 4K on a Shogun recorder. The camera was housed in a Nauticam custom underwater housing with dome port (Settings: 30FPS; blue light; 124,000-260,000 iso; manual focus). A two foot meter

stick attached to the camera housing was used to keep video footage of displays in focus. Displays were included in analysis if clean first and last pulses were captured and in focus. 10 out of 30-100 recorded displays were analyzed for individual mating display characteristics (number of pulses, pulse duration, and inter-pulse interval). Pulse duration was quantified as time (seconds) from initial observed luminescence to no luminescence observed. Inter-pulse interval was quantified from the end of 1 pulse (no observed luminescence) to the start of the following pulse.

DNA Preparation and Genotyping

Male *P. annecohenae* from each population (N=12) (except for 1.5N Columbus Cay and 3N upward display) were dissected, and stomach contents were removed to reduce DNA contamination. Genomic DNA (gDNA) was extracted using the Qiagen protocol for blood and animal tissue (modified only in that the first elute was 100 µl instead of 200 µl). Extracted gDNA was quantified using the Qubit dsDNA HS assay kit (Invitrogen) and samples were only kept in the analysis if they yielded 3.5 ng/µl or higher concentrations of gDNA. Hind-III (New England Biolabs) digests were assayed on a 1% agarose gel to test the digestive quality of the DNA (i.e. size and ability to ligate [>95%]). Two 96-well plates with a total of 190 gDNA extracts (15-16 individuals x 12 sites) were prepared and sent to the University of Wisconsin-Madison Biotechnology Center (UWBC). Double-digest RAD-sequencing libraries were prepared by UWBC, with enzymes PstI and MspI selected based on *in vitro* optimization. Two libraries contained 95 individuals barcoded with a unique 4-9 nucleotide sequence. Single-read, 100-bp target length sequencing on two lanes of an Illumina HiSeq2500 platform conducted at the UWBC.

One library was demultiplexed using the *process_radtags* program in the STACKS bioinformatic pipeline (Catchen et al. 2013). Polymorphic SNPs were identified on reads truncated to 100 bp and filtered for overall quality. RAD loci were allowed a maximum of three nucleotide mismatches between two alleles of a heterozygote sample (M = 3) which was identified as an optimum threshold based on the method developed by Catchen et al. (2013). We selected a minimum stack depth of three to control for the number of identical reads required to initiate putative alleles (m = 3) among reads with variable sequences (*ustacks* module in Stacks, default parameters selected). Reads were then aligned de novo with each other to create a catalog of putative RAD tags (*cstacks* module in Stacks, default parameters selected). In the *populations* module of stacks we retained SNPs in 70%, 75%, 80%, and 85% of the individuals and sampling sites (r). Homeologs were excluded by removing markers showing heterozygosity >0.70 within samples. Polymorphisms with a minor allele frequency (MAF) > 0.05 on average across sampling sites were kept to avoid bias in baseline differentiation and reduce any sequencing error from the SNP data set. Sequences were also assembled using ipyrad v. 0.6.8 (Eaton, 2014). Reads were clustered within and between samples at 85% identity with 50% missing data, and all other parameters were set at default values. VCF (variant call format) files were generated from both STACKS and ipyrad to be used for statistical analyses in R v3.0.6. PCA (principal component analysis) and DAPC (discriminant analysis of principal components) analyses were run in R using procedures outlined by Tabima et al. https://grunwaldlab.github.io/Population_Genetics_in_R/TOC.html) and Fst was calculated using the stampp function in R (following procedures of Kolář 2017; https://botany.natur.cuni.cz/hodnocenidat/Lesson_05_tutorial.pdf)

Differences in character traits across sample sites were evaluated using one-way ANOVA analyses using R v. 3.6.0 on each morphological character trait (Length, Height, Eye, Keel) and one behavioral character trait (number of pulses). We performed repeated measure ANOVA analyses using Systat v. 4.0 on the remaining behavioral character traits (pulse duration and inter-pulse interval) across all sites to evaluate differences in character traits with nested properties (pulse 1-5 nested within pulse duration; start, middle, end nested within inter-pulse interval). Significant groupings were assigned based on sites that significantly differed at $p<0.05$ using Tukey's post-hoc tests.

Primer v. 6.1.10 was used to create Euclidean dissimilarity matrices for morphological character traits (L, H, E, K) (N = 15) and behavioral character traits (pulse duration, inter-pulse interval, number of pulses) (N = 10). We calculated geographical distance (N = 13) between each sample site using Google Earth (Mountainview, California) to create our geographic distance matrix. Behavioral character traits (pulse duration, inter-pulse interval and pulse number) were independently evaluated for dissimilarity then averaged together into one dissimilarity matrix. Our genetic matrix represents a matrix of pairwise Fst calculations based on our ipyrad data in R v. 3.6.0. To evaluate the relationship between geography, morphology, behavior, and genetic divergence, we performed a series of Mantel tests using GenAlEx v. 6.51 b2 (Mantel, 1967).

Results

Morphological traits (length, height, eye and keel) differed significantly between sites (all traits, $p < 0.05$, Tukey post hoc results of significantly different groups, $p < 0.05$, represented on graphs). In general, length and height decreased from south to north. The exception is site 4N which is more similar in size to the southern populations, 3S-6S.

Three significant groupings of sizes occur: (1) 6S-3S and 4N, (2) 2S-1N, and (3) 2.NU, 2.5ND, and 3N (Fig. 2A, B). Eye size was significantly greater than all other populations at site 3S and significantly smaller than all other populations at site 3N for the downward displaying individuals collected from that site (Fig 2C.).

