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ORIGINAL PAPER

Wide-ranging phylogeographic structure of invasive red lionfish in the Western Atlantic and Greater Caribbean

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Abstract The red lionfish (*Pterois volitans*) is an invasive predatory marine fish that has rapidly expanded its presence in the Western Hemisphere. We collected 214 invasive red lionfish samples from nine countries and territories, including seven unpublished locations. To more comprehensively evaluate connectivity, we compiled our d-loop sequence data with 846 published sequences, resulting in 1,060 samples from 14 locations. We found low nucleotide diversity ($\pi = 0.003$) and moderate haplotype diversity ($h = 0.59$). Using haplotype population pairwise Φ_{ST} tests, we analyzed possible phylogeographic breaks that were previously proposed based on other reef organisms. We found support for the Bahamas/Turks/Caicos versus Caribbean break ($\Phi_{ST} = 0.12$) but not for the Northwestern Caribbean, Eastern Caribbean, or US East Coast versus Bahamas breaks. The Northern Region had higher variation and more haplotypes, supporting introductions of at least five haplotypes to the region. Our wide-ranging samples showed that a lower-frequency haplotype in the Northern Region dominated the Southern Region and suggested multiple introductions, possibly to the south. We tested multiple scenarios of phylogeographic structure with analyses of molecular variance and found support for a Northern and Southern Region split at the Bahamas/Turks/Caicos versus Caribbean break (percentage of variation among regions = 8.49 %). We found that Puerto Rico clustered with the Southern Region more strongly than with the Northern Region, as opposed to previous reports. We also found the rare haplotype H03 for the first time in the southern Caribbean (Panama), indicating that either secondary releases occurred or that the low-frequency haplotypes have had time to disperse to extreme southern Caribbean locations.

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Introduction

Widely distributed in the Pacific Ocean, red lionfish (*Pterois volitans* [Linnaeus 1758]) were first reported within the Western Hemisphere, in Florida in 1985, and have since been documented throughout the US East Coast and Greater Caribbean (Fig. 1; Schultz 1986; Whitfield et al. 2002; Schofield 2009; Morris and Akins 2009). As with most coastal marine species, lionfish primarily disperse in their larval form and show high site-fidelity as adults (Jud

