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RESEARCH ARTICLE

Phylogeography and genetic diversity of the commercially-collected Caribbean blue-legged hermit crab (*Clibanarius tricolor*)

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Abstract

Shifts from ornamental fish tanks to functional reef ecosystem aquaria has led to unprecedented pressure on marine invertebrate fisheries. The Caribbean blue-legged hermit crab (*Clibanarius tricolor*) is among the species targeted for functional reef tanks, valued for its role as an aquarium cleaner. Little is known about the biology of the species or the genetic landscape in which the increased collecting is happening. Here, we investigate the phylogeographic history and genetic diversity of *C. tricolor* through analysis of mitochondrial (CO1, 16S) and nuclear (H3) DNA. We test whether phylogeographic breaks for other invertebrates structure the genetic diversity of *C. tricolor* and explore additional factors that may govern structure, such as reproductive strategy, life history, habitat preference, adult mobility, demographic history, and vicariance events. Based on these three markers, we find high genetic diversity and connectivity and find no evidence to support the tested barriers as relevant to gene flow for *C. tricolor*. Rather, mitochondrial and nuclear markers infer high genetic diversity, panmixia, and demographic expansion during the Pleistocene. Our finding of panmixia makes it difficult to identify source or sink populations, but the absence of hierarchical structure inferred from mtDNA and nuDNA markers we use, high levels of genetic diversity and homogeneity for these same markers, and advantageous life history traits suggest *C. tricolor* is not currently at special risk; however, geographically restricted haplotypes and limitations within our study prevent us from making a strong conclusion about the sustainability of the fishery. Our work on the Caribbean blue-legged hermit crab highlights the importance of acquiring basic information on exploited species and reiterates that common regional forces may not equally impact connectivity among co-distributed species.

Keywords *Clibanarius tricolor* · Marine invertebrate · Phylogeography · Aquarium trade · Caribbean · Population genetics

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Introduction

Analysis of genetic data can reveal historic and contemporary factors responsible for the geographic distribution of intraspecific genetic variation (Avise et al. 1987). For marine animals, these approaches have the power to counteract the difficulties of studying small, highly mobile organisms in a fluid environment in which barriers to gene flow are difficult to recognize. Historically, the absence of conspicuous barriers and the presence of a pelagic larval stage were presumed to slow genetic differentiation and provide high potential for passive dispersal and population connectivity for many marine organisms (Palumbi 1994). However, phylogeographic analyses of diverse marine taxa have contradicted this view, finding gene flow structured in ways that do not correspond to currents or that suggest barriers to dispersal (Palumbi 1994; Taylor and Hellberg 2006; Sa-Pinto et al. 2012; Titus and Daly 2015; DeBiasse et al. 2016). Factors

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suspected to influence structure and dispersal include life-history traits, ecological requirements, vicariance events, and oceanographic processes (reviewed in Baums et al. 2006; Ludt and Rocha 2015). While some factors are well studied for some species, information for numerous others are lacking (Calado et al. 2003). This paucity of data is of particular concern for exploited species, as identifying the processes influencing gene flow between intraspecific populations is essential for designing management plans that are effective and sustainable.

Among the most vulnerable and exploited species are those collected for the aquarium trade. Little is known about the impact of harvesting for most commercially collected species, as stock assessments, sustainable harvest levels, and data on population dynamics and life history are inadequate or remain unknown (Calado et al. 2003; Dee et al. 2014). Recent shifts in the industry from ornamental fish tanks to functional reef ecosystems has led to unprecedented pressure on the marine invertebrate fishery (Rhyne et al. 2009; Murray et al. 2012): the West Atlantic has experienced a two-fold increase in invertebrate catch size from 1994 to 2007, with 9 million invertebrates, including 6 million ecologically important grazing invertebrates, collected from Florida's coasts alone. This has unleashed a wave of concern within the scientific community, as culture techniques and vital data on larval biology of a majority of these newly targeted species are unknown (see Calado et al. 2003). Without proper understanding and sustainable management of the newly targeted, yet poorly studied invertebrate species, overexploitation, collapse, and altered community structure (e.g. shifts to 'unhealthy' algae-dominated reefs; predators changing prey; loss of biodiversity, etc.) are potential outcomes (Calado et al. 2003; Dee et al. 2014).

The Caribbean blue-legged hermit crab (*Clibanarius tricolor*) is among those invertebrate species newly targeted by the aquarium trade, prized for its role as an aquarium cleaner (Rhyne et al. 2009). *Clibanarius tricolor* is a small (cephalothorax length: 6–9 mm) hermit crab found throughout the West Atlantic Ocean and Caribbean Sea (Provenzano 1959) (Fig. 1). Their bright blue color, occurrence in accessible habitats, and their dietary breadth makes them an appealing aquarium pet (Provenzano 1959; Hazlett 1966).

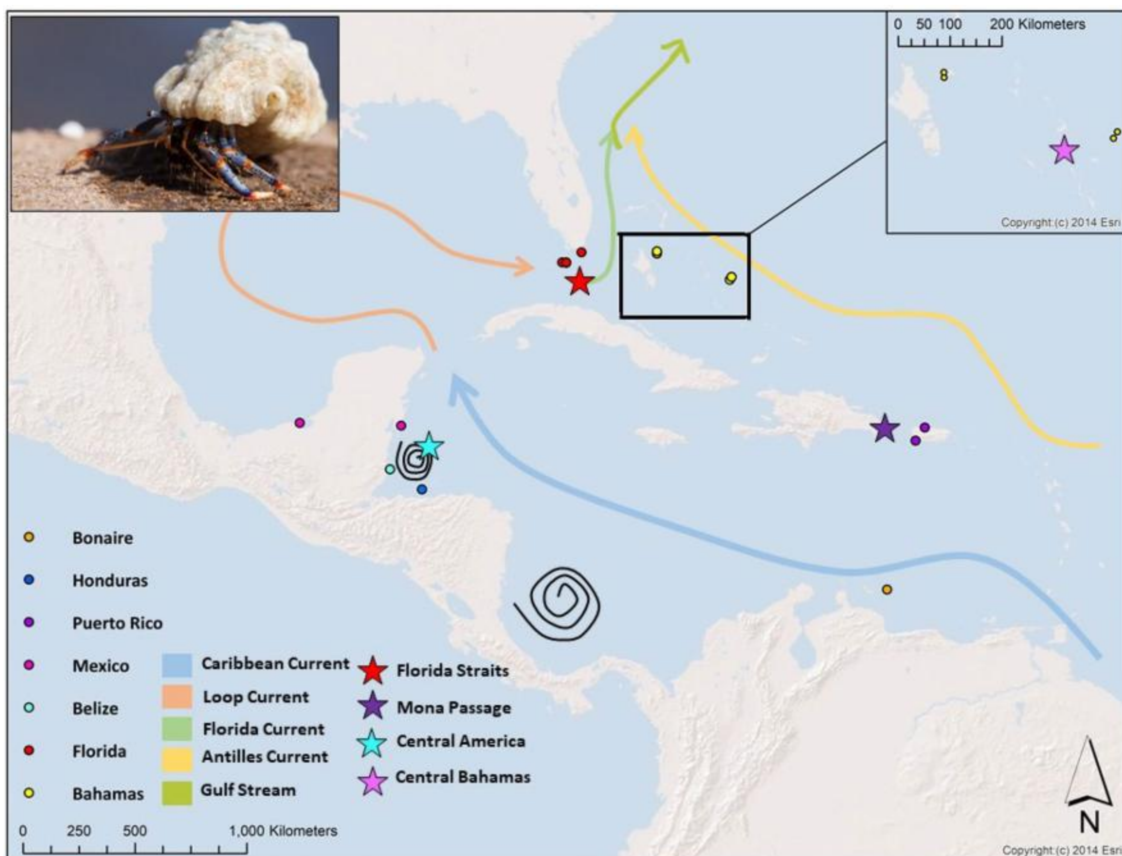

Fig. 1 Collection localities of *Clibanarius tricolor* (Caribbean blue-legged hermit crab). Major currents driving circulatory patterns in the Caribbean and Tropical West Atlantic are represented with colored arrows. Previously identified phylogeographic breaks are represented with colored stars and gyres of interest are represented with swirl icons. Details about collection localities can be found in Table 1. Photo credit: Matt Baskett

They fulfill the same ecological role as grazers in tanks as in natural ecosystems, where they provide resilience against algae overgrowth on reefs (Hazlett 1966; Denny and Gaines 2007; Rhyne et al. 2009). Although most abundant in the shallow rocky intertidal and subtidal [< 3 m] zones, they also inhabit *Thalassia* sp. seagrass beds and mudflats (Hazlett 1966; Bauer 1985).