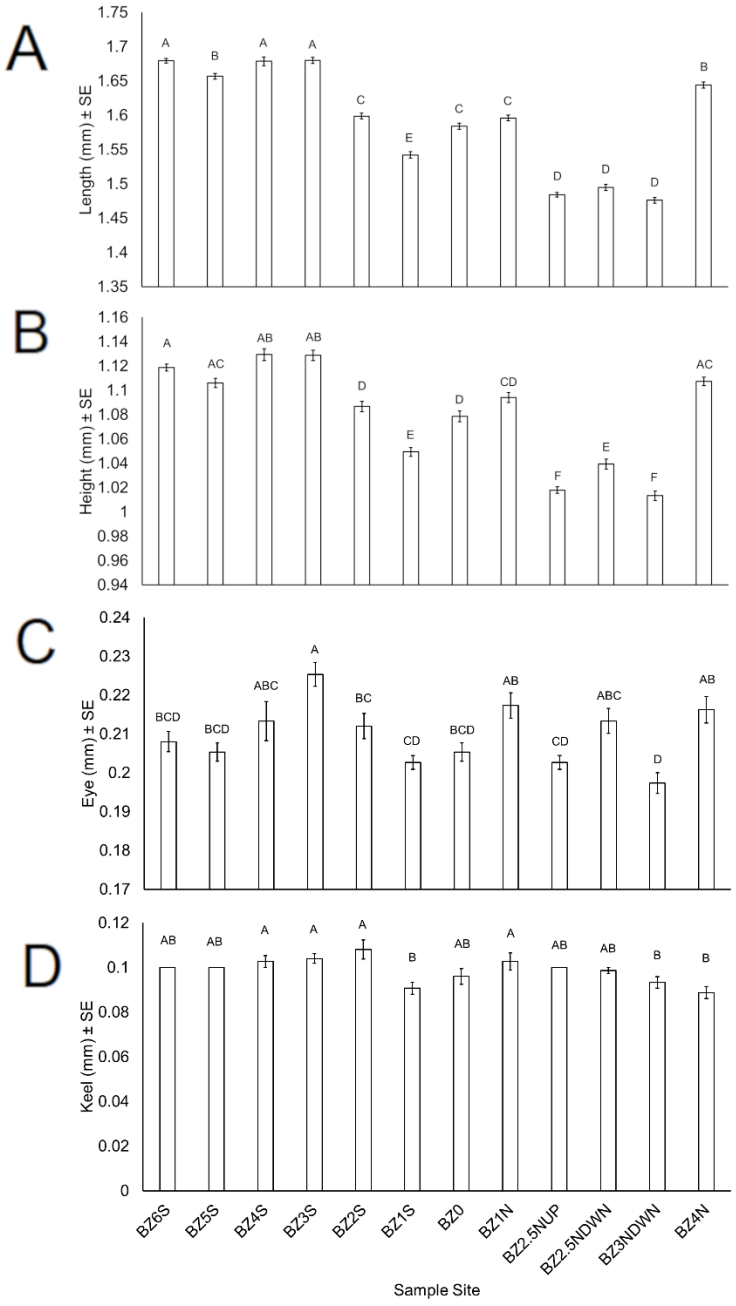

Figure 2. Mean measured morphological characters (mm) compared across sampling sites. (A) Length (N=30) ($df = 11$, $F = 307.1$, $p < 0.05$).; (B) Height (N=30) ($df = 11$, $F = 111.6$, $p < 0.05$).; (C) Eye (N=15) ($df = 11$, $F = 6.837$, $p < 0.05$).; (D) Keel (N=15) ($df = 11$, $F = 5.058$, $p < 0.05$). Letters indicate significant groupings assigned based on sites that differed significantly based on post-hoc Tukey analyses.

Differences in courtship display behaviors were observed during collections. At most sites, male-produced courtship displays that travelled in an upward direction and followed the general trend described by Torres and Morin (2007) and Rivers and Morin (2008). At sites 2.5N and 3N some individuals displayed in a downward direction and these displays occurred side-by-side with individuals displaying in an upward direction.

Downward displays began at the same height within the water as upward displays and often moved below the top of the seagrass blades as they travelled downward. At the southern sites (3S-6S) individuals occasionally "skipped" a pulse during the last half of their display in which there was a long (almost twice as long) inter-pulse interval between two pulses prior to the ending "trill" of their display. Courtship display traits (number of pulses, inter-pulse interval and pulse durations of displays) differed significantly across sample sites ($p<0.05$). The number of pulses/display was greatest at site 3N. Individuals from southern sites (2S, 4S-5S) produced significantly greater number of pulses/display than individuals from northern sites (Fig 3A). Inter-pulse interval values show similar trends to pulse duration. Sites 3S-6S, 1N and 4N had shorter pulse durations and inter-pulse intervals, while sites 1.5N-3N had the longest pulse durations and inter-pulse intervals, and sites 2S-0SW fell between these two extremes (Fig 3B, C). Pulse durations differed significantly from pulse 1 to pulse 5 ($p < 0.05$) with the first pulse having the longest duration and each sequential pulse generally decreasing in duration. Inter-pulse intervals differed significantly from start to middle to end ($p < 0.05$) with the first starting intervals being the greatest and the middle and end intervals being variable across sites. Since pulses and intervals were nested within sites for the repeated measure ANOVA, no interaction term was tested.

