Mitochondrial DNA (mtDNA) Haplotype Diversity of the Invasive Lionfish (*Pterois volitans*) in Barbados

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ABSTRACT

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Shekira Sealy

The 1990s saw the introduction of the Indo-Pacific lionfishes (*Pterois volitans and P. miles*) into the Western Central Atlantic (WCA). Their subsequent spread from the US east coast to Bermuda, Bahamas and the entire Caribbean region crossing several marine connectivity barriers has sparked significant amounts of research. A strong founder effect has been noted in the WCA populations of both species which show low genetic diversity when compared to the native population of Western Indonesia. A secondary founder had been observed with only three or four d-loop haplotypes found in the north-western and central Caribbean populations (Grand Cayman, San Andrés, Santa Marta) compared to nine in the northern WCA group (North Carolina, Bahamas and Bermuda). This study therefore represents the first report of the application of DNA sequencing to determine the species and haplotypic composition of the invasive lionfish in the Eastern Caribbean (EC).

Lionfish were collected in Barbados from the first reported arrival (24 Nov, 2011) until September 2013. DNA was extracted from muscle tissue and subjected to mitochondrial d-loop sequencing. As expected, the 178 sequences all aligned to those of the red lionfish (*Pterios volitans*) confirming our expectation that to date, Barbados has only been invaded by a single species. Consistent with other WCA populations, the genetic variation of the Barbados population was low (haplotype diversity = 0.4780), with the population comprised of six haplotypes (H01-H05 and H07), with haplotypes H01 and H02 dominant (30.3% and 65.7% respectively), which is also consistent with other WCA populations. The remaining haplotypes were comparably rare, each being found in less than 3% of the sample population.

AMOVA analyses indicated that there was no significant difference between the Year 1 pioneer (comprising H01, H02 and H04) and Year 2 established (comprising H01, H02, HO3, H04, H05 and H07) populations in Barbados ($p = 0.91007$), nor among the island's three coastal regions ($p = 0.69208$), indicating a single homogenous population. Additionally, statistical analyses showed significant differences between both the established Barbados population and the Bahamas and Santa Marta populations ($p = 0.00000$ and $p = 0.04008$, respectively), suggesting that neither population served as the sole source population. Significant differences ($p = 0.00098$) were also observed between the pioneer population and the Bahamas population, while on the other hand there was no significant difference when compared to the Santa Marta population ($p = 0.32356$), indicating that the initial invasion likely originated from the south with a subsequent invasion pulse from the north. A more extensive genetic analysis using samples from more locations within the EC is required to better resolve the route of invasion and relatedness of island populations.

Key words: Lionfish, invasive species, mitochondrial control region, *Pterois volitans*, Barbados

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1 INTRODUCTION

An invasive species is a species that is not native to a habitat and whose introduction is likely to result in economic or environmental harm or harm to human health. Alien species are recognized as major threats to ecosystems, causing dramatic effects on biodiversity and habitat composition (Mack et al. 2000). Invasive species are a leading cause of biodiversity loss and while invasive marine fishes are fairly uncommon and their ecological effects largely unknown, introductions of predatory freshwater fishes have often proven to be devastating to native communities (Helfman 2007 cited by Albins and Hixon 2008).

The oceans offer various means to disperse individuals within and among populations with eggs or larvae being the primary dispersal phase for most species, while in others both juveniles and adults disperse. Combining dispersal and factors leading to survival of the dispersed organisms results in the concept of population connectivity (Cowen and Sponaugle 2009). Cowen, Paris, and Srinivasan (2006) developed biophysical models to identify connectivity patterns for the larvae of reef fishes and the proposed connectivity scenarios included: between the east coast of the US and Bermuda by way of the Gulf Stream; high connectivity in the northern Caribbean in the area of the Bahamas and the Turks and Caicos Islands with minor exchange with northern Cuba and Hispaniola; a north-western Caribbean barrier, isolating the area of the Mesoamerican Barrier Reef System, southern Cuba, and the Cayman Islands from the rest of the Caribbean; the Eastern Caribbean break which occurs in the north between Puerto Rico and Hispaniola and Colombia in the south; and isolation of the reefs along the Panama-Colombia Gyre from the rest of the Caribbean. Population connectivity through larval dispersal has produced biogeographic patterns within the Caribbean and four defined regions of connectivity according to Cowen, Paris, and Srinivasan (2006) have emerged: the Eastern Caribbean, the Bahamas and the Turks and Caicos Islands (TCI), the western Caribbean, and the region at the border of the Colombia-Panama Gyre. The area of Hispaniola and Jamaica is a zone of mixing among many of the above regions. In a 2009 study, Cowen and Sponaugle revealed the degree of connection between Caribbean populations through larval dispersal using a geographical representation. The region has fairly confined levels of successful dispersal leading to population connectivity (Figure 1; Appendix 1), with various source locations (the Bahamas, TCI, Jamaica, Nicaragua, Panama, Colombia and the Lesser Antilles).