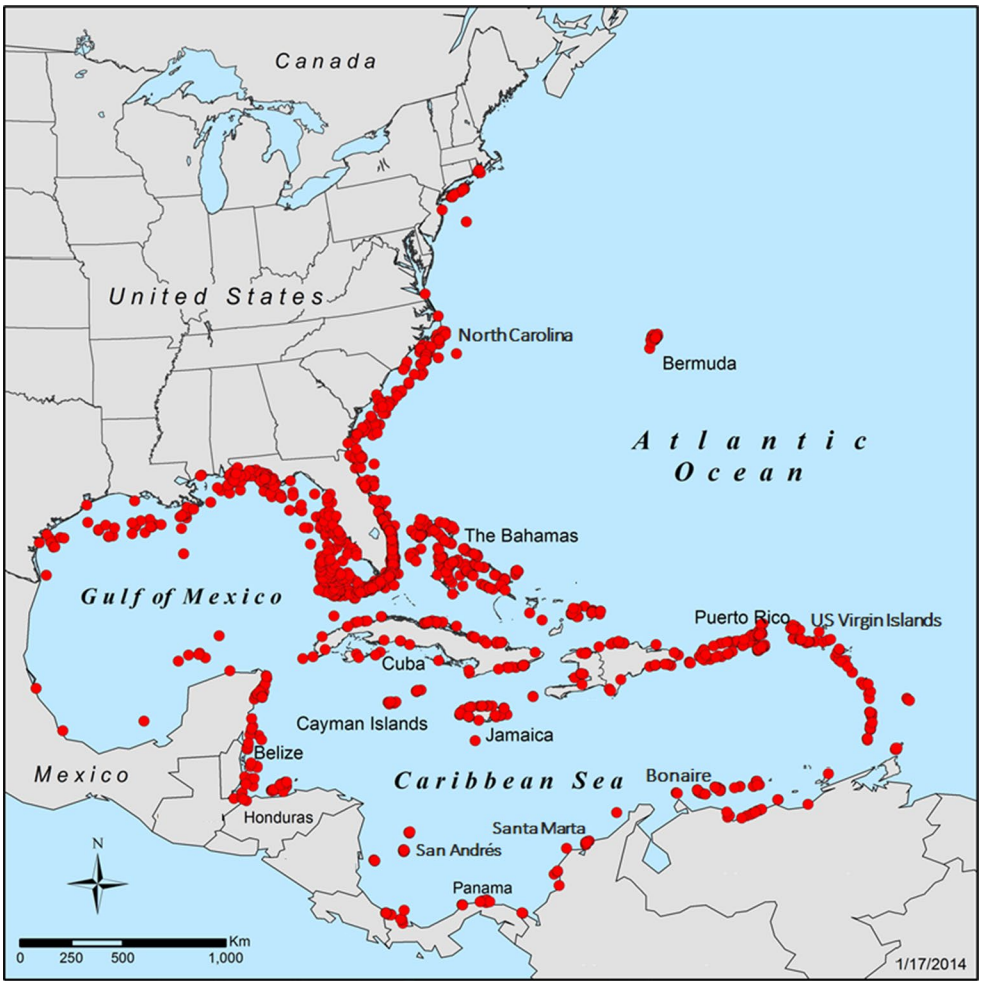

Fig. 1 Map of the invasive lionfish range, including both *Pterois volitans and P. miles*. *Red dots* indicate location of lionfish sightings between 1985 and January 17, 2014. Data are from the USGS–NAS database (Nonindigenous Aquatic Species information resource for the United States Geological Survey)

and Layman 2012). Female lionfish can spawn two floating egg masses approximately every 4 days, resulting in more than two million eggs in a year (Morris 2009). Spawning is followed by a 20–35 day larval stage that provides sufficient opportunity for long-distance dispersal via ocean currents (Ahrenholz and Morris 2010). After introduction and establishment, lionfish most likely dispersed northward along the US East Coast by ocean currents, such as the Gulf Stream, and more slowly southward against currents.

Studying population connectivity with phylogenetic markers provides essential information on parental populations, genetic relationships between neighboring populations, and gene flow. Knowledge of the genetic structure of lionfish in the Caribbean is useful for tracking dispersal and provides insight into possible introduction locations. Previous invasive lionfish genetic studies found evidence of strong founder effects, supported by low levels of mitochondrial cytochrome *b* (cyt *b*) and d-loop haplotype diversity in invasive populations (three and nine haplotypes, respectively) and high levels of haplotype diversity in native regions (25 and 36 haplotypes, respectively; Hamner et al. 2007; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). Previous studies analyzed seven locations and found two main groups: one of higher haplotype diversity to the north (Bahamas, North Carolina, Puerto Rico, and Bermuda) and a second with lower haplotype diversity to the south (Santa Marta, Grand Cayman, and San Andrés; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). The studies revealed that more samples from the western, southern, and eastern parts of the invasive range are needed to test proposed connectivity breaks (Betancur-R et al. 2011; Toledo-Hernández et al. 2014). Here we analyzed the mitochondrial d-loop of lionfish collected in new areas of the western range to gain better insight into the invasion. To more comprehensively evaluate connectivity, we assessed previously proposed regional and phylogeographic breaks after compiling our data with published sequences.

Materials and methods

Red lionfish samples ($n = 214$) were opportunistically provided by fishermen from nine locations: Belize (Belize

City, Hol Chan Marine Reserve, and Caye Caulker Marine Reserve; $n = 59$; March–October 2009), US Virgin Islands (St. Croix; $n = 10$; April 2009), Bonaire ($n = 21$; June 2009), Jamaica (Negril; $n = 37$; August 2011), Puerto Rico (multiple locations on all coasts; $n = 20$; February–March 2012), Bahamas (San Salvador; $n = 23$; March 2012–March 2013), Cuba (Guantanamo Bay; $n = 24$; January–February 2013), Honduras (Roatán and Santa Elena; $n = 15$; May 2013), and Panama (Portobelo; $n = 5$; May 2013). Fin clips or muscle tissue were stored in 95 % ethanol or dimethyl-sulfoxide tissue buffer. DNA extraction of lionfish samples was done using Qiagen DNAeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). We performed PCR amplification of the mitochondrial d-loop using final reaction concentrations of 0.5 ng/μL DNA, 800 μM DNTP's, 3 mM $MgCl_2$, 0.5 mg/μL Bovine Serum Albumin (BSA), and 0.25 μM each LionA_H (5′-CCA TCT TAA CAT CTT CAG TG-3′) and LionB_L (5′-CAT ATC AAT ATG ATC TCA GTAC-3′) by denaturing DNA at 94 °C for 3 min, followed by 34 cycles of denature for 1 min at 94 °C, annealing for 1 min at 57 °C, and extension for 1 min at 72 °C, followed by 10 min extension at 72 °C (Freshwater et al. 2009). We used ExoSap-IT® (Affymetrix, Santa Clara, California, USA) for PCR cleanup and sequenced following the BigDye Terminator v3.1 Cycle Sequencing Kit protocol using Applied Biosystems 3130xl genetic analyzer (Life Technologies Corporation, Carlsbad, California, USA). Sequences were aligned and edited in Geneious 5.4.7 (Biomatters, Ltd., Auckland, New Zealand).