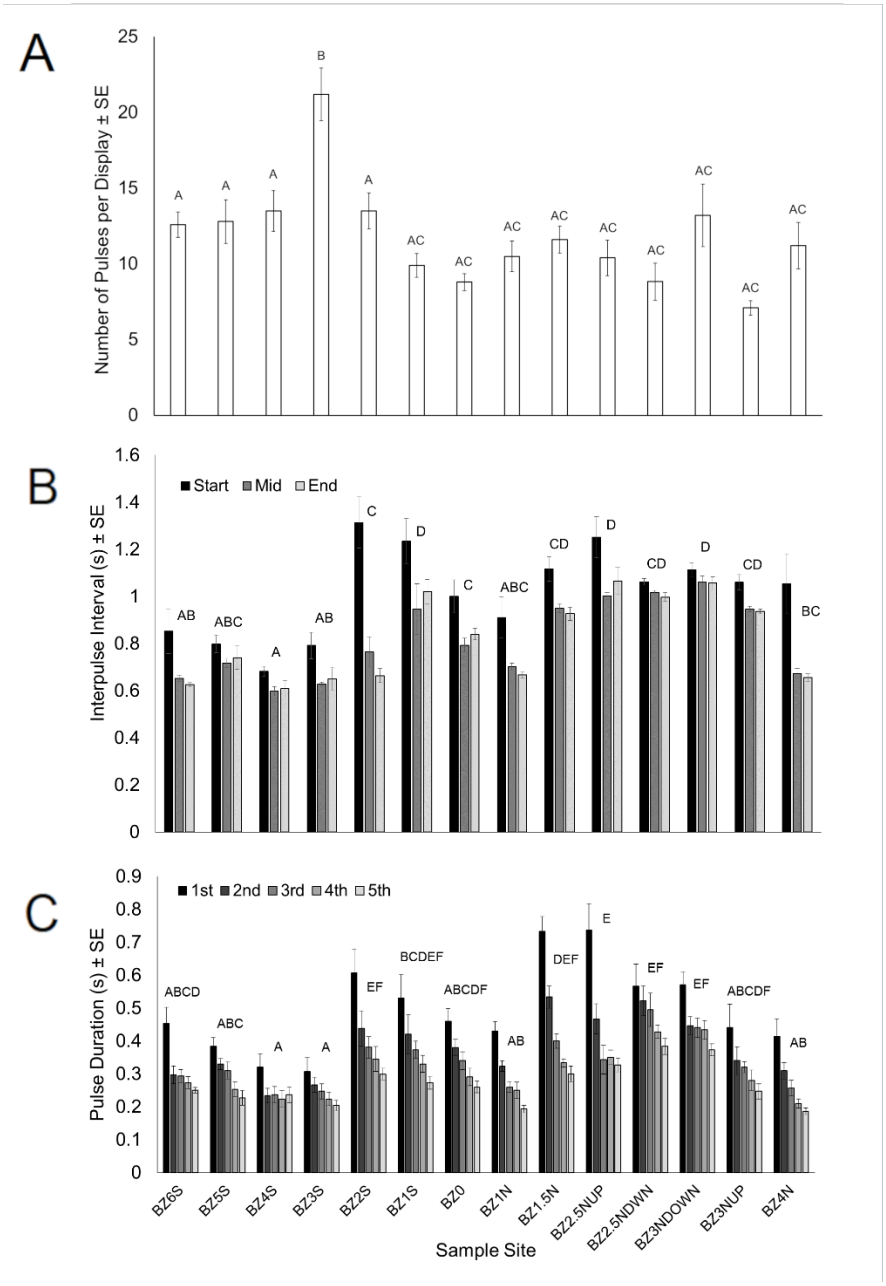

Figure 3. Mean courtship display behavior characteristics compared across sites; (N=10) for all sampling sites except BZ2.5NDWN (N=6) and BZ3NUP (N=5). (A) Number of pulses per display ($df = 13$, $F = 8.192$, $p < 0.05$); (B) Mean start, middle, and end inter-pulse interval ($df = 13$, $F = 27.017$, $p < 0.05$); (C) Mean pulse duration of pulses 1-5 ($df = 13$, $F = 27.651$, $p < 0.05$). Letters indicate significant groupings assigned based on sites that differed significantly based on post-hoc Tukey analyses.

Cluster dendrograms of morphological and behavioral similarities and paired Fst between all sites show two groupings which herein will be considered southern (3S, 4S, 5S, 6S) and northern (2S, 1S, 0, 1N2.5NUP, 2.5NDOWN, 3N, and 4N). The southern group consistently clustered together across all dendrograms (Fig. 4). The northern group consistently clustered into two sub-groups with a few anomalies. Sites 2S, 1S, 0, and 1N clustered together in the morphological and genetic dendrograms. While site 1N fell into the southern group of the behavioral dendrogram (Fig. 4A). The other sub-group, sites 2.5ND, 2.5NU, and 3N clustered together in all three dendrograms. In the morphological dendrogram they were the outermost group (Fig. 4B). Site 4N clustered in the southern group in the morphological and behavioral dendrograms and clustered in the northern group in the genetic dendrogram (Fig. 4). A dendrogram of courtship display traits (Fig. 4A.) show two clusters: (1) 6S, 5S, 4S, 3S, 1N, 4N; (2) 2S, 1S, 0, 2.5NDOWN, 2.5NUP, 3N. While the morphological character trait dendrogram (Fig. 4B) is similar, but not identical to genetic distance (Fig. 4C): (1) 6S, 5S, 4S, 3S, 4N (2) 2S, 1S, 0, 1N. Sites 3N, 2.5NUP, 2.5ND were the outermost cluster in the dendrogram and may be due to the fact that they were much smaller bodied than their far northern or southern counterparts at sites 6S, 5S, 4S, 3S, and 4N. Two clusters were identified in genetic distance (Fig. 4C), sites (1) 6S, 5S, 4S, 3S; (2) 2S, 1S, 0, 1N, 2.5NUP, 2.5NDOWN, 3N, 4N; suggesting strong differentiation between these two clusters.