The functional and aesthetic value of *C. tricolor* in ornamental aquaria has led to them being one of the most heavily traded marine invertebrate species in the western Atlantic (FWC landing data, 2003–2016; Matos-Caraballo and Mercado-Porrata 2008; Baeza and Behringer 2017; Rhyne et al. 2017; Aquarium Trade Data—Marine Aquarium Biodiversity and Trade Flow: https://www.aquariumtradedata.org/). Despite this, only Florida has a record of regulations on their collection outside of established protected areas (Crabtree and Schwaab 2011). The Florida Fish and Wildlife Conservation Commission requires specific permits for commercial collection and current daily bag limits for *C. tricolor* are one quart per person or vessel (whichever is less) (Florida Department of State 2010). Although Florida has established limits, lack of monitoring and enforcement leave room for over-collection and false landing reports (Thornhill 2012; Dee et al. 2014). In addition, unsuccessful attempts to rear them in captivity and the lack of size restrictions or seasonal closures on the fishery further increase pressure on wild populations (Calado et al. 2003; Rhyne et al. 2009). Despite their importance and economic value, little is known of the early life history traits (e.g. larval dispersal period), genetic diversity, or population structure of *C. tricolor* (Calado et al. 2003; Baeza and Behringer 2017). Because no previous studies on population dynamics or structure exist, the effects of heavy collection on wild *C. tricolor* populations remain unknown.

Within the range of *C. tricolor*, phylogeographic patterns and connectivity have been primarily defined by largescale vicariance events like the formation of the Isthmus of Panama, and oceanographic processes like hydrodynamic currents (Palumbi 1994; Lessios 2008). The major currents in the Caribbean and tropical West Atlantic (Fig. 1) are unidirectional and expected to aid in the passive dispersal of pelagic larvae, playing a significant role in distribution patterns for marine species (Cowen et al. 2007; Andras et al. 2013). However, historical factors and smaller-scale oceanographic processes can also disrupt connectivity and cause fine- and large-scale genetic discordance (Lobel and Robinson 1986; Palumbi 1994). Previous studies have identified several key phylogeographic breaks in the region. For example, the Florida current runs north between the Florida peninsula and the Bahamas; this fast current over deep water may create a barrier to dispersal between Florida and the Bahamas, despite being a mere ~ 300 km away (Baringer and Larsen 2001; Carlin et al. 2003; Lee and Foighil 2004; Andras et al. 2013; Debiasse et al. 2016). Within the Bahamas, a proposed central Bahamas break runs east of Long Island and west of San Salvador Island and is theorized to be due to lowered sea level during the Pleistocene and gyres in the Exuma Sound (Taylor and Hellberg 2006). Approximately 2000 km away lies another well supported break, the Mona Passage, where a deep, swift current and seasonal small-scale eddies support an Eastern-Western division in the broader Caribbean (Baums et al. 2006; Taylor and Hellberg 2006; Díaz-Ferguson et al. 2010; Foster et al. 2012; Andras et al. 2013; Debiasse et al. 2016). In Central America, isolated populations in Honduras Bay and significant genetic differences between populations in Belize, Honduras and Panama compared to the wider Caribbean are probably caused by seasonal freshwater influx from rivers, deep water between adjacent reefs, and gyres off the shores of Panama and Colombia and in Honduras Bay (Foster et al. 2012; Andras et al. 2013; Debiasse et al. 2016). Identifying genetic breaks and understanding the complex processes influencing connectivity and divergence for *C. tricolor* and for other exploited marine taxa are fundamental for developing sustainable management plans. Here we investigate the genetic structure and phylogeography of *C. tricolor* through analysis of mtDNA and nuDNA sequence variation to understand (1) the degree of diversity and connectivity in contemporary *C. tricolor* populations, and (2) what phylogeographic breaks have contributed to its population structure. Answering these questions will provide basic information on the genetic structure of this heavily traded, yet poorly studied organism; knowledge that is vital for its sustainable management (Calado et al. 2003; Baeza and Behringer 2017). We investigate (2a) if phylogeographic breaks found in *C. tricolor* coincide with previously identified breaks for other coral reef species within the Caribbean basin and west Atlantic Ocean (i.e. Florida Straits, Mona Passage, Central America, central Bahamas) and (2b) explore additional factors that may govern phylogeographic structure.

Materials and methods

Sample collection

Specimens of *C. tricolor* from 16 sites to 7 countries (n = 167, Table 1; GenBank accession numbers: Appendix, Table 1) were assessed to infer genetic diversity, population connectivity, and phylogeographic structure. All specimens were collected by hand and via shallow SCUBA diving (3 m) and preserved in 100% EtOH for genetic analysis at The Ohio State University. Sample collections were approved by the Florida Fish and Wildlife Commission (# SAL-17-1919-SR), the NOAA Office of

Table 1 Detailed collection information for specimens used in Sanger sequencing analysis (n = 167), including site ID and number of specimens per sample location

Population	Pop site ID	n
Florida	FL	41
Lower Keys (LK)	LKFL	15
Bahia Honda (BH)	BH	15
Middle Keys (MK)	MKFL	13
Duck Key (DK)	DK	11
Sombrero Key(SK)	SK	2
Upper Keys (UK)	UKFL	13
Harry Harris BP (HHBP)	HHBP	13
Bahamas	BHS	37
New Providence Island	NPI	17
Coral Harbor (NPI)	CH	8
Rock Point (NPI)	RP	9
San Salvador Island	SSI	20
Fernandez Bay (SSI)	FB	9
North Point (SSI)	NP	11
Puerto Rico	PR	27
Arecibo (PRA)	PRA	15
Laurel (PRL)	PRL	12
Bonaire	BON	10
Lagun Bonaire		10
Honduras	HON	27
Coral View Resort (CV)	CV	13
Eco Marine Diver (EMD)	EMD	14
Belize	BEZ	4/9
Wee Wee Key (WWK)		4/9
Mexico	MX	16
Yucatan (YUC)	YUC	10
Mahahual (MAH)	MAH	6

For the purpose of analyzing proposed phylogeographic breaks, specimens from geographically close regions were grouped together (e.g. Coral Harbor and Rock Point are both within New Providence Island, Bahamas). For Belize, nuDNA H3 was successfully sequenced for four out of nine specimens. GenBank accession numbers can be found in the Appendix Table 1

National Marine Sanctuaries (# FKNMS-2017-067), the Mexico's Comision Nacional de Acuacultura y Pesca (CONAPESCA-SEMARNAT PPF/DGOPA-294/17), the Bahamas Department of Marine Resources (# MAMR/FIS/17), the College of Veterinary Doctors of Honduras (cert#68182), and the National Health and Food Safety Service of the Government of the Republic of Honduras. Specimens from all other sites were provided by colleagues at various institutions and museums via loans or gifts (see Acknowledgments). Specimens collected by the authors are vouchered at the Florida Museum of Natural History.

DNA extraction and marker selection

Hermit crabs were removed from their gastropod shells with a small vise. DNA extractions from abdominal tissue followed manufacturer protocols for the DNeasy Blood and Tissue Kits (QIAGEN Inc.). DNA extractions were visualized on a 0.6% agarose gel and stored in a − 20 °C freezer pending amplification and sequencing.

We determined through preliminary work that traditional mitochondrial (CO1, 16S-rDNA) and nuclear markers (H3) were sufficient for capturing genetic variation and structure in *C. tricolor*, and that more expensive high throughput sequencing methods (e.g. ddRAD) were not necessary for the scale of this study (Stark, 2018). In brief, the patterns we saw with preliminary ddRAD for samples from Florida and the Bahamas did not suggest that there was additional, cryptic variation in *C. tricolor*. Pursuing genomic methods would have reduced the geographic scope of the work to only those populations sampled recently, as these methods require higher quality material (i.e. disqualifying inclusion of museum specimens) and cost more than PCR-directed Sanger sequencing, which would have limited the number of samples that could be included. Further, our mtDNA and nuDNA markers show high individual-level diversity, which emphasizes both their appropriateness for the questions being asked and the importance of sampling more individuals and populations to uncover population connectivity (Nei and Chesser 1983; Kalinowski 2005) and thus to understand patterns of dispersal and gene flow in this commercially and ecologically important hermit crab. The three markers chosen, mitochondrial Cytochrome C Oxidase subunit 1 (CO1), mitochondrial 16S-rDNA, and nuclear Histone 3 (H3) have been used extensively in studies of crustacean population diversity (e.g., Porter et al. 2005; Zakšek et al. 2009; Malay and Paulay 2010; Negri et al. 2012; Titus and Daly 2015; Veglia et al. 2018; Guzik et al. 2019; Palacios Theil and Felder 2019; Ni et al. 2020; Nishikawa et al. 2021).

The highly variable CO1 gene, which has been proposed as a universal 'barcoding' gene for molecular species identification, generally evolves at a rate that differentiates between intra- and inter-specific genetic variation, allowing distinction between closely-related species, as well as recently diverged intra-specific phylogeographic groups (Hebert et al. 2003; Chu et al. 2009; Morhbeck et al. 2015). The slower-evolving 16S-rDNA gene is commonly used in addition to CO1 because of its ability to resolve higher level relationships and thus to contextualize species-level diversity within a broader phylogeny (Bilodeau et al. 2005; Porter et al. 2005; Chu et al. 2009; Malay and Paulay 2010; Negri et al. 2012; Titus and Daly 2015).

Although mitochondrial DNA is a valuable tool for identifying cryptic structure and assessing phylogeographic history (reviewed by Bowen et al. 2014), mitochondrial

data are limited because the mitochondrion is a single, non-recombining locus that may be vulnerable to directional evolutionary changes (Naylor and Brown 1998; Shaw 2002; Ballard and Whitlock 2004). To address these limitations, we sequenced the conserved nuclear Histone 3 (H3) gene, as previous studies (Porter et al. 2005) have shown that it is suitable for resolving decapod taxonomic relationships.