We compiled our d-loop sequences from nine locations ($n = 214$) with the published sequences (Genbank) from seven locations ($n = 846$) from the studies by Freshwater et al. (2009), Betancur-R et al. (2011), and Toledo-Hernández et al. (2014), for a total of 1,060 samples in fourteen locations. The seven previously published locations were Bermuda ($n = 45$), Bahamas ($n = 127$), North Carolina ($n = 264$), Grand Cayman ($n = 79$), San Andrés ($n = 47$), Puerto Rico ($n = 118$), and Santa Marta ($n = 166$). While Bahamas overlapped with the previously published location, our Bahamas samples were collected from San Salvador, at the extreme eastern edge of the country. The Northern Region included Bahamas, North Carolina, and Bermuda. The Southern Region included Belize, Bonaire, Grand Cayman, Cuba, Honduras, Jamaica, Panama, Puerto Rico, San Andrés, Santa Marta, and US Virgin Islands. We assessed individual countries, regions, and all samples combined with summary statistics of nucleotide diversity (π), haplotype diversity (h), sequence diversity (k), and the standard neutrality test, Tajima's D, using dnaSP version 5.0 (Table 1; Librado and Rozas 2009). We calculated haplotype population pairwise Φ_{ST} (100,000 permutations) and exact tests of population differentiation (100,000 Markov chain steps; 10,000 dememorization steps; Table 2) between the Northern and Southern Regions and each location using Arlequin 3.5 (Excoffier and Lischer 2010).

Using our expanded data set, we tested four previously proposed phylogeographic breaks by calculating population pairwise Φ_{ST} values between locations proximal to the break at different geographic scales. To assess the proposed Bahamas/Turks/Caicos versus Caribbean phylogeographic break (A in Fig. 2; Table 3; Cowen et al. 2006; Betancur-R et al. 2011), we compared samples from Bahamas versus Southern Region. To narrow the geographic scope and allow us to more strictly evaluate the break, we additionally tested Bahamas versus Cuba/Jamaica grouping. For the Bahamas versus US East Coast break (B in Fig. 2; Table 3; Schultz and Cowen 1994; Hare et al., 2002; Betancur-R et al. 2011), we compared Bahamas versus North Carolina. For the Northwestern Caribbean break (C in Fig. 2; Table 3; Cowen et al. 2006; Salas et al. 2010; Betancur-R et al. 2011), we compared groupings of Honduras/Belize/Grand Cayman versus Jamaica/San Andrés/Cuba. To test the Eastern Caribbean break (D in Fig. 2; Table 3; Cowen et al. 2006; Taylor and Hellberg 2003, 2006; Baums et al. 2005; Betancur-R et al. 2010, 2011), we compared groupings of Santa Marta/Cuba/Jamaica versus Puerto Rico/US Virgin Islands/Bonaire.

Genetic differentiation was tested with an analysis of molecular variance (AMOVA) in Arlequin 3.5, using the Jukes and Cantor (JC) model of DNA sequence evolution, as selected by the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) in jModelTest 2.1.4 (Guindon and Gascuel 2003; Excoffier and Lischer 2010; Darriba et al. 2012). We combined individual locations into groups based on proposed phylogeographic breaks of lionfish and other reef organisms from previous studies, with a goal of maximizing the percentage of variance among regions (Table 4). Scenario 1 included the Northern and Southern Region division at the Bahamas/Turks/Caicos versus Caribbean break. Scenario 2 and 3 tested three-region scenarios that kept the Northern Region intact and divided the Southern Region into two regions, eastern and western, with either Northwestern or Eastern Caribbean breaks dividing them. Scenario 4 had four regions and kept the Northern Region intact, while dividing the Southern Region into western, central, and eastern regions at both the Northwestern Caribbean break and the Eastern Caribbean break. To test Scenario 5 proposed by Toledo-Hernández et al. (2014), Scenario 1 was updated to include Puerto Rico in the Northern as opposed to the Southern Region.