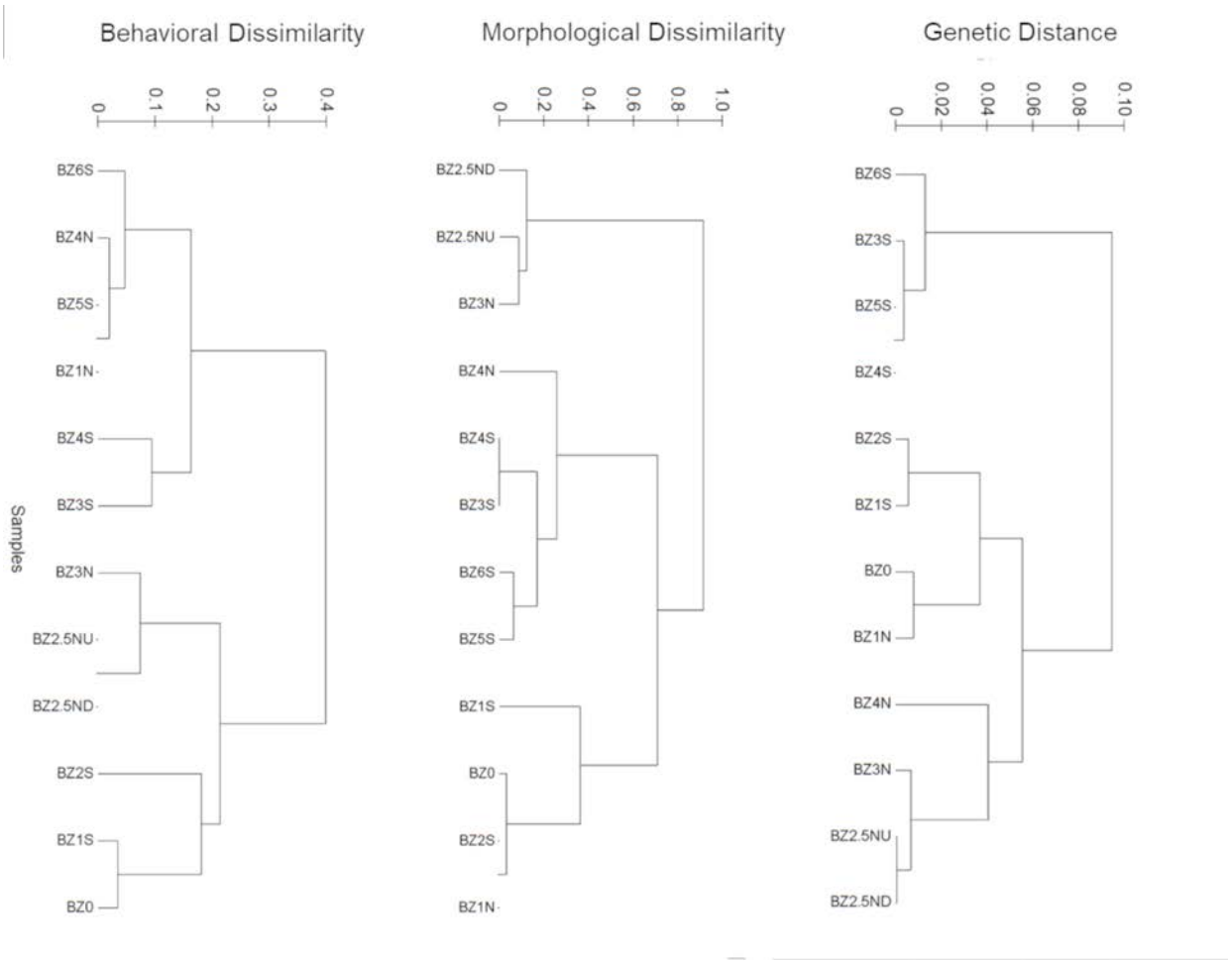

Figure 4. Cluster dendrograms based on Euclidean distance matrices. (A) pairwise dissimilarity of courtship behavior characters (N = 12); (B) pairwise dissimilarity of morphological characters (N = 12); (C) pairwise Fst (N = 12).