Each 25-mL PCR reaction consisted of a GE Healthcare illustra™ puReTaq Ready-To-Go PCR bead (stabilizers, BSA, 250-mM of each deoxyribonucleotide triphosphate: dATP, dCTP, dGTP, dTTP, 10 mM Tris–HCL, 50 mM KCl, 1.5 mM $MgCL_2$, and ~ 2.5 U of puReTaq DNA polymerase & reaction buffer), 0.5 µL of each primer, 22 µL of autoclaved H_2O, and 2 µL of template DNA. PCRs were completed in an Eppendorf Mastercycler. Protocols for amplification followed Folmer et al. (1994) and Santos (2006) (CO1); Titus and Daly (2015) (16S); and Malay and Paulay (2010) and Pérez-Losada et al. (2004) (H3). Samples were sequenced in both directions via automated Sanger sequencing at TACGen DNA Sequencing in Richmond, CA, USA. Sequences were viewed and edited in Geneious 7.1.9 (http://www.geneious.com, Kearse et al. 2012). To verify that we had not sequenced nuclear pseudogenes, the CO1 sequences were translated into amino acids to detect any stop codons within the frames. Consensus sequences with ≥ 90% HQ for mtDNA and ≥ 80% for nuDNA were aligned using ClustalW with default parameters (Thompson et al. 1994). After alignment, CO1 and 16S sequences were concatenated and analyzed together because mtDNA is a single locus. Mitochondrial and nuclear DNA were analyzed separately and together in multilocus analyses.

Genetic diversity and structure

We estimated standard genetic diversity metrics for mtDNA and nuDNA markers in DnaSP 5.1.0 (Librado and Rozas 2009) following Nei (1987). We constructed haplotype networks in TCS1.21 (Clement et al. 2000) to visualize the relationships between haploid genotypes for CO1 and 16S mtDNA markers. We assessed genetic differentiation and structure among populations in ARLEQUIN 3.11(Excoffier et al. 2005) and Genepop 1.2 (Raymond and Rousset 1995) using pairwise ΦST estimated between populations defined by sample site and country of collection. Models of nucleotide substitution for analyses were selected in jMODELTEST 2.1.10 (Darriba et al. 2012) using the Akaike Information Criterion (AIC). The AIC identified General Time Reversible + Invariable sites + Gamma distribution (GTR + I + G) (Felsenstein and Churchill 1996) as the best-fit model for mtDNA and TPM2 (Kimura 1981) as the best-fit model for the nuclear H3 gene. Because these models are not available in ARLEQUIN, we chose the most similar model available, Tamura and Nei (TrN: Tamura and Nei 1993). ARLEQUIN input files were created in DnaSP or manually.

We used analyses of molecular variance (AMOVA, Excoffier et al. 1992) to evaluate the hierarchical subdivision of genetic diversity within populations (ΦST), among populations in the same geographic region (ΦSC), and between geographically separated populations (ΦCT). For mtDNA and nuDNA, we defined groups a priori to test our phylogeographic break hypotheses: the Florida Straits, Mona Passage, Central America, and central Bahamas. We tested geographic breaks proposed in previous studies of the region, with sites of collection grouped into one of two populations on either side of each proposed break (Baringer and Larsen 2001; Carlin et al. 2003; Lee and Foighil 2004; Baums et al. 2006; Taylor and Hellberg 2006; Díaz-Ferguson et al. 2010; Foster et al. 2012; Andras et al. 2013; Debiasse et al. 2016). AMOVA tests were run in ARLEQUIN and computed with molecular distance based on TrN distances among haplotypes. Pairwise ΦST and AMOVA analyses (ΦST, ΦSC, ΦCT) were performed using 10,000 permutations.

We conducted spatial analyses of molecular variance (SAMOVA) tests for mtDNA and nuDNA using SAMOVA 2.0 software (Dupanloup et al. 2002). SAMOVA tests can detect genetic barriers by defining geographically homogenous populations that maximize among group variation. We used them to explore alternative groupings of populations that do not coincide with our phylogeographic hypotheses (Dupanloup et al. 2002). Analyses were conducted by sample site and country of collection, following models specified by AIC in jMODELTEST2. Country of collection is an artificial unit, but within our sampling regime, "countries" represent units for which the distance between them exceeds the distance between the populations within them, and so allows us to cluster and compare populations. Populations were defined into 2–6 groups when analyzed with 7 populations (i.e. country) and 2–15 when with 16 populations (i.e. by sample site). The number of predefined groups cannot be a single group (i.e.1) or the maximum possible number of groups (i.e. the number of "populations"). To ensure the output of K was not affected by the initial configuration value, we did 100 initial configurations (Dupanloup et al. 2002). Finally, we conducted a Mantel test (Mantel 1967) in Alleles in Space (AIS; Miller 2005) to test the null hypothesis of no significant correlation between genetic and geographic distance (i.e. isolation by distance; IBD) for mtDNA and nuDNA. If structure is present, we expect to find statistically significant (p value < 0.05) positive values for r which would suggest a pattern concurrent with IBD (Wood and Gardner 2007). Distance was defined as "geographic distance" in km calculated from latitude and longitude values for each specimen.

We used POWSIM 4.1 (Ryman and Palm 2006) to determine the statistical power with which significant genetic

differentiation could be determined using sample sizes and mtDNA and nuDNA markers assessed in this study. Two markers had more than 50 haplotypes (CO1: 156 haplotypes, 16S: 54 haplotypes), exceeding the maximum number allowed for analysis in POWSIM. Per the author's instruction, rare haplotypes were lumped together, reducing the total number to 26 haplotypes across three markers. Parameter values followed recommended defaults (Ne of 2000; 10 generations of drift; 1000 runs).

Historical demography

We used ARLEQUIN to conduct neutrality tests and mismatch distribution analyses as an indication of recent population expansion, with 10,000 permutations per analysis. Neutrality indices, Tajima's D (Tajima 1989), and Fu's F statistic (Fu 1997) were calculated for each sample site and country to assess deviation from the null hypothesis of a neutral model of evolution (Hri; Harpending 1994). The mismatch analysis provides a tau (τ) value that is used to estimate the time since expansion (t) (Slatkin and Hudson 1991; Rogers and Harpending 1992). We calculated the time of expansion for all specimens in coalescent units ($4N_e$*generation length) using Tajima's D (TJ's D = − 2.56) and nucleotide diversity (π = 0.0165) in an Approximate Bayesian Computation (ABC) analysis with 100,000 permutations computed in Python. The mean of the closest 100 points (points in which the distance between the parameter priors and empirical data are minimized) were used to calculate the overall time of expansion in the following equation: (average time of expansion)(4)(N_e)(generation time) (Appendix Table 2). Effective population size (N_e) was computed with theta (θ) we estimated in DnaSP, using the equation $\theta = 4N_e{*}u$, where u is the neutral mutation rate.

Results

Genetic diversity and structure

Final sample sizes differed for mitochondrial and nuclear markers due to low sequence quality for a few amplifications. For Belize, H3 was successfully sequenced for only four of nine available specimens. Therefore, final sample size for mtDNA and nuDNA analyses were N = 167 and N = 162, respectively.

Mitochondrial markers revealed high levels of genetic diversity across all biogeographic regions and populations. The 621 bp CO1 fragment contained 234 polymorphic sites, 128 singleton and 106 parsimony informative sites, defining 156 different haplotypes across the 167 specimens sampled. Standard genetic diversity indices (S, nh, h, π) for CO1 show extremely high haplotype diversity (h = 0.997–1.000) with low nucleotide diversity values that vary only slightly across sites and samples (π = 0.0096–0.0721) (Table 2; Appendix Table 3). For CO1, all countries have nearly double the number of segregating sites as they do samples, with the exception of Belize, which has 20× as many segregating sites (S = 182; N = 9). Further investigation into this anomaly exposed the culprit, a single specimen (BZ10) which carries an extremely distinct haplotype. Following unsuccessful attempts to classify the specimen as a hybrid or a different species, we opted to run all population structure analyses with and without this rare specimen and haplotype included. The 432 bp 16S fragment revealed 38 polymorphic sites, with 27 singleton haplotypes and 11 parsimony informative sites. Similar to CO1, 16S exhibited high haplotype diversity (0.333–1.000) and low nucleotide diversity (0.0008–0.0069) (Table 2; Appendix Table 3). Each country has a moderate number of segregating sites, and haplotypes, with Honduras (h = 0.960) and Puerto Rico (h = 0.926) as the

Table 2 Genetic diversity indices, neutrality test statistics, and Harpending raggedness index for mtDNA when analyzed by country

	Site ID	n	S		nh		h		π		TJ's D		Fu's F		HRI	
Florida	FL	41	69	13	40	18	0.999	0.913	0.0123	0.0044	− 1.895*	− 1.171	− 24.959*	− 13.628*	0.006	0.086
Bahamas	BHS	37	73	14	36	18	0.998	0.878	0.0147	0.005	− 1.756*	− 1.139	− 24.762*	− 12.992*	0.006	0.042
Puerto Rico	PR	27	57	11	26	15	0.997	0.926	0.0112	0.0047	− 2.017*	− 0.94	− 22.009*	− 10.791*	0.03	0.08
Bonaire	BON	10	39	5	10	5	1	0.756	0.0156	0.0033	− 1.442	− 0.783	− 3.574	− 1.393	0.034	0.069
Honduras	HON	27	63	15	26	17	0.997	0.96	0.0141	0.0056	− 1.780*	− 1.293	− 18.881*	− 12.988*	0.009	0.067
Belize	BEZ	9	182	6	9	7	1	0.917	0.0721	0.0045	− 1.721*	− 0.52	− 0.125	− 3.892	0.068	0.123
Mexico	MX	16	45	7	16	8	1	0.758	0.013	0.003	− 1.704*	− 1.366	− 9.739*	− 4.614	0.024	0.085

Statistics analyzed by sample site and region are in the Appendix Table 3

For each text, values for CO1 are in bold; values for 16S are in plain text

n # samples, *S* # segregating sites, *nh* # haplotypes, *h* haplotype diversity index, *π* nucleotide diversity index, *TJ's D* Tajima's D, *Hri* Harpending raggedness index

Significant p values indicated with a*

most genetically diverse regions and Bonaire (h = 0.756) and Mexico (h = 0.758) as the least diverse. No rare haplotype was found among specimens for 16S (including within the BZ10 individual).