Results

We successfully sequenced 679 base pairs of the mitochondrial d-loop from our collected samples and observed

Table 1 Invasive red lionfish d-loop haplotype diversity indices and tests of neutrality for 1,060 samples at 14 locations

Source	*n*	*hap*	*h*	π	*k*	H01	H02	H03	H04	H05	H06	H07	H08	H09
N[a,b,c]	459	9	0.60	0.0032	2.16	194	172	24	19	14	19	15	1	1
BAH[a,b]	150	8	0.63	0.0032	2.14	74	53	8	4	5	1	4	1	0
NC[b]	264	8	0.70	0.0038	2.54	102	98	15	15	9	14	10	0	1
BER[c]	45	5	0.63	0.0030	2.02	18	21	1	0	0	4	1	0	0
S[a,c,d]	601	4	0.47	0.0026	1.73	159	405	3	34	0	0	0	0	0
BEL[a]	59	3	0.49	0.0025	1.72	17	39	0	3	0	0	0	0	0
BON[a]	21	2	0.32	0.0014	0.97	4	17	0	0	0	0	0	0	0
CAY[c]	79	4	0.43	0.0021	1.41	21	56	1	1	0	0	0	0	0
CUB[a]	24	3	0.41	0.0022	1.46	5	18	0	1	0	0	0	0	0
HON[a]	15	3	0.63	0.0037	2.53	5	8	0	2	0	0	0	0	0
JAM[a]	37	3	0.40	0.0022	1.49	7	28	0	2	0	0	0	0	0
PAN[a]	5	2	0.40	0.0030	2.00	0	4	1	0	0	0	0	0	0
PR[a,d]	138	4	0.45	0.0022	1.49	39	95	1	3	0	0	0	0	0
SA[c]	47	3	0.56	0.0029	1.98	18	26	0	3	0	0	0	0	0
SM[c]	166	3	0.52	0.0031	2.09	42	106	0	18	0	0	0	0	0
VI[a]	10	3	0.38	0.0025	1.71	1	8	0	1	0	0	0	0	0
Total	1,060	9	0.59	0.0031	2.10	353	577	27	53	14	19	15	1	1

The Northern Region (*N*) represents North Carolina, Bermuda, and Bahamas. The Southern Region (*S*) represents Grand Cayman, Jamaica, and Cuba, Belize, Honduras, Puerto Rico, United States Virgin Islands, Bonaire, San Andrés, Panama, and Santa Marta

BER Bermuda, *BAH* Bahamas, *CAY* Grand Cayman, *NC* North Carolina, *SA* San Andrés Islands, *SM* Santa Marta, *PR* Puerto Rico, *BEL* Belize, *JAM* Jamaica, *VI* US Virgin Islands, *PAN* Panama, *CUB* Cuba, *HON* Honduras, *BON* Bonaire, *n* number of individuals, *hap* number of haplotypes, *h* haplotype diversity, π nucleotide diversity, *k* sequence diversity

[a] This study

[b] Freshwater et al. (2009)

[c] Betancur-R et al. (2011)

[d] Toledo-Hernández et al. (2014)

four haplotypes (H01–H04) in seven new locations ($n = 171$) and two previously assessed locations ($n = 43$) for nine total locations ($n = 214$). We added 23 Bahamas sequences of three previously found haplotypes (H01, $n = 13$; H02, $n = 9$; H03, $n = 1$) to formerly published Bahamas sequences of eight haplotypes ($n = 127$). We also added 20 Puerto Rico sequences of three previously found haplotypes (H01, $n = 5$; H02, $n = 14$; H03, $n = 1$) to formerly published Puerto Rico sequences of four haplotypes ($n = 118$; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). We found no *P. miles* sequences in our collected samples.

Combining sequences with the published data resulted in the majority of samples (87.74 %) being one of two haplotypes (H01, $n = 353$; H02, $n = 557$; Table 1; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). Panama and the US Virgin Islands had ten or fewer samples, while Bahamas, North Carolina, Puerto Rico, and Santa Marta had more than 100 samples. There was a minimum of two haplotypes in each location (Panama and Bonaire) and a maximum of eight haplotypes in any one location (North Carolina and Bahamas). There were more than twice as many haplotypes in the Northern Region than in the Southern Region. Our diversity tests across all samples resulted in $h = 0.59$, ranging from 0.32 (Bonaire) to 0.70 (North Carolina), $\pi = 0.003$, ranging from 0.0014 (Bonaire) to 0.0038 (North Carolina), and sequence diversity ($k = 2.10$) ranging from 0.97 (Bonaire) to 2.54 (North Carolina). Tajima's D was insignificant for the tests of neutrality at all locations and both regions, corresponding with Betancur-R et al. (2011).