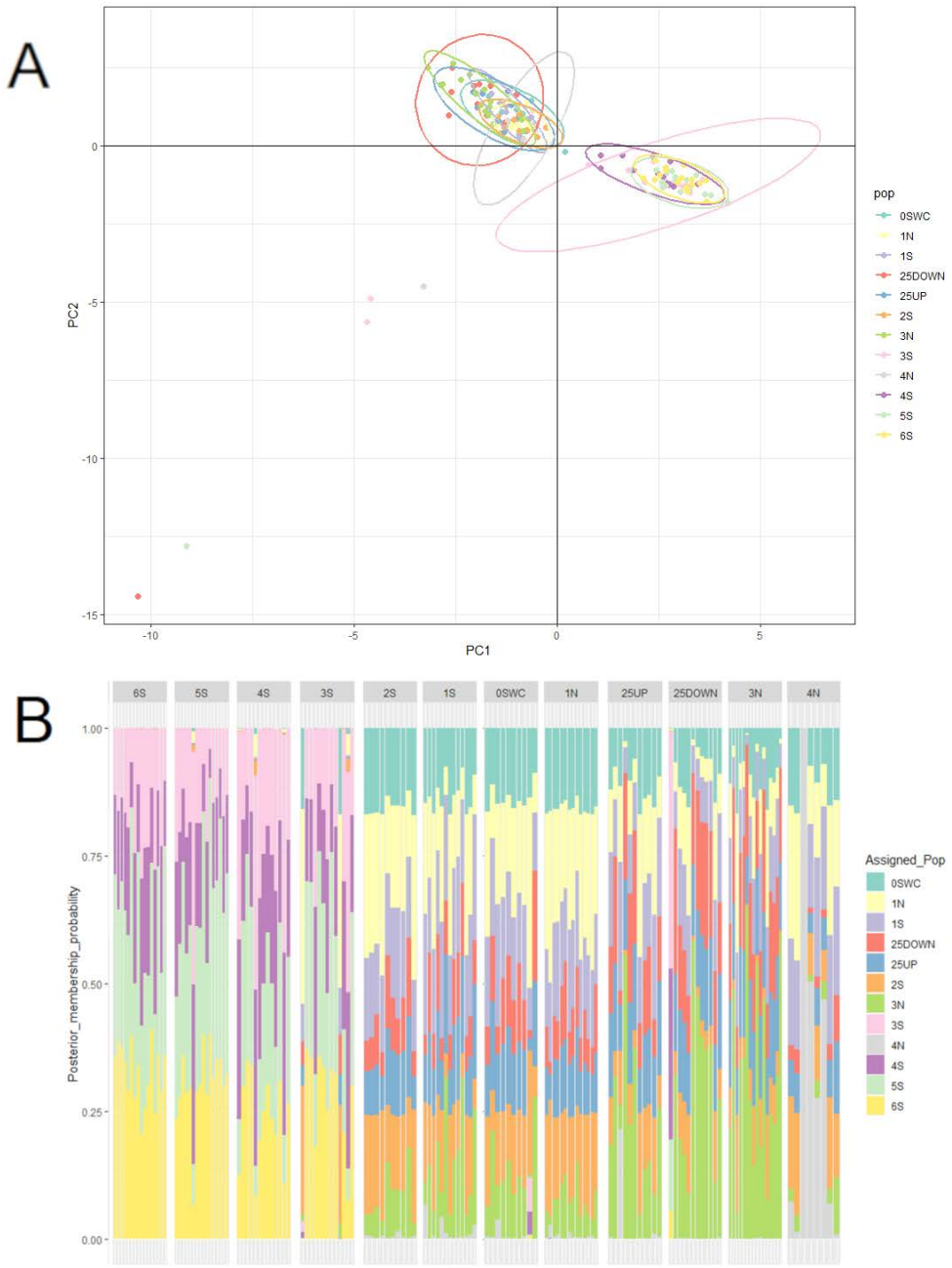

Figure 5. *Photeros annecohenae* population structure (except for individuals from Columbus Caye and upward displaying individuals from Middle Long Caye). (A) Principal component analysis (PCA) and (B) assignment probabilities (DAPC) using data from the ipyrad bioinformatic pipeline.

In total, we obtained an average of 2.75 + 0.99 (SD; standard deviation) million reads per individual, 2.74 + 0.98 million reads retained after quality filtering. We recovered 994 loci per individual which contained a total of 20 SNPs, after applying a <50% missing data filter. Using demultiplexed data in ipyrad individual population assignment (DAPC) and principal component analyses (PCA) support that the southern and northern populations of *P. annecohenae* are genetically diverging (Fig 5). DAPC and PCA using demultiplexed data in STACKS showed near identical results to ipyrad. Both populations present admixture among sites. The differences among the two populations are accompanied by consistent differences in morphology and courtship behavior. Site 4N had the lowest amount of admixture, which is most likely due to poor library preparation and is unlikely due to genetic differentiation from the other sites.

There is significant, strong correlation between genetic distance and geographic distance ($p = 0.01$, $R^2 = 0.6241$) (Fig. 6A) and significant, weak correlation between geographic distance and morphological similarity ($p = 0.02$, $R^2 = 0.095$) (Fig. 6B). There was no significant correlation between geographic distance and courtship display traits ($p = 0.100$, $R^2 = 0.0359$) (Fig. 6C). There is a significant, moderate correlation between genetic distance and morphological similarity ($p = 0.01$, $R^2 = 0.2155$) (Fig. 6E) and weak correlation between genetic distance and behavioral similarity ($p = 0.04$, $R^2 = 0.0644$ respectively) (Fig 6F). Sites with similar courtship displays are also morphologically similar ($p = 0.01$, $R^2 = 0.4562$) (Fig. 6D), suggesting a tight linkage between morphology and courtship behavior.