The final fragment length of nuclear H3 was 304 bp and this contained 65 polymorphic sites for our 162 samples. Genetic diversity indices were only computed for samples from the Bahamas, Puerto Rico, Bonaire, and Belize because samples from Florida, Honduras, and Mexico had no polymorphic sites. This is not unexpected, as the slower mutation rate causes nuclear DNA to be less variable and generally less informative than mtDNA for intraspecific-level studies (Schubart 2009). Haplotype diversity was much lower in the nuclear marker, ranging from 0.000 to 0.500, while nucleotide diversity was higher, ranging from 0.000 to 0.105 (Table 3; Appendix Table 4). Interestingly, as was found for CO1, the number of segregating sites for Puerto Rico and Belize were very high relative to other countries and their samples sizes (S = 64 vs. 0–1). Further investigation of this occurrence revealed two specimens responsible, one from Puerto Rico (PRLLP8) and another from Belize (BZ10); the latter is the same specimen whose haplotype differed greatly for CO1. This potentially explains the high nucleotide diversity for Belize. To avoid bias and inflated differentiation between Belize and Puerto Rico versus the other populations, subsequent analyses were computed with and without these two specimens included.

TCS haplotype networks for CO1 and 16S (Fig. 2) did not reveal a geographic pattern in haplotype distribution. The CO1 network had 156 haplotypes (N = 167), with only a few haplotypes shared by multiple individuals. The number of nucleotide differences between CO1 haplotypes varied from one to many. The 16S network showed a number of common haplotypes that differed from less frequent haplotypes by one or two nucleotide differences. The most common 16S haplotype (Hap1) was found in 46 individuals (~ 28%) and the second most common (Hap53) was found in 21 individuals (~ 13%); these two common 16S haplotypes were found in individuals from all seven countries studied.

Pairwise ΦST comparisons based on mtDNA and nuDNA across all seven countries and 16 sample localities indicated genetic homogeneity. For mtDNA, no comparisons were significant when comparisons were made by country or site, with values ranging from 0.000 to 0.001 (BHS vs. FL; BHS vs. PR; BHS vs. BON; BHS vs. BEZ; and BHS vs. MX) and 0.00 (all comparisons except YUC vs. PRL) to 0.01 (YUC vs. PRL), respectively (Appendix Tables 5 and 7). For H3, there were moderate differences between Bonaire and other localities when analyzed by country (ΦST = 0.00–0.174; Appendix Table 6), and between Belize and others when analyzed by sample site (ΦST = 0.00–0.36; Appendix Table 8). However, all differences were insignificant ($P > 0.05$), and low sample sizes ($N_{BON} = 10$; $N_{BEZ} = 4$) and inclusion of the distinct BZ10 haplotype in analyses may have inflated the inferred values for differentiation. Neither pairwise ΦST comparisons in which the rare allele was *excluded* nor pairwise ΦST across all three markers supported inferences of significant structure (no comparisons had significant values) (Appendix Tables 9–16). Overall, these analyses do not provide sufficient evidence to support phylogeographic structure between populations at either the site or aggregate (country or island) level.

AMOVA tests of the mitochondrial (CO1 + 16S) and nuclear (H3) data sets found that genetic variation was entirely distributed within populations (ΦST = 97–100%), as opposed to among populations in the same geographic region (ΦSC), or between geographically separated populations (ΦCT) (Table 4). These findings agree with those based on pairwise ΦST comparisons, suggesting the Florida Straits, Mona Passage, Central America, and central Bahamas phylogeographic breaks are not factors that contribute to population structure for *C. tricolor*.

Table 3 Genetic diversity indices, neutrality test statistics, and Harpending raggedness index for nuDNA H3 when analyzed by country

	Site ID	n	S	nh	h	π	TJ's D	Fu's F	Hri
Florida	FL	41	0	1	0	0	na	na	na
Bahamas	BHS	37	1	2	0.105	0	− 0.82	− 0.621	0.635
Puerto Rico	PR	27	64	2	0.074	0.016	− 2.740*	11.586	0.868
Bonaire	BON	10	1	2	0.2	0.001	− 1.112	− 0.339	0.4
Honduras	HON	27	0	1	0	0	na	na	na
Belize	BEZ	4	64	2	0.5	0.105	− 0.869*	8.786	0.75
Mexico	MX	16	0	1	0	0	na	na	na

Tajima's D and Fu's F cannot be computed for populations with a single haplotype. For many populations, the variance of the mismatch distribution is too small, preventing demographic parameters from being estimated. These situations are designated with "na". Statistics analyzed by sample site and region are in the Appendix Table 4

n # samples, *S* # segregating sites, *nh* # haplotypes, *h* haplotype diversity index, *π* nucleotide diversity index, *TJ's D* Tajima's D, *Hri* Harpending raggedness index

Significant p-values indicated with a *

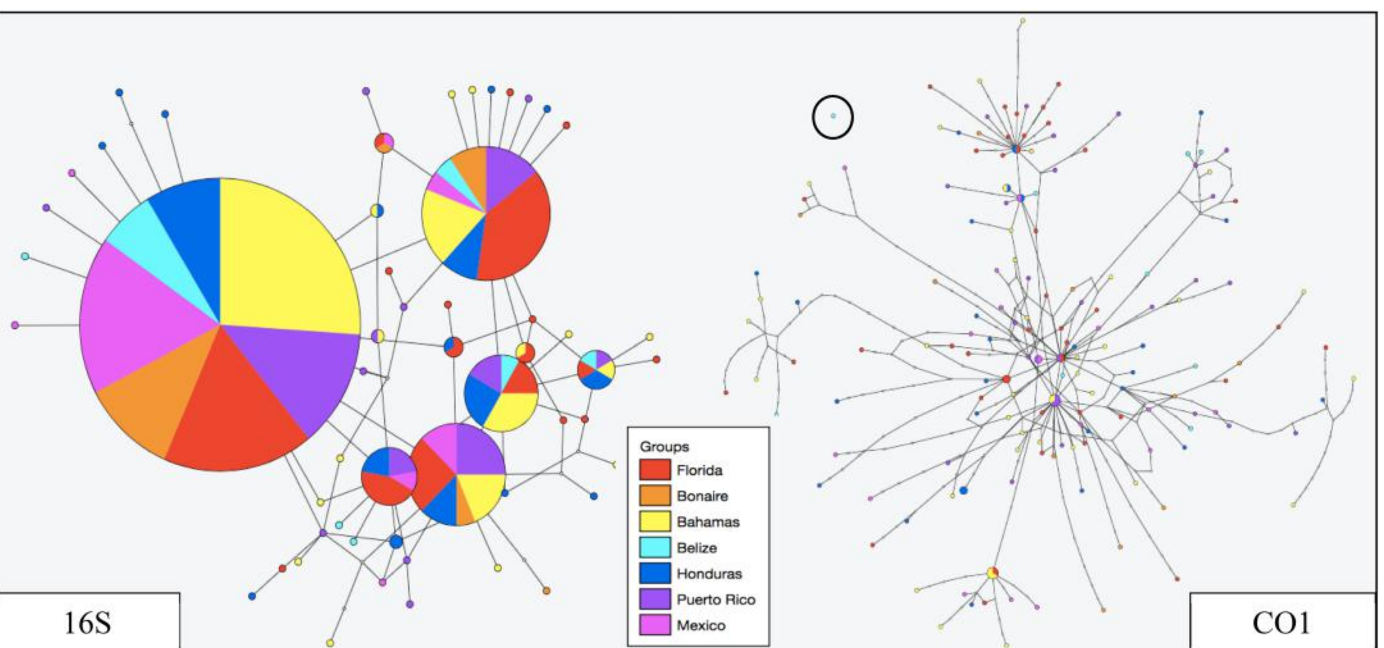

Fig. 2 TCS haplotype networks of 16S (left) and CO1 (right) mtDNA. Each circle represents a haplotype. The color(s) of the circle corresponds to the location in which that haplotype was found, and the size of each circle relates to its frequency. The rare BZ10 haplotype is included in the CO1 network (circled)