The Bahamas/Turks/Caicos versus Caribbean break (A in Fig. 2) was significant when comparing Bahamas versus Southern Region ($\Phi_{ST} = 0.12$, p value <0.01) and Bahamas versus Jamaica/Cuba grouping ($\Phi_{ST} = 0.16$, p value <0.01; Table 3). Of the previously proposed breaks, three were not supported by population pairwise Φ_{ST} values, including Bahamas versus US East Coast (B in Fig. 2), Northwestern Caribbean (C in Fig. 2), and Eastern Caribbean (D in Fig. 2).

The AMOVA resulted in a maximum percentage of variation among regions when grouping locations into Northern and Southern Regions (8.49 %; Table 4). Differing from a previous study, percentage of variation among

Table 2 Invasive red lionfish d-loop haplotype population pairwise Φ_{ST} using the Jukes and Cantor model (below diagonal) and exact tests of population differentiation p values (above diagonal; p value <0.05*) including both the current study and previously published haplotypes (n = 1,060; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014)

Source	*N*	BAH	NC	BER	S	CAY	SA	SM	BEL	JAM	VI	PAN	PR	CUB	HON	BON
N[a,b,c]		0.41	0.94	0.53	0.00*	0.00*	0.18	0.00*	0.00*	0.00*	0.18	0.23	0.00*	0.10*	0.53	0.07
BAH[a,b]	0.00		0.11	0.06	0.00*	0.00*	0.09	0.00*	0.00*	0.00*	0.09	0.13	0.00*	0.03*	0.34	0.03*
NC[b]	0.00	0.00		0.46	0.00*	0.00*	0.12	0.00*	0.00*	0.00*	0.25	0.26	0.00*	0.09	0.72	0.07
BER[c]	0.00	0.02	0.00		0.00*	0.00*	0.05	0.00*	0.01*	0.00*	0.10	0.12	0.00*	0.07	0.21	0.08
S[a,c,d]	0.09*	0.12*	0.08*	0.05*		0.24	0.32	0.11	0.96	0.61	0.39	0.03	0.32	0.88	0.30	0.57
CAY[c]	0.09*	0.13*	0.09*	0.06*	0.00		0.10	0.02*	0.49	0.41	0.24	0.14	0.98	0.62	0.09	0.73
SA[c]	0.02	0.03*	0.01	0.00	0.01	0.03		0.22	0.55	0.14	0.21	0.07	0.17	0.32	0.73	0.11
SM[c]	0.07*	0.10*	0.06*	0.04*	0.00	0.01	0.01		0.47	0.43	0.61	0.04*	0.01*	0.60	0.65	0.21
BEL[a]	0.06*	0.09*	0.06*	0.03	−0.01	−0.01	0.00	0.00		0.54	0.33	0.09	0.68	0.83	0.40	0.43
JAM[a]	0.11*	0.16*	0.10*	0.09*	0.00	−0.01	0.00	0.01	0.00		0.69	0.18	0.41	1.00	0.23	0.87
VI[a]	0.13*	0.17*	0.11*	0.10	−0.01	−0.01	0.04	−0.02	−0.01	−0.05		0.74	0.23	0.67	0.50	0.53
PAN[a]	0.13	0.18*	0.10*	0.11	0.02	0.02	0.07	0.01	0.02	−0.02	−0.08		0.06	0.21	0.16	0.20
PR[a,d]	0.08*	0.12*	0.08*	0.05*	0.00	−0.01	0.02	0.01	−0.01	0.00	0.00	0.03		0.57	0.12	0.68
CUB[a]	0.10*	0.14*	0.09*	0.07*	−0.01	−0.02	0.03	0.00	−0.02	−0.03	−0.05	−0.02	−0.01		0.34	1.00
HON[a]	0.00	0.02	−0.01	0.00	0.00	0.04	−0.03	−0.02	−0.01	0.03	0.01	0.03	0.02	0.02		0.08
BON[a]	0.14*	0.18*	0.13*	0.11*	0.01	−0.01	0.07	0.03	0.01	−0.02	−0.03	0.01	0.00	−0.03	0.09	
Count[a,b,c,d]	459	150	264	45	601	79	47	166	59	37	10	5	138	24	15	21