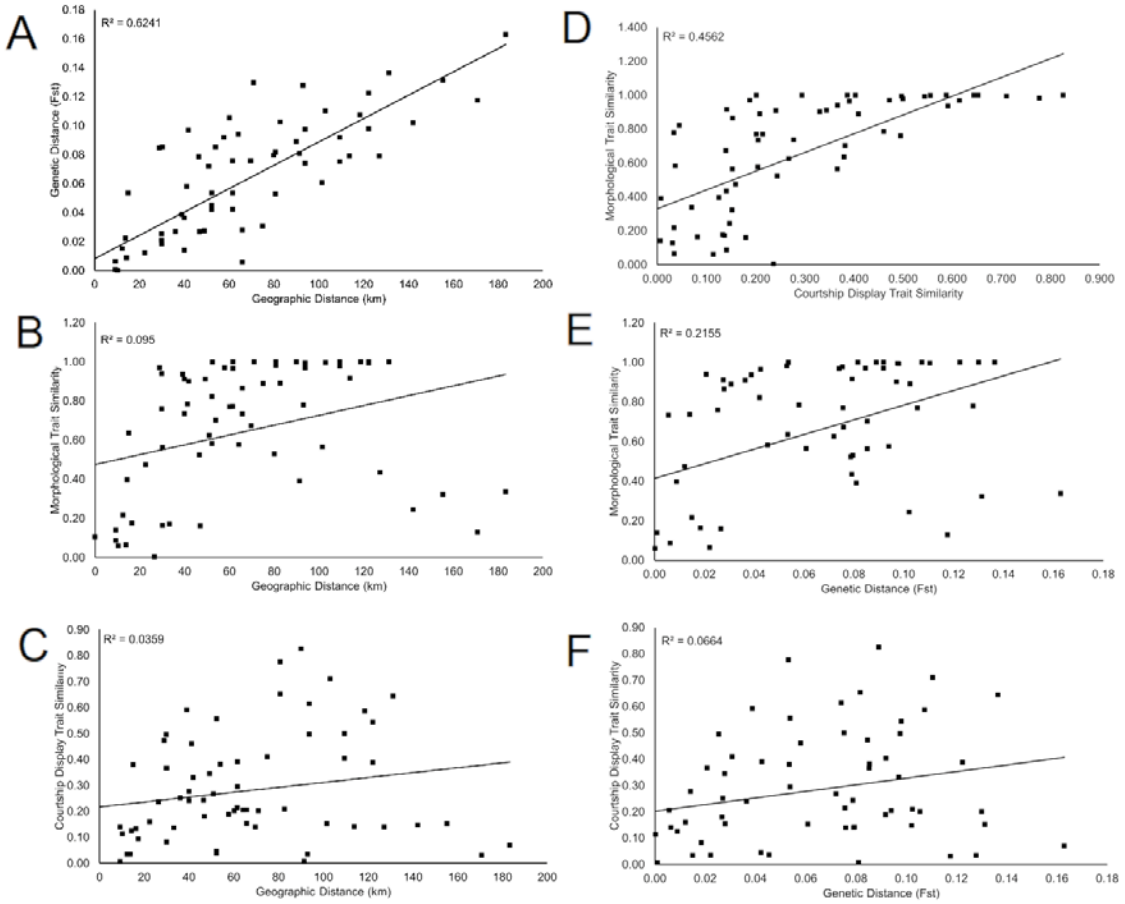

Figure 6. Mantel correlation plots; (A) genetic isolation by distance (y = 774.8x + 18.05, p = 0.01); (B) morphological trait similarity by geographic distance (y = 0.0025x + 0.4754, p = 0.02); (C) courtship display trait similarity by geographic distance (y = 0.0009x + 0.2175, p = 0.10); (D) morphological trait similarity by courtship display similarity (y = 1.1053x + 0.3306, p = 0.01); (E) morphological trait similarity by genetic distance (y = 3.7047x + 0.4137, p = 0.01); (F) courtship display trait similarity by genetic distance (y = 1.2566x + 0.2025, p = 0.04).

DISCUSSION

One of the first steps in identifying a speciation event is recognizing differences in phenotypic traits. Reproductive isolation occurs when diversification of morphology (e.g. colour morphology, genitalia) and/or behavior (e.g. frequency shifts in frog auditory display characteristics or flash signaling in fireflies) reduces mate recognition and/or preference. While morphological or behavioral changes can act independently to generate reproductive isolation, it is more common that both are observed in a divergence event. In the tropical hamlet fish genus *Hypoplectrus* only morphological color pattern differences define sympatric sibling species (Fischer, 1980). But, among sympatric species of the bluehead wrasse, *Thalassoma*, females recognize male mates based on their color pattern and chose their mates through species-specific mate recognition behaviors (Warner and Schultz, 1992). European flounders of the Baltic Sea exhibit two reproductive behaviors where one group reproduces through demersal spawning in a low salinity environment and the other utilizes pelagic spawning (Momigliano et al., 2017). We observe clear differences in phenotypes, both behavioral and morphological, for *P. annecohenae*. Pulse duration (Fig 3B), inter-pulse interval (Fig 3C), length (Fig 2A) and height (Fig 2B) all significantly change between sites 2S and 3S where a large gap in the reef may act as a geographic barrier to gene flow. And, these phenotypic changes take place in the same region where we observe significant genetic differences (Fig 5). While the genetics data for *P. annecohenae* supports observed phenotypic differences between sites 2S and 3S, the up vs. down behaviors at site 2.5N and morphological differences between 3N and 4N were not correlated with strong genetic differences. The lack of genetic signal may indicate that the observed phenotypic shifts are simply phenotypic variants within a

population that come and go over time. Or, the variants are recent enough that the associated reproductive isolation has not had time to yield a significant genetic signal.

In species that have complex courtship behaviors selection can act on multiple axes of variation in which slight deviations in character traits can result in reproductive isolation. Taxon that utilize sexual selection, specifically complex courtship displays, have some of the highest diversification rates (Ritchie, 2007; Ellis and Oakley 2015). The auditory call is an integral component for mate recognition systems in insects. To reduce courtship arena noise, sympatric communities partition their "acoustic niches" temporally (mean call time) and acoustically (e.g. frequency). Differences in insect "acoustic niches" or auditory courtship song is the main diagnostic tool to delimiting species when in sympatry (Tishechkin and Vedenina, 2016). In the acridid grasshopper genus *Chorthippus*, species are nearly identical in their morphology and in their habitat specialization, yet their acoustic signals are so distinct one can easily distinguish their courting song (Tishechkin and Vedenina, 2016). Similarly, sexual selection has been a major evolutionary force in lampyrid fireflies that use visual luminescent displays for courtship (Lewis and Cratsley, 2008). Firefly species are often delimited through differences in male genitalia and behavioral courtship signals (Green and McElroy, 1956; Lloyd, 1966). Behavioral courtship displaying fireflies follow a 'one habitat, one time, one signal, one species rule', providing more axes of divergence in space and time (Lloyd, 1981). Display trait differences are how researchers first catalogue and identify luminescent ostracod species in the field (Morin, 1986; Cohen and Morin, 2003; Rivers and Morin, 2008). Over a single reef habitat, luminescent courtship displays of male cypridinid ostracods from several species occur. Each species displays over a specific