Table 4 AMOVA testing the four proposed phylogeographic breaks with mtDNA and H3

Phylogeographic break	DNA	Source of variation	d.f	Sum of squares	Variance components	% variation	Fixation index
	mtDNA	Among groups	1	0.514	0.005	0.10	$F_{CT}=0.001$
		Among populations within groups	6	2.973	− 0.000	− 0.10	$F_{SC}=-0.001$
Florida straits		Within populations	70	35.000	0.500	100.00	$F_{ST}=0.000$
FL	H3	Among groups	1	0.057	0.001	4.29	$F_{CT}=0.043$
vs		Among populations within groups	6	0.094	− 0.001	− 4.18	$F_{SC}=-0.044$
BHS		Within populations	70	1.798	0.026	99.89	$F_{ST}=0.001$
	mtDNA	Among groups	1	0.473	− 0.001	− 0.29	$F_{CT}=-0.003$
		Among populations within groups	2	1.000	0.000	0.00	$F_{SC}=0.000$
Central Bahamas		Within populations	33	16.500	0.500	100.29	$F_{ST}=-0.003$
NPI	H3	Among groups	1	0.092	0.005	9.26	$F_{CT}=0.093$
vs		Among populations within groups	2	0.002	− 0.006	− 10.86	$F_{SC}=-0.120$
SSI		Within populations	33	1.798	0.054	101.60	$F_{ST}=-0.016$
	mtDNA	Among groups	1	0.491	− 0.000	− 0.03	$F_{CT}=-0.000$
		Among populations within groups	14	6.986	− 0.000	− 0.02	$F_{SC}=-0.000$
Central America		Within populations	151	75.500	0.500	100.05	$F_{ST}=0.000$
BEZ-HON	H3	Among groups	1	0.014	− 0.000	− 1.54	$F_{CT}=-0.015$
vs		Among populations within groups	14	0.504	− 0.001	2.05	$F_{SC}=0.020$
All Others		Within populations	146	4.365	0.030	99.48	$F_{ST}=0.005$
	mtDNA	Among groups	1	0.489	− 0.000	− 0.05	$F_{CT}=-0.001$
		Among populations within groups	6	3.000	0.000	0.00	$F_{SC}=0.000$
Mona Passage		Within populations	81	40.500	0.500	100.05	$F_{ST}=-0.001$
BON-PR	H3	Among groups	1	0.019	− 0.001	− 2.78	$F_{CT}=-0.028$
vs		Among populations within groups	6	0.331	0.002	6.09	$F_{SC}=0.059$
BEZ-HON-MX		Within populations	76	2.567	0.034	96.68	$F_{ST}=0.033$
	mtDNA	Among groups	1	0.504	0.000	0.02	$F_{CT}=0.000$
BHS-FL		Among populations within groups	9	4.487	− 0.000	− 0.03	$F_{SC}=-0.000$
vs		Within populations	104	52.000	0.500	100.00	$F_{ST}=-0.000$
PR-BON	H3	Among groups	1	0.019	− 0.000	− 0.44	$F_{CT}=-0.004$
		Among populations within groups	9	0.253	− 0.001	− 1.93	$F_{SC}=-0.019$
		Within populations	104	3.615	0.035	102.37	$F_{ST}=-0.024$

The Mona Passage is evaluated in two ways, reflecting previous concepts of this break. Hierarchical subdivision of genetic variation for *C. tricolor* populations is organized within populations (ΦST), between populations in the same geographic region (ΦSC), and between geographically separated populations (ΦCT). Geographic regions were determined by findings from previous studies and specimens from localities of interest to the hypothesis were grouped into a geographic region on either side of the predefined break. None of the p values are significant at $p=0.005$. Negative F_{ST} should be interpreted as zero (0)

SAMOVA tests for mtDNA and for H3 sequences detected four and three groups, respectively, when the rare Belize/Puerto Rico haplotype was included and data were analyzed by country (Table 5). The F_{CT} value for the H3-based grouping was much higher than that of mtDNA but the findings based on mtDNA and nuDNA were similar in clustering Belize and Bonaire separately from other populations. These findings were not unexpected, as Belize harbors the unusual haplotype and is an area of interest and isolation due to oceanographic processes and Bonaire is at the edge of our sample range, thus it may be explained by isolation by distance. SAMOVA tests run with the distinct haplotype excluded favored three (mtDNA) and six groups (H3) (Table 5). Findings based on mtDNA and nuDNA with the distinct haplotype excluded consistently clustered Bonaire separately from other populations. The validity of groupings suggested by the SAMOVAs were tested with pairwise ΦST comparisons. Resulting ΦST values were low to moderate, yet insignificant across all groups when analyzed by country (Appendix Table 17).

Population clusters were also analyzed by sample site, with and without the distinct haplotypes (Table 6). As in the results for analysis by country, a majority of the suggested groupings showed little differentiation and were insignificant. However, when testing the validity of groupings suggested by the SAMOVAs with pairwise ΦST comparisons,

Table 5 mtDNA and H3 SAMOVA groupings with the highest significant F_{CT} (maximum variation between defined populations) when analyzed by country (K = 2–5)

DNA	Rare included?	Result	#Defined	Groups	% variation	F-statistic	p value
mtDNA	Yes	Highest F_{CT} and significant	4	(BEZ) (BON) (MX) (FL-BHS-HON-PR)	$\Phi_{CT}=17.58$ $\Phi_{SC}=-2.15$ $\Phi_{ST}=84.57$	$F_{CT}=0.274$ $F_{SC}=-0.028$ $F_{ST}=0.254$	0.022* 0.583 0.085
H3	Yes	Highest F_{CT} and significant	3	(BEZ) (BON) (FL-BHS-HON-PR-MX)	$\Phi_{CT}=42.18$ $\Phi_{SC}=-0.61$ $\Phi_{ST}=58.43$	$F_{CT}=0.422$ $F_{SC}=-0.011$ $F_{ST}=0.416$	0.046* 0.162 0.013*
mtDNA	No	Highest F_{CT} and significant	3	(BON-MX) (BEZ) (FL-BHS-HON-PR)	$\Phi_{CT}=3.19$ $\Phi_{SC}=-0.51$ $\Phi_{ST}=97.32$	$F_{CT}=0.032$ $F_{SC}=-0.005$ $F_{ST}=0.027$	0.013* 0.798 0.083
H3	No	Highest F_{CT} and significant	6	(BON) (BHS) (FL) (HON) (PR) (BEZ-MZ)	$\Phi_{CT}=21.28$ $\Phi_{SC}=-19.42$ $\Phi_{ST}=98.15$	$F_{CT}=0.213$ $F_{SC}=-0.247$ $F_{ST}=0.019$	0.048* 1.000 0.123
H3	No	2nd Highest F_{CT} and significant	3	(BON) (BHS) (FL-BEZ-HON-PR-MZ)	$\Phi_{CT}=11.23$ $\Phi_{SC}=-4.47$ $\Phi_{ST}=93.24$	$F_{CT}=0.112$ $F_{SC}=-0.050$ $F_{ST}=0.068$	0.046* 1.000 0.173

Whether groupings include rare haplotypes (BZ10 and PRLLP) is indicated

Significant p values indicated with a*

Table 6 mtDNA and H3 SAMOVA groupings with the highest significant F_{CT} (maximum variation between defined populations) when analyzed by site (K = 2–16)

DNA	Rare included?	Result	#Defined	Groups	% variation	F-statistic	p value
mtDNA	Yes	Highest F_{CT} and significant	3	(BEZ) (SK) (BH-DK-HHBP-BON-CH-RP-FB-NP-CV-EMD-PRA-PRL-YUC-MAH)	$\Phi_{CT}=38.90$ $\Phi_{SC}=-4.66$ $\Phi_{ST}=65.76$	$F_{CT}=0.453$ $F_{SC}=-0.082$ $F_{ST}=0.408$	0.008* 0.006* 0.315
H3	Yes	Highest F_{CT} and significant	3	(BEZ) (SK) (BH-DK-HHBP-BON-CH-RP-FB-NP-CV-EMD-PRA-PRL-YUC-MAH)	$\Phi_{CT}=61.93$ $\Phi_{SC}=-1.68$ $\Phi_{ST}=39.76$	$F_{CT}=0.619$ $F_{SC}=-0.044$ $F_{ST}=0.602$	0.008* 0.494* 0.066*
mtDNA	No	Highest F_{CT} and significant	2	(RP) (BH-DK-HHBP-SK-BON-CH-FB-NP-BEZ-CV-EMD-PRA-PRL-YUC-MAH)	$\Phi_{CT}=11.22$ $\Phi_{SC}=0.78$ $\Phi_{ST}=88.00$	$F_{CT}=0.112$ $F_{SC}=0.009$ $F_{ST}=0.120$	0.049* 0.086 0.011*
H3	No	Highest F_{CT} and significant	2	(BON-FB-NP) (BH-DK-HHBP-SK-CH-RP-BEZ-CV-EMD-PRA-PRL-YUC-MAH)	$\Phi_{CT}=22.94$ $\Phi_{SC}=-8.61$ $\Phi_{ST}=85.67$	$F_{CT}=0.229$ $F_{SC}=-0.112$ $F_{ST}=0.143$	0.002* 1.000 0.432

Whether groupings include rare haplotypes (BZ10 and PRLLP) is indicated

Significant p values indicated with a*

there were two exceptions, both with H3 (Appendix, Table 18). When defined as three populations and when the distinct haplotype was included, Belize was significantly different from all other sample locations, except Sombrero Key (BEZ vs. all others except SK: $\Phi ST = 0.445$; $p = 0.036$). When the distinct haplotype was excluded, Lagun Bonaire, Fernandez Bay, and North Point were significantly different from all other samples (BON-FB-NP vs. all others: $\Phi ST = 0.214$; $p = 0.000$).