Count refers to the number of sequences represented at that location

BER Bermuda, *BAH* Bahamas, *CAY* Grand Cayman, *NC* North Carolina, *SA* San Andrés, *SM* Santa Marta, *PR* Puerto Rico, *BEL* Belize, *JAM* Jamaica, *VI* United States Virgin Islands, *PAN* Panama, *CUB* Cuba, *HON* Honduras, *BON* Bonaire

* p value <0.05 (significant)

[a] This study

[b] Freshwater et al. (2009)

[c] Betancur-R et al. (2011)

[d] Toledo-Hernández et al. (2014)

Table 3 Proposed invasive red lionfish d-loop phylogeographic breaks, tested using population pairwise Φ_{ST} values calculated with sampled locations closest to phylogeographic break including current study samples and previously published haplotypes (n = 1,060; p value <0.05*; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014)

Phylogeographic break	Φ_{ST}	p value	References
(A) Bahamas/Turks/Caicos versus Caribbean			Cowen et al. (2006), Betancur-R et al. (2011)
Bahamas versus Southern Region	0.12*	<0.01	
Bahamas versus Cuba/Jamaica	0.16*	<0.01	
(B) Bahamas versus US East Coast			Schultz and Cowen (1994); Hare et al. (2002), Betancur-R et al. (2011)
Bahamas versus North Carolina	0.00	0.18	
(C) Northwestern Caribbean			Cowen et al. (2006), Salas et al. (2010), Betancur-R et al. (2011)
Honduras/Belize/Grand Cayman versus Jamaica/San Andrés/Cuba	−0.01	0.95	
(D) Eastern Caribbean			Cowen et al. (2006), Taylor and Hellberg (2003, 2006), Baums et al. (2005), Betancur-R et al. (2010, 2011)
Santa Marta/Cuba/Jamaica versus Puerto Rico/US Virgin Islands/Bonaire	−0.01	0.09	

The southern region (S) represents Grand Cayman, Jamaica, Cuba, Belize, Honduras, Puerto Rico, United States Virgin Islands, Bonaire, San Andrés, Panama, and Santa Marta. Location of phylogeographic breaks (A–D) are indicated on Fig. 2

regions in the AMOVA was maximized by including Puerto Rico in the Southern versus in the Northern Region (Toledo-Hernández et al. 2014). Northern versus Southern Region haplotype population pairwise differentiation tests (Φ_{ST} = 0.09; Table 2) and exact tests of population differentiation (p value <0.01) were both significant.

Table 4 Invasive red lionfish d-loop haplotype analysis of molecular variance (AMOVA) testing combinations of proposed breaks in different scenarios, with current study samples and previously published haplotypes (n = 1,060; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p value
Scenario 1: two regions divided at A					
Among regions	1	50.13	0.09	8.49	<0.01
Among populations within regions	12	14.45	0.00	0.29	0.15
Within populations	1,046	1,049.43	1.00	91.22	<0.01
Total	1,059	1,114.01	1.09		
Scenario 2: three regions divided at A/C					
Among regions	2	50.43	0.07	6.74	<0.01
Among populations within regions	11	14.16	0.00	0.43	0.11
Within populations	1,046	1,049.43	1.00	92.83	<0.01
Total	1,059	1,114.01	1.08		
Scenario 3: three regions divided at A/D					
Among regions	2	51.76	0.07	6.89	<0.01
Among populations within regions	11	12.82	0.00	0.26	0.19
Within populations	1,046	1,049.43	1.00	92.85	<0.01
Total	1,059	1,114.01	1.08		
Scenario 4: four regions divided at A/C/D					
Among regions	3	52.75	0.07	6.14	<0.01
Among populations within regions	10	11.84	0.00	0.30	0.19
Within populations	1,046	1,049.43	1.00	93.56	<0.01
Total	1,059	1,114.01	1.07		
Scenario 5: two regions divided at A with Puerto Rico included in Northern Region					
Among regions	1	29.27	0.05	4.31	<0.01
Among populations within regions	12	35.31	0.03	2.77	<0.01
Within populations	1,046	1,049.43	1.00	92.93	<0.01
Total	1,059	1,114.01	1.08		

Proposed phylogeographic breaks: A, Bahamas/Turks/Caicos versus Caribbean break (A in Fig. 2; Cowen et al. 2006; Betancur-R et al. 2011); C, Northwestern Caribbean break (C in Fig. 2; Cowen et al. 2006; Salas et al. 2010; Betancur-R et al. 2011); D, Eastern Caribbean break (D in Fig. 2; Cowen et al. 2006; Taylor and Hellberg 2003, 2006; Baums et al. 2005; Betancur-R et al. 2010, 2011)

d.f. degrees of freedom

p value < 0.05 is significant

Discussion

Here we report the most geographically comprehensive genetic characterization of the red lionfish in the Western Hemisphere to date. Using 14 locations and 1,060 sequences, we found high differentiation between the Northern and Southern Regions (Betancur-R et al. 2011; Toledo-Hernández et al. 2014). The regional differences were due to five private northern haplotypes and different dominant haplotypes frequencies in the two regions. Phylogeographic breaks created by currents and dispersal potential, as seen in other reef species, are likely to have caused the regional differentiation (Cowen et al. 2006; Betancur-R et al. 2011).