microhabitat and the timing for initiation of displays for each species differs (Gerrish et al., 2016). During the display train each courting male dispenses a sequence of bioluminescent pulses as they swim through the water column. Most displays begin with an initiation phase where pulse durations are longer, followed by a series of pulses before the display ends with a rapid series of short pulses known as the trill phase (Morin, 1986; Morin and Cohen, 1991; Rivers and Morin, 2008). Intraspecific male courtship displays are driven by male-male competition and/or cooperation, a high operational sex ratio and two alternative mating tactics, entrainment and sneaking (Morin 1986; Morin and Cohen, 1990). Entrainment (Morin, 1986) occurs when non-signaling males cue off an adjacent signaling male and initiate a display in synchrony with or adjacent to the signaler. Sneaking is when a non-signaling male swims silently (without producing pulses of light) adjacent to a signaling male in hopes that he will intercept an incoming female that is receptive to the signalers display (Morin, 1986; Rivers and Morin, 2009). The sneaker will continue his tactic until the display train ends and then either become an initiator, an entrainer, or a sneaker (Rivers and Morin, 2009). Unlike with fireflies, female *P. annecohenae* do not luminesce in response to male signals but responds by redirection and swimming to intercept signaling males (Morin, 1986; Morin and Cohen, 1991, Rivers and Morin 2008). Rivers and Morin (2008) reported that *P. annecohenae* flash characteristics are highly conserved. In the findings reported here we observe differences up to 50% in inter-pulse interval and up to 40% in pulse duration between southern and northern populations (Fig. 3). These differences are similar to the findings observed between *P.annecohenae* and its sister taxa described in Belize, *P. morini* (Torres and Cohen, 2005; Torres and Morin, 2007; Gerrish and Morin, 2016). Beside differences in

standard display characters, pulse duration and inter-pulse interval, slightly aberrant behaviors were observed in isolated locations. During the display train, males seemingly skipped a pulse in the latter portion of their sequence of pulses, where the distance and inter-pulse interval (duration/time) between two sequential pulses were nearly twice as long as the prior and subsequent pulses .The reproductive benefit of this "skipped" pulse could potentially reduce "sneakers" intercepting an incoming female, the cost may be that the female also perceives the male has terminated their display train. This observation was not made in any northern sample sites. Display direction is one trait that often differs between closely related taxa (Gerrish et al., in preparation) and may provide a phenotypic shift that initiates species divergence. The direction flip observed at sites Gator Caye (2.5N) and Middle Long Caye (3N), could reinforce the fact the directional variation and pulse duration variation observed in *P. annecohenae* would encompass the traits observed for their sister-taxa *P. morini*, a clearly separate species found in the same geographic region.

While behavior plays an important role in reproductive isolation, divergent morphology can also act to reinforce post-mating reproductive isolation. For decades, taxonomy was limited to morphological characters for taxa descriptions. To date, morphology is still a useful tool to identify species-specific characters when paired with the evaluation of genetic and/or ecological traits. Barriers to reproduction occur when differences in morphology restrict copulation and the formation of zygotes. This concept comes from the lock and key hypothesis, where the structural differences in genitalia prevents the hybridization of species (Dufour, 1844). Lock and key reproductive isolation functions in two ways, which are not mutually exclusive: 1) structural differences result

in mechanical incompatibilities that reduce successful copulation (Eberhard, 1992) 2) differences in genital characters are recognized and one or both sexes produce a behavioral or physiological response to reduce reproductive fitness or eliminate mating attempts (De Wilde, 1964). Wojcieszek and Simmons (2012) found that divergence in genital shape and structure led to "lock and key" mechanical difficulties and reduced reproductive success when isolated populations of the millipede *Antichiropus variabilis* were experimentally mated. In the millipede genus *Parafontaria*, mismatched genitalia and body size differences were associated with the termination of copulation without insemination because of preliminary intromission failure (Tanabe and Sota, 2008). In some cases, male genitalia are more variable and evolve more rapidly than non-genitalia morphological traits as a result of sexual selection (Arnqvist, 1997; Hosken & Stockley, 2004; Eberhard, 2010). Male genital traits of 25 Caribbean *Anolis* lizard species were found to evolve ~six times faster than non-genital traits (Klaczko et al., 2015). Our data shows that there is a ~25% difference in length and height between the largest individuals of the southern population and the smallest individuals of the northern population. The difference between these two populations is comparable to the differences observed between *P. annecohenae* and *Photeros morini*, its Belize sister taxa (Torres and Cohen, 2005; Torres and Morin, 2007; Morin and Cohen, 2010; Morin, 2019). In addition to the large difference in overall size there may also be fine morphological differences between the two populations that act to reinforce reproductive isolation. Species within the genus *Photeros* have highly variable copulatory organs across species (Cohen and Morin 2010) and it has been suggested that they may play a role in mechanical reproductive isolation among luminescent species (Cohen and Morin, 1990).