We performed an Isolation By Distance (IBD) analysis with a Mantel test to investigate minor signatures of differentiation between samples collected from Bonaire and Belize and samples collected from other localities (Fig. 3; Appendix Fig. 1). IBD analyses generally revealed negative correlation coefficient (r) values for mtDNA and nuDNA, indicating that factors other than isolation by distance are needed to explain the genetic structure. Although r was slightly positive for H3 when the distinct haplotype was included ($R = 0.014$; $p = 0.149$), the value was not significant, and so this haplotype cannot be confidently explained through IBD (Wood and Gardner 2007).

The POWSIM analysis indicated that our dataset had relatively low statistical power, with a 17% probability of detecting a true F_{st} as low as 0.0025, using the chi-square approach. However, statistical power of our markers increased after modifying parameters to allow detection of less conservative Fst values, with a high (100%) probability of detecting a true Fst of 0.05.

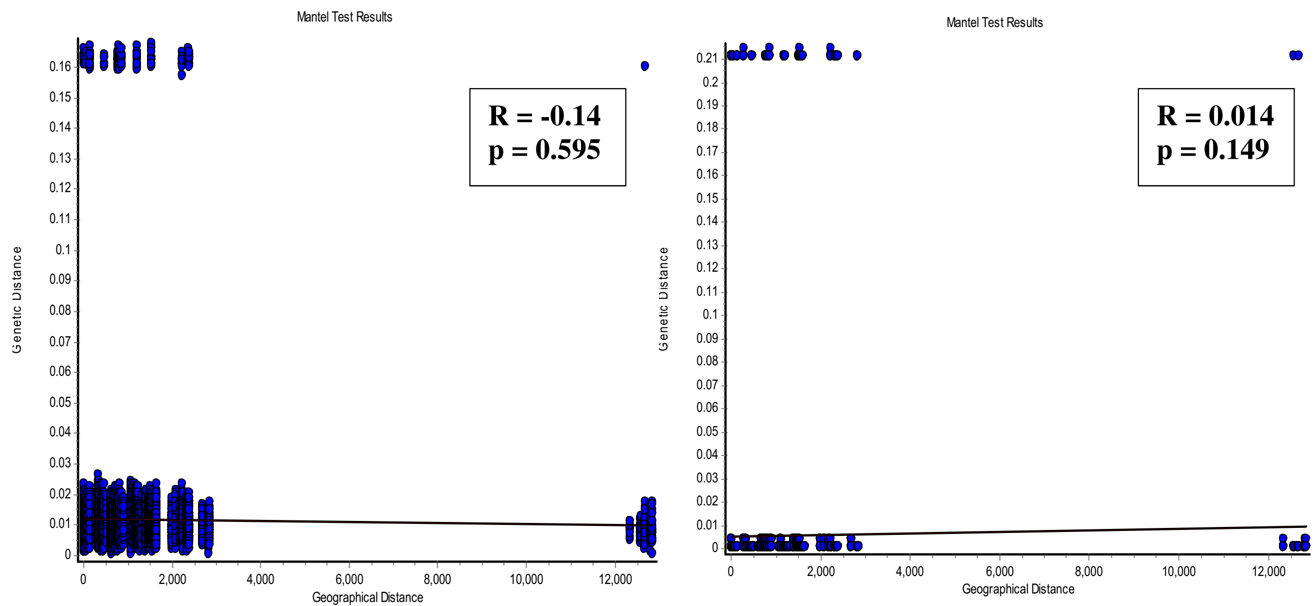

Fig. 3 Scatterplot from isolation by distance (IBD) analysis showing the relationship between genetic and geographic distance for the mtDNA (left) and H3 (right) loci for all specimens. The distinct BZ10 and PRLLP haplotypes are included. Distance was defined as "geographic distance" in km calculated from latitude and longitude values for each specimen. Scatterplots for the IBD analysis without the distinct haplotypes are in Appendix, Fig. 1

Historical demography

Neutrality tests and mismatch distribution analyses suggest that *C. tricolor* has experienced a recent population expansion. For CO1, Tajima's D was significantly negative for Florida, Bahamas, Puerto Rico, Honduras, Belize, and Mexico ($p < 0.05$), and Fu's F was significantly negative for the same localities, with the exception of Belize ($p < 0.02$) (Table 2). Bonaire had negative but insignificant values for both neutrality indices. For 16S, Fu's F was significant and negative for all countries except Bonaire, Belize, and Mexico (Table 2). In contrast to CO1, however, for 16S, Tajima's D was negative but not significant for any locations. H3 had significant negative values of Tajima's D for Puerto Rico and Belize (Table 3). Due to the limited sequence variation in H3, Tajima's D and Fu's F could not be calculated for Florida, Honduras, or Mexico. Hri values from the mismatch distribution analyses for mitochondrial and nuclear markers were not significant for any populations, further supporting the null hypothesis of sudden population expansion (Tables 2, 3).

These results are bolstered by TCS haplotype networks and genetic diversity indices. TCS haplotype networks for both mitochondrial markers exhibit a starburst pattern indicative of population expansion, with the most common haplotypes separated by less frequent haplotypes by only a small number of nucleotide differences (Fig. 2). This pattern, in concert with high haplotype diversities and low nucleotide diversities across all three markers (Tables 2, 3), confirms inferences made based on neutrality tests and mismatch distribution analyses of relatively recent expansion.

Based on τ (tau) for all specimens included in the study, the estimated time since expansion for *C. tricolor* is 280,000–380,000 years before present (ybp) (Table 7). When samples were grouped into populations by country, time since expansion ranges from 200,000–360,000 ybp (1.7% mutation rate) to 150,000–265,000 ybp (2.3% mutation rate), with Florida being most recent and Bonaire being oldest and having the greatest accumulation of mutations. The ABC analysis suggested a time since expansion of ~ 220,000 to 300,000 ybp. This ABC analysis used a mean time (in coalescent units) of expansion of 0.0627, a generation time of 1 year, and respective N_e values to compute the time of expansion values. We used the generic mutation rate for arthropod CO1 of 1.7–2.3% per million years (Brower 1994; Williams and Knowlton 2001). These analyses agree on a timeframe for expansion of 150,000–360,000 ybp, during the mid to late Pleistocene.

Discussion

We hypothesized that the phylogeographic patterns for *C. tricolor* would coincide with those of other marine taxa in the Tropical West Atlantic. However, our analyses inferred from mtDNA and nuDNA data from specimens across the region led us to reject the hypothesis that the previously identified phylogeographic breaks at the Florida Straits,

Table 7 Tau values from mismatch distribution analysis and estimated time since expansion (t) for all specimens (K = 1) and populations, defined by site and country

Country	Site	Tau	$t_{1.7\%}$	$t_{2.3\%}$
Florida, US	Florida	4.219	199,763	147,693
	Bahia Honda	3.170	150,137	110,971
	Duck Key	6.145	291,039	215,115
	Sombrero Key	na	–	–
	Harry Harris BP	3.688	174,671	129,105
Bahamas	Bahamas	5.287	250,331	185,080
	Coral Harbor	3.924	185,848	137,366
	Rock Point	11.889	563,086	416,194
	Fernandez Bay	10.076	477,219	352,727
	North Point	5.818	275,552	203,669
Puerto Rico	Puerto Rico	7.154	338,731	250,438
	Arecibo	7.328	347,068	256,529
	Laurel	5.822	275,741	203,809
Bonaire	Lagun Bonaire	7.592	359,572	265,770
Honduras	Honduras	6.604	312,689	231,184
	Coral View	8.527	403,855	298,502
	Eco Marine Divers	5.490	260,017	192,187
Belize	Wee Wee Key	5.281	250,118	184,870
Mexico	Mexico	4.988	236,174	174,613
	Yucatan	5.105	241,783	178,709
	Mahahual	9.453	447,712	330,918
Belize w/out BZ10	Wee Wee Key	5.838	276,499	204,369
All w/ BZ10		7.9	374,159	276,553
All w/out BZ10		5.4	255,754	189,036

We used the generic mutation rate for arthropod CO1 of 1.7–2.3% per million years (Brower 1994; Williams and Knowlton 2001) to bracket the estimates

Mona Passage, Central America, and central Bahamas influence the structure of *C. tricolor* populations. Rather, our analyses are concordant in showing high genetic diversity across the region, with signatures that support an interpretation of panmixia and demographic expansion during the Pleistocene.

What is the degree of genetic diversity and connectivity in contemporary C. tricolor populations?