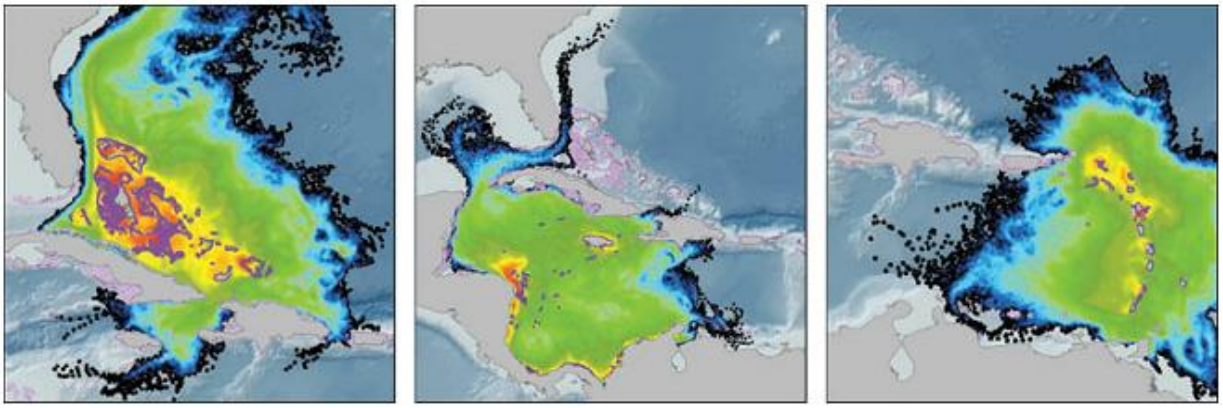


Figure 1. Geographical representation of dispersal in the Caribbean (Cowen and Sponaugle 2009). Red and orange areas represent ecologically significant levels of exchange while green and blue show areas of very low levels of genetically relevant exchange. Source locations are shown in purple, and potential receiving areas are shown in pink.

The models produced in these studies may provide insight into the larval dispersal of lionfish and may be valuable for predicting the spread of lionfish, as according to Cowen, Paris, and Srinivasan (2006), the rates, scale, and spatial structure of successful exchange or connectivity among local marine populations, drives population replenishment and therefore, have profound implications for the spread of invasive species.

1.1 The Invasion

Lionfishes, the first non-native marine fishes to invade the Caribbean, have the potential to add additional stress to a coral reef environment already compromised by overfishing, pollution and global climate change (Schofield 2010). The Caribbean lionfish invasion is unprecedented not only because they are the first non-native marine fishes to invade and become established in the region, but because of the remarkable speed with which they have spread. This invasion illustrates the speed with which non-native marine fishes are able to spread through new coastal systems Johnston and Purkis (2011), used the lionfish reported sightings (recorded by the USGS) to show that the invasion apparently occurred in three stages (Figure 2). Stage one began in south Florida and then spread to North Carolina, Bermuda and finally New York and New Jersey until the end of 2004 when they were first sighted in the Bahamas. The source of dispersal to the Bahamas likely originated from the east coast of Florida populations as indicated by genetic analyses which have linked the two populations (Freshwater et al. 2009). Stage two ensued in 2004 in a south and easterly direction from the central Bahamas and once lionfish were established in the Bahamas, dispersal carried on southward into the entire Caribbean including South America by 2009 and stage three began when recruitment occurred in the Florida Keys. After lionfish were first sighted in Aruba in 2009, they dispersed in a west and easterly direction to Bonaire, Curaçao just off the Venezuelan coast, to off the Columbian coasts and Panama (Figure 2). However, the subsequent invasion of the up-wind, up-current Lesser Antilles island chain is not considered by Johnston and Purkis (2011) (Figure 2) and the source of this phase is unknown. In 2010, lionfish were first sighted in the British Virgin Islands and dispersal