Capturing speciation in action and delineating new species from populations is one of the most challenging aspects of evolutionary biology. Speciation patterns and processes for some model organisms, *Drosophila, Caenorhabditis elegans, Danio rerio, Mus musculus,* African cichlid fish, and three-spine sticklebacks, have been relatively well documented but testing speciation in non-model systems has remained difficult because of the breadth of required understanding. Next-Generation Sequencing (NGS) techniques are revolutionizing our ability to study divergence in non-model organisms. In the reef-building coral *Acropora* single-nucleotide polymorphisms were mapped to a genome to parse out phylogenetic relationships and build a foundation for resolving regions of the genome that influence spawning time (Porto-Hannes et al., 2014; Rosser et al., 2017). NGS is also providing for small non-model organisms, for which there is no reference genome, the ability to identify population-level genetic variation. Fine population structure was identified across a small spatial scale in the lotic diving water beetle *Exocelina manokwariensis* of New Guinea, that would otherwise go unnoticed using traditional markers (e.g. microsatellite loci). In divergent populations of the New Zealand marine isopod *Isocladus armatus*, a large number of high-quality SNPs were used to identify loci under putative selection for color (Wells and Dale, 2018). With the recent expansion and increased applications for NGS techniques our understanding of ecological and evolutionary processes has improved. As with application of any new methodology, there is still skepticism in the biological inferences being made (Vijay et al., 2012; Chaisson,Wilson & Eichler, 2015). Whether studies use reference-based or *de novo* approaches, the use of multiple downstream pipelines ensures a robust outlook on the population genetic and demographic inferences (Shafer et al., 2017). In this study we

applied two pipelines, iPyrad and Stacks, to our ddRAD sequencing data to evaluate population structure along a ~200km range for a non-model species. Both pipelines confirm that *P. annecohenae* is genetically diverging into two genetically distinct lineages and that there is an overall pattern of isolation by distance.

NGS tools can provide insights at multiple scales of genetic divergence using different analyses on a single data set. Whether identifying barriers to reproductive isolation through whole-genome scans or population structure, inferences made on geographic and genetic divergence are now becoming more accessible to non-model organisms. A principal target of investigation in genomic speciation research is identifying the barrier loci involved in reducing gene flow and causing reproductive isolation (Ravinet et al., 2018). Whole-genome sequencing has identified multiple genomic regions of high differentiation across the genome of marine and freshwater three-spined stickleback ecotypes (*Gasterosteus aculeatus*) (Jones et al., 2012), greater mean Fst values between geographically separated populations of walking stick ecotypes (*Timema cristinae*) (Soria-Carrasco et al., 2014), and higher differentiation and lower gene flow at loci under divergent wing patterns of *Heliconius sp.* butterflies (Martin et al., 2013). Reduced-representation NGS techniques have identified lower overall genetic differentiation (Fst) of sympatric *Helianthus* sunflowers (Renaut et al., 2013), identified positive correlations of mean Fst values, outlier region size and linkage disequilibrium with morphological differentiation in benthic and dwarf limnetic whitefish ecotypes (*Coregonus clupeaformis*) (Gagnaire et al., 2013), and found barrier loci associated with population level differentiation between annual and perennial yellow monkeyflower ecotypes (*Mimulus guttatus*) (Twyford and Friedman, 2015). With increased use of reduced-

representation techniques, the framework for understanding the ecological and evolutionary mechanisms of speciation based on these types of data is improving. Ravinet et al. (2018) proposed a road map for elucidating genomic landscapes of species in 6 steps: (1) know the study system by understanding the ecology, reproductive biology, life history strategies, and geographic distribution; (2) establish the extent of gene flow and understand the demographic history by sampling a study system where divergent populations or species meet; (3) capture the best possible picture of the genomic landscape through NGS *de novo* or whole-genome assembly; (4) measure genomic factors that contribute to landscape differentiation; (5) reliably identify potential signatures of divergent selection or candidate barrier loci, while taking modifying factors into account; (6) use evidence independent from genomic data by directly testing for signatures of selection on a given locus or genetically map linkage between genotype and phenotype. Our study captured steps 1-3 and provides an opportunity to identify potential morphological and behavioral barrier loci under divergent selection in luminescent ostracods of the Caribbean Sea.

The highly connected yet heterogenous structure of coral reef systems provides complexity when considering the genomic landscape of reef organisms. For marine metapopulations, extrinsic barriers to gene flow are assumed permeable (Bowen et al. 2013) and should exhibit low levels of genetic differentiation (Lessios, 1998). Many studies support this prediction within the Mesoamerican barrier reef system where evidence supports high gene flow in metapopulations of corals (Porto-Hannes et al., 2015), fishes (Hepburn et al., 2009), lobster (Truelove et al., 2015), and across many other taxa. The concept that the ocean is homogenous challenges our understanding of the

origin and maintenance of distinct genetic lineages. Yet there is still growing evidence that there are distinct genetic lineages across many marine taxa (Payo et al., 2013) and across small to large spatial scales (Payo et al., 2013; Martinez-Takeshita et al., 2015). There are multiple lines of evidence suggesting that courtship displaying bioluminescent ostracods of the Caribbean Sea are exhibiting increased differentiation. The first line of evidence is the large number of undescribed species being uncovered within this group (Morin 2019). Secondly, the level of genetic structure paired with behavioral and morphological differentiation observed in this study at a small spatial scale suggests strong reproductive isolation and the potential for increased population divergence. Although there is support for a pattern of genetic isolation by distance (IBD) among sampled populations, we suggest that isolation by barrier (IBB) has had a strong influence on the genetic, morphological, and behavioral break identified between Gladdens Caye (2S) and Queens Caye (3S). The IBB observed between these two sites may due to a large cut in the forereef causing an intense ocean current. This heavy flow prevents the establishment of coral structures and associated fauna. The potentially large cut may further reduce the already limited dispersal capacity of *P. annecohenae* and promote population divergence between these two sites.

CONCLUSION

Our understanding of the multi-faceted interactions between evolutionary processes (e.g. barrier loci) and ecological opportunity are beginning to unravel as new genomic technologies arise. Furthermore, NGS tools are make it accessible for non-model systems to ask the primary questions in ecological and genomic speciation. Here we have strong evidence suggesting that because we observed *P. annecohenae* diverging in morphology, behavior, and genetics at a short geographic scale acting on multiple barriers to gene flow, there is an origin event occurring along the speciation continuum.

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