Neither the mitochondrial nor the nuclear markers we examined show significant genetic structure in *Clibinarius tricolor*. We find high haplotype diversity and low nucleotide diversity (Tables 2, 3) with the greatest number of variable sites in CO1 (234/621 or 0.37) followed by H3 (65/304 or 0.21) and then 16S-rDNA (38/432 or 0.09). We attribute the higher perceived diversity in the slower-evolving nuclear H3 gene to a distinct haplotype found in two specimens, one from Belize (BZ10) and one from Puerto Rico (PRLLP8). However, despite more than 60 nucleotide differences between this and other H3 haplotypes, we find no evidence that either of these specimens is a hybrid or cryptic species. BZ10 also carries a distinct haplotype for CO1, whereas PRLLP8 does not, meaning the rare haplotypes sort independently. Furthermore, we compared the rare *C. tricolor* CO1 and H3 haplotype sequences to those available for other *Clibanarius* species in GenBank and found no greater affinity between these specimens and other species of *Clibanarius*. This, in addition to our limited sampling in Belize and the southern Caribbean, suggest it is merely a rare genotype, possibly remnant of past structure during the Pleistocene, during which Belize saw repeated fluctuations in sea level and currents (Ludt and Rocha 2015). The absence of this genotype in more heavily sampled populations in the northwestern Caribbean basin (e.g. Florida and Bahamas) and its rare occurrence in the southeastern region strongly argue for additional sampling in peripheral locations in the southern Caribbean. As marine and coastal environments continue to rapidly change, the maintenance of this rare genotype in the larger population may be advantageous for adaptation to future environmental changes. Contrary to expectations, H3 proved to be more informative for inferring patterns of genetic structure than either mitochondrial marker, showing more distinction among sites when data were analyzed without rare haplotypes.

Haplotype networks reveal largescale uniformity in genetic diversity across geography, with individuals bearing the most common haplotypes found in all populations studied (Fig. 2). Genetic diversity networks and haplotype indices suggest high dispersal potential and connectivity throughout the region. We did not find a clear directional pattern of gene flow despite the expected potential for passive dispersal of pelagic larvae from upstream (south eastern) source populations to downstream (north western) sink populations via unidirectional surface currents (Andras et al. 2013). Although the variation in 16S approximates the expectations of current-based dispersal at a broad scale, with the southern point of the range in Bonaire having the lowest levels of haplotype diversity and Florida having among the highest levels of diversity, CO1 haplotype diversity is high for all populations and uninformative for distinguishing patterns in gene flow. Furthermore, we lack samples from Bermuda (the northern extent of the range of *C. tricolor*) and localities between these two ends exhibit diversity measures that discount source/sink dynamics; more sampling of these key sites may provide additional insight into the evolutionary history of *C. tricolor* (Tables 2, 3). Greater sampling depth and geographic coverage is required to resolve this ambiguity, particularly as these findings exhibit the limited power of mitochondrial markers for determining direction of gene flow between *C. tricolor* populations.

Our findings inferred from mtDNA and nuDNA markers suggest populations of *C. tricolor* are highly connected across all four phylogeographic breaks tested, with high genetic diversity and lack of significant population structure. Pairwise ΦST values were non-significant or negative (interpreted as zero) between countries and sites of collection (Appendix Tables 5–16). Similarly, AMOVA tests of previously identified phylogeographic breaks showed that genetic variation was primarily distributed *within* populations, as opposed to among populations (Table 4). Consequently, based on the markers and samples we have analyzed, we cannot reject the null hypotheses of no significant genetic difference between populations.

SAMOVA analyses inferred from mtDNA and nuDNA markers further bolster support for no geographic structure of genetic diversity for the populations of *C. tricolor* we have sampled. Pairwise ΦST values between suggested groups were small and statistically insignificant (Appendix Tables 17 and 18). However, there were a few exceptions to these conclusions. Moderate but nonsignificant levels of differentiation for H3 between Belize and other sites likely reflect the distinct H3 haplotype of the BEZ10 sample. The only significant differentiation we see in our SAMOVA analyses are BEZ vs. all others (except SK) and BON-FB-NP vs. all others (Appendix, Table 18). The first includes the distinct haplotype which may inflate inferred values for differentiation between samples from Belize and other locations and therefore is most likely due to bias, and the second does not align with any previously suggested phylogeographic pattern or process. Given the relative stability of the currents in the Tropical West Atlantic since the closing of the Isthmus of Panama ~ 3 mya (Haug and Tiedmann 1998; Carlin et al. 2003), it is highly unlikely for Bonaire and sites within the Bahamas to be a single population that is genetically distinct from a second population encompassing samples from other sites in the Bahamas, unless the BON-FB-NP group represented a cryptic species, and we see no evidence of cryptic speciation in any of our analyses. Furthermore, these exceptions were found only in the H3 data set and not in either of the larger and more genetically diverse mitochondrial data sets.

The SAMOVA results for H3 highlight the consistent differentiation we found between Bonaire and other populations and also suggest isolation for the Bahamas (Tables 5, 6). While the majority of these findings were not statistically significant, moderate pairwise ΦST and p-values (Appendix Table 17) prompted further exploration with an isolation by distance analysis. We found a weak and insignificant relationship (Mantel test p-value > 0.05), implying gene flow is not restricted by physical distance for *C. tricolor* (Fig. 3; Appendix Fig. 1). The genetic isolation of Bonaire is also seen in intertidal gastropods (Diaz-Ferguson et al. 2010; Diaz-Ferguson et al. 2011) and could be due to habitat disruption from the formation of the Colombian Santa Marta Mountains during the Pleistocene, freshwater influx from the Magdalena River, or cold water upwelling off the coast of Venezuela, any of which might reduce the success of physiologically intolerant coral reef-dwelling organisms (Baums et al. 2005; Debiasse et al. 2016). In our study, Bonaire is one of two locations sampled on the eastern side of the well-supported East–West Caribbean break (Baums et al. 2006; Vollmer and Palumbi 2002; Díaz-Ferguson et al. 2010; Debiasse et al. 2016) and its distinctiveness might diminish with greater sampling in the eastern Caribbean. In the Bahamas, samples from Fernandez Bay exhibit the highest intraspecific genetic distances (ΦST = 0.00–0.06), but the high level of sampling we completed within the archipelago (N = 37) and insignificant differentiation *between* samples within the Bahamas supports interpreting this as a reflection of historic rather than present structure, possibly from isolation of the Exuma Sound during the Pleistocene (Taylor and Hellberg 2006; Foster et al. 2012). Additional sampling in the Eastern Caribbean and other areas of interest (i.e. Belize and Puerto Rico) may further resolve patterns discussed here.

Absence of significant genetic differentiation in a region where phylogeographic barriers have been detected for other marine invertebrates called for additional analyses to substantiate our findings. The POWSIM results indicate limited power for these markers to detect a F_{st} as low as 0.0025, but they had high (100%) probability of detecting a true Fst of 0.05, a value comparable to those in a study on the pantropical sea urchin *Tripneustes* (see Lessios et al. 2003) and lower than that reported for other widely distributed crustaceans (e.g. Baeza et al. 2019; Desiderato et al. 2019). Still, detection of lower statistically significant levels of differentiation in other marine invertebrate studies analyzing mtDNA (Stamatis et al. 2004; Veglia et al. 2018) suggests it is possible that markers we used limited our ability to detect low but significant levels of differentiation. Future work on *C. tricolor* phylogeography should evaluate a larger number of molecular markers from more individuals and from additional geographic locations to improve statistical power, resolve ambiguities, and to improve the resolution of genetic population structure in *C. tricolor* (Mamoozadeh et al., 2018).

Taken together, the population genetic structure analyses inferred from CO1, 16S, and H3 support an absence of phylogeographic structure and bolster support for *C. tricolor* as a single, large panmictic population with high dispersal potential. We do not have sufficient evidence to reject the null hypothesis of panmixia and our findings suggest previously defined Florida Straits, Mona Passage, Central America, and central Bahamas phylogeographic breaks are not factors that shape the geographic distribution of genetic variation for *Clibanarius tricolor*. However, we acknowledge the limitations of our markers for detecting

fine-scale genetic differentiation and admit application of high throughput sequencing methods (e.g. ddRAD) may yield different results.

What additional factors may contribute to phylogeographic patterns?

Findings inferred from mitochondrial and nuclear markers suggest *Clibanarius tricolor* has the ability to permeate phylogeographic breaks that act as barriers for other marine taxa. This begs the question of what attributes of this species might be correlated with panmixia versus structure. Oceanographic processes (currents, gyres, etc.) may not exert strong influence on the structure of contemporary *C. tricolor* populations. However, processes such as weather, reproductive strategy, life history, habitat preference, adult mobility, demographic history, and vicariance events have been proposed to shape geographic patterns of genetic variation in the Caribbean and West Atlantic (reviewed in Baums et al. 2006; Ludt and Rocha 2015).