Only nine haplotypes have been identified in invasive red lionfish populations, compared to 36 haplotypes in their native region (Freshwater et al. 2009; Betancur-R et al. 2011). The low variation in the invasive population implies a limited number of introductions, a bottleneck of fish released by the pet trade, and/or selection of superiorly adapted fish (Kawecki and Ebert 2004). In the Southern Region as a whole, three haplotypes (H01, H02, and H04), comprised the vast majority of samples (n = 598), while haplotype H03 was found in only three samples (Table 1).

The initial lionfish introductions likely included haplotype H01 and H02, due to their high representation at all locations. Interestingly, haplotypes H01 and H02 were found in different proportions in the Northern (H01: 42.3 %; H02: 37.5 %) and Southern Region (H01: 26.5 %; H02: 67.4 %; Table 1; Fig. 2). Given the lower proportion of haplotype H02 in the Northern Region, it may be possible that the introduction of this haplotype occurred in the Southern Region and dispersed north with ocean currents (Betancur-R et al. 2011).

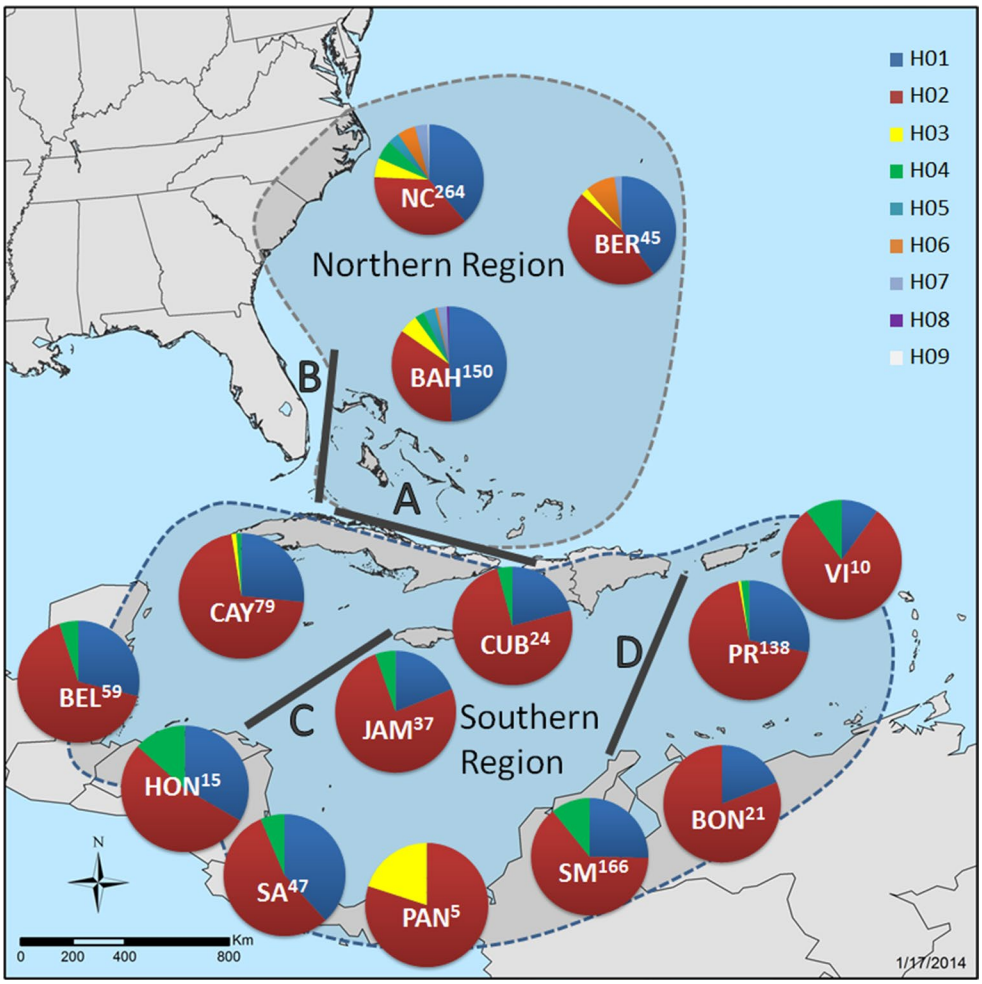

Fig. 2 Map of red lionfish d-loop haplotypes for 1,060 samples at 14 locations ($n = 1{,}060$; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). *A* Bahamas/Turks/Caicos versus Caribbean break; *B* US East Coast versus Bahamas break; *C* Northwestern Caribbean break; *D* Eastern Caribbean break (Schultz and Cowen 1994; Hare et al., 2002; Taylor and Hellberg 2003, 2006; Baums et al. 2005; Cowen et al. 2006; Salas et al. 2010; Betancur-R et al. 2010, 2011). *Superscript* indicates number of samples analyzed. *BER* Bermuda, *BAH* Bahamas, *CAY* Grand Cayman; *NC* North Carolina, *SA* San Andrés, *SM* Santa Marta, *PR* Puerto Rico, *BEL* Belize, *JAM* Jamaica, *VI* US Virgin Islands, *PAN* Panama, *CUB* Cuba, *HON* Honduras, *BON* Bonaire

Alternatively, H02 may have dispersed from the Northern Region and become established in the Southern Region prior to or in greater numbers than haplotype H01. Environmental differences between the Northern and Southern Regions may have created selective pressures that would explain why haplotype H02 was able to successfully disperse southward prior to haplotype H01 (Johannesson and André 2006). Temporal separation of sample collections (newly sequenced samples in this study were collected between March 2009 and May 2013) and/or sampling bias may also explain the disparity between haplotype frequencies. Additional sampling and genetic analysis of the populations to the north, particularly along the eastern US coast and Bahamas, would help to determine whether a shift in haplotype frequencies has occurred and to further investigate introduction locations.