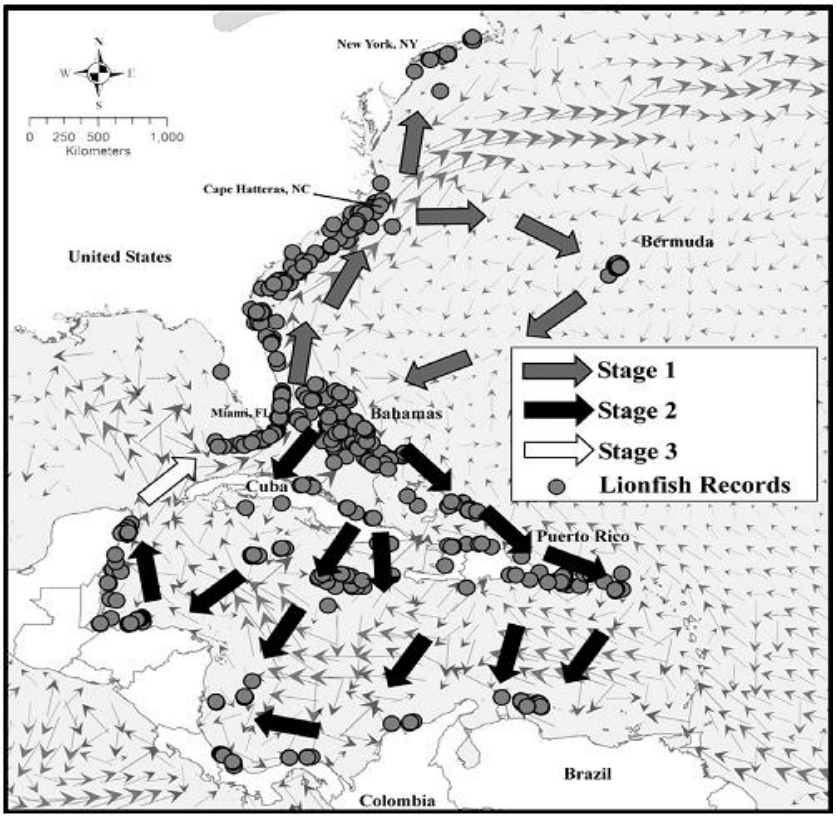


Figure 2. Three Stage Invasion Map (Johnston and Purkis 2011). Stage one was mainly driven by currents (dark grey arrows), stage two was more circular and proximity based (black arrows), and stage three (white arrow) was current driven and represents a return back to the likely point of origin.

proceeded southward into the Lesser Antilles, with the entire island chain being invaded over the following two years. The last two reported invasions occurred in the most easterly islands of Barbados in November 2011 and Tobago in February 2012.

The two closely related lionfish species, *Pterois volitans* [Linnaeus, 1758] and *Pterois miles* [Bennett, 1828], are upper trophic level predators, both native to the Indo-Pacific including the Red Sea, the Indian Ocean, and the western Pacific, and are currently considered amongst the most successful marine invaders in the history of aquatic invasions (Albins and Hixon 2008). The global aquarium trade has been identified as the source of the invasive lionfishes in the Western Central Atlantic (Freshwater et al. 2009). They are thought to have been introduced in the early 1990s, although it appears that the first lionfish was in fact caught in 1985 north of Miami (Morris and Akins 2009).While lionfishes are conspicuous and easy to spot in the field, *P. miles* and *P. volitans* are indistinguishable morphologically (González et al. 2009).

Lionfishes have significant dispersal capabilities as they release free-floating gelatinous egg masses which develop into planktonic larvae (Morris et al. 2009; Betancur et al. 2011). Surface ocean currents during this early life stage are responsible for their dispersal (Freshwater et al. 2009) as it is the link between larval dispersal and population connectivity as ocean currents not only control the dispersal of individuals but also connect populations. Freshwater et al. (2009), suggested that the floating egg mass may not only increase the efficiency and extensive dispersal of the species but it may also improve their survivorship by reducing predation. The characteristic floating egg mass coupled with year-round spawning is likely a major contributor to the rapid and widespread dispersal of lionfish and the resulting invasion of the United States east coast and Caribbean (Bariche, Torres, and Azzurro 2013; Freshwater et al. 2009) (Figure 3). Less than 30 years since it was first sighted off the coast of Florida, the non-native red lionfish, *P. volitans*, has covered most of the Western Central Atlantic ocean (Santander-Monsalvo et al. 2012). The invasion across the Caribbean has been well documented, yielding the most recent invasion map (Figure 3). Freshwater et al. (2009) and Santander-Monsalvo et al. (2012) agree that the continued expansion may be naturally self-sustaining through larval dispersal, since its larval phase lasts 25 to 40 days.

The first known sighting of lionfish in Barbados was November 15th, 2011and within eight months there were ten reported sightings and today over one dozen sighting are made weekly ([1]S. Browne. pers. comm.). Using sea surface temperature as the only limiting factor, lionfish distribution models predict that its range in the Western Central Atlantic (WCA) will continue southward reaching southern Brazil (Morris and Whitfield 2009) although this prediction has recently been questioned (Luiz et al. 2013). The US Geological Survey Nonindigenous Aquatic Species database (USGS-NAS; http://nas.er.usgs.gov/) has documented the chronology and extent of the lionfish invasion based on confirmed occurrences and as of 2013, lionfish have invaded the entirety of coastal waters throughout the wider Caribbean, Gulf of Mexico and the Southeast United States of America (Figure 3).

[1] Browne, S (June 12th 2013). Hightide Watersports.