Reproductive strategy may shape population structure (e.g. Kanciruk and Herrnkind 1976; Foster et al. 2012; Andras et al. 2013). *Clibanarius tricolor* produces multiple clutches during their 6-month reproductive season (May to November), which overlaps with hurricane season (McDermott 2002). During such extreme events, larvae may become trapped in ephemeral counter-flow currents and gyres connecting otherwise separated populations. The duration of pelagic larval development (PLD) sometimes correlates with dispersal potential of marine species, with a shorter pelagic dispersal period generally corresponding to significant genetic structure (e.g. Palumbi 2003; Diaz-Ferguson et al. 2010; Ludt and Rocha 2015). However, studies in the Caribbean and West Atlantic have exhibited both strong and weak correlations between these components (e.g. Taylor and Hellberg 2003; Barcia et al. 2005; Baums et al. 2005; Taylor and Hellberg 2006; Diaz-Ferguson et al. 2010; Eytan and Hellberg 2010; Richards et al. 2015). While the duration of planktonic larval development for *C. tricolor* is unknown, high levels of genetic diversity and inferred lack of significant structure over large distances (e.g. 1700 km) from mtDNA and nuDNA data demonstrates potential for long range dispersal and thus for a relatively long PLD (Calado et al. 2003). However, inconsistencies across taxa and weak correlations between PLD and structure suggest other factors play a larger role in shaping phylogeographic structure of marine organisms. Dispersal may also be impacted by adult mobility. Hermit crabs like *C. tricolor* are mobile as adults, and this may increase their dispersal potential. *Clibanarius tricolor* may be found 20 km offshore, suggesting they may migrate further than their proposed 2 m home range (Hazlett 1983), and raising the possibility that they, like the panmictic *P. argus* spiny lobsters, mate offshore (Lyons 1981).

The combined effect of vicariance events and demographic history result in isolation and diversification of populations (Carlin et al. 2003; Ludt and Rocha 2015). Although we find no significant genetic structure for *C. tricolor* populations, small discordant genetic signatures in Bonaire and the Bahamas may reflect a combination of these process. During the Pleistocene, climatic oscillations (2.5 Ma–11.7 ka) led to repeated fluctuations in sea level that highly influenced phylogeography of most marine taxa (Ludt and Rocha 2015). The complexity introduced by these fluctuations may provide an explanation for the overall homogeneity in population structure between sites while still preserving weak differentiation within the nuclear H3.

Demographic analyses for the sampled populations of *C. tricolor* reject a neutral model of evolution and support the null sudden expansion model, bolstering inference of a recent population expansion following a population bottleneck (Tables 2, 3). High haplotype diversity, low nucleotide diversity, and starburst haplotype networks indicative of population expansion also align with this interpretation (Fig. 2). Time since expansion was estimated to be 280,000–380,000 ybp, during the Pre-Illinoian interglacial period where sea level was similar to that of present day (Hansen et al. 2007). Opportunity to colonize newly submerged reef habitat may explain the high number of marine population expansions, including that of *Clibanarius tricolor*, during this period in the Caribbean (Eytan and Hellberg 2010; Johnston et al. 2012; Baeza and Fuentes 2013). Our estimates of time since expansion varied for our sites, with Bonaire interpreted as the oldest, Florida as the youngest, and other populations as intermediate (Table 7). These dates correlate with expansion out of Bonaire via surface currents approximately 300,000 ybp (Fig. 4) after sea level began to rise.

Conclusions

Findings based on mitochondrial and nuclear DNA support the conclusion that the Caribbean blue-legged hermit crab is a genetically diverse, highly connected, panmictic marine invertebrate that has experienced no significant barrier to dispersal since expansion ~ 300,000 ybp. The ability of *Clibanarius tricolor* to persist through multiple glaciations and the absence of structure across long distances suggests high dispersal potential. Although contemporary oceanographic processes act as barriers for some ecologically similar species that share a distribution with *C. tricolor*, minor differences in larval behavior and life history traits may shape demographic responses to vicariance events, impacting presence or absence of structure (Ludt and Rocha 2015). Differences in phylogeographic histories among co-distributed taxa suggests population genetic studies on poorly studied exploited species would be beneficial,

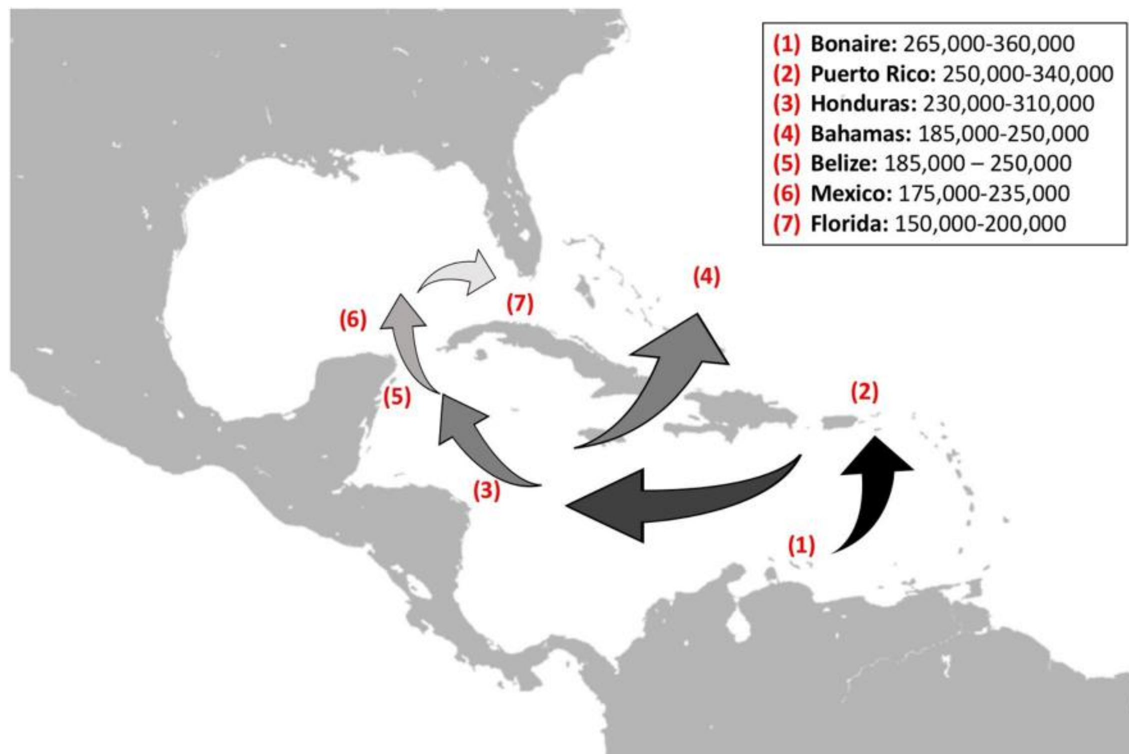

Fig. 4 Map illustrating the pattern of *C. tricolor* expansion (in years before present) as indicated by mismatch distribution analyses, from older to more recent expansions (numbered 1 to 7). Older and more recent expansions are represented with darker and lighter arrows, respectively

rather than extrapolating from findings from other species as a proxy. Our finding of panmixia makes it difficult to identify source or sink populations, but the absence of genetic structure, high levels of genetic diversity and homogeneity, and advantageous life history traits (i.e. inferred high dispersal potential, large brood size, long reproductive season, multiple clutches per season) suggest the *C. tricolor* fishery is not at special risk from human activities (Hazlett 1966; McDermott 2002). However, we did identify geographically restricted haplotypes and recognize limitations within our study that prevent us from making a strong conclusion.

Heavy collection of *C. tricolor* for the aquarium trade in Florida and Haiti have decreased, most likely due to the 2010 Haiti earthquake and overall decreased monetary value of the species (FWC landing data, 2003–2016; Rhyne et al. 2017). Ongoing harvest may also potentially be mediated by migrants from "upstream" populations. While we find no detectable structure and see no immediate cause for concern about this species, the lack of efficient regulation and the many open questions about its biology still poses a threat to this and other popular aquarium trade species (Rhyne et al. 2017). Furthermore, although our conclusions are robust, they are data dependent: methodological limitations, sample and data limitations, and gaps in the scientific literature may limit our perception of the genetic relationship among populations of *C. tricolor*. Although we determined through preliminary work across the well-documented Florida Straits phylogeographic break that fine-scale genomic techniques (i.e. ddRADseq) were not necessary for detecting population structure throughout the entirety of *C. tricolor*'s range (Stark 2018), this covers only a fragment of *C. tricolor*'s broad range, and inclusion of specimens from other regions in fine-scale genomic data analyses may have yielded different results. Furthermore, for the markers used here, denser sampling in the Eastern Caribbean and other areas of interest (i.e. Belize and Puerto Rico) might add haplotypes that connect and differentiate populations of *C. tricolor*. Finally, we note that the lack of differentiation we see might reflect historical factors rather than contemporary connectivity.

In finding that *C. tricolor* has high genetic diversity and low genetic structure across the Caribbean, our results contrast with other studies of Caribbean invertebrates that find genetic differentiation across the Florida Straits or Mona Passage. While this may be due to the low power of our markers for detecting fine-scale genetic differentiation, it also highlights the possibility that common regional forces may not equally impact connectivity among co-distributed species. Although we identify aspects of the reproductive biology of *C. tricolor* that might explain the genetic patterns we find, the framework for comparing and interpreting these is less robust than for geographic factors because the data are much less comprehensive and their interplay much less well

appreciated. The inferred panmixia of *C. tricolor* contrasts with what has been reported for other species in the region, but most studies of genetic structure in the Caribbean have been based on reef-dwelling species rather than species that are habitat generalists or primarily intertidal, and these habitat differences may be important factors.

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