Interestingly, the rare H03 haplotype (2.5 % of samples) was detected for the first time in the extreme southern Caribbean Sea (Panama, $n = 1$). Haplotype H03 was previously found only in the Northern Region (Bahamas, Bermuda, and North Carolina; $n = 24$) and in the northern portion of the Southern Region (Grand Cayman, $n = 1$; Puerto Rico, $n = 1$; Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). Haplotype H03 may have been newly introduced and has not yet increased in number, or has a low presence in the Southern Region, allowing it to evade detection. The sequence for haplotype H03 differs from the three other haplotypes (H01, H02, and H04) found in the Southern Region by at least four base pairs, making it unlikely to have been a recent mutation from another similar haplotype. Like H03, five low-frequency haplotypes (H05–H09) have been found only in the Northern Region and may not have had the opportunity to disperse out of the region or they simply have not been detected elsewhere (Freshwater et al. 2009; Betancur-R et al. 2011; Toledo-Hernández et al. 2014). Other possibilities include poor adaptation to southern environmental and ecological conditions or prevention of dispersal by gene flow boundaries. Additional samples from Panama, Gulf of Mexico, Lesser Antilles, and South America would help to inform the overall invasion pathway and timeline for the Southern Region. Low sample numbers (e.g., Panama, US Virgin Islands, and Honduras) may limit detection of rare haplotypes and produce low haplotype population pairwise differentiation Φ_{ST} values and insignificant p values; increased sample sizes are recommended.

Sequencing a larger region of the mitochondrial genome, in addition to nuclear DNA analyses, such as microsatellites or restriction-site associated DNA sequencing (RAD-seq), may provide more comprehensive identification of diversity, fine-scale genetic relatedness, and dispersal pathways (Schultz et al. 2013).

Knowledge of genetically connected populations and directions of gene flow may improve removal of lionfish, as targeting source populations (e.g., upstream of ocean currents) could help to prevent continuous replenishment of sink locations (e.g., downstream of ocean currents; Barson et al. 2009; De León et al. 2013; Green et al. 2014). Although range expansion seems to be unhindered at present, continued releases of lionfish would likely increase genetic diversity. New adaptive alleles could result in the release of bottleneck pressures associated with low diversity and even further spread through increased fitness or fecundity (Reed and Frankham 2003; Da Silva et al. 2005; Roman and Darling 2007). The Western Hemisphere lionfish invasion has highlighted the negative impact that predatory invasive species can have on biodiversity (Albins and Hixon 2008). Insights from the invasion could be useful in guiding response and control measures for future and continuing exotic species introductions.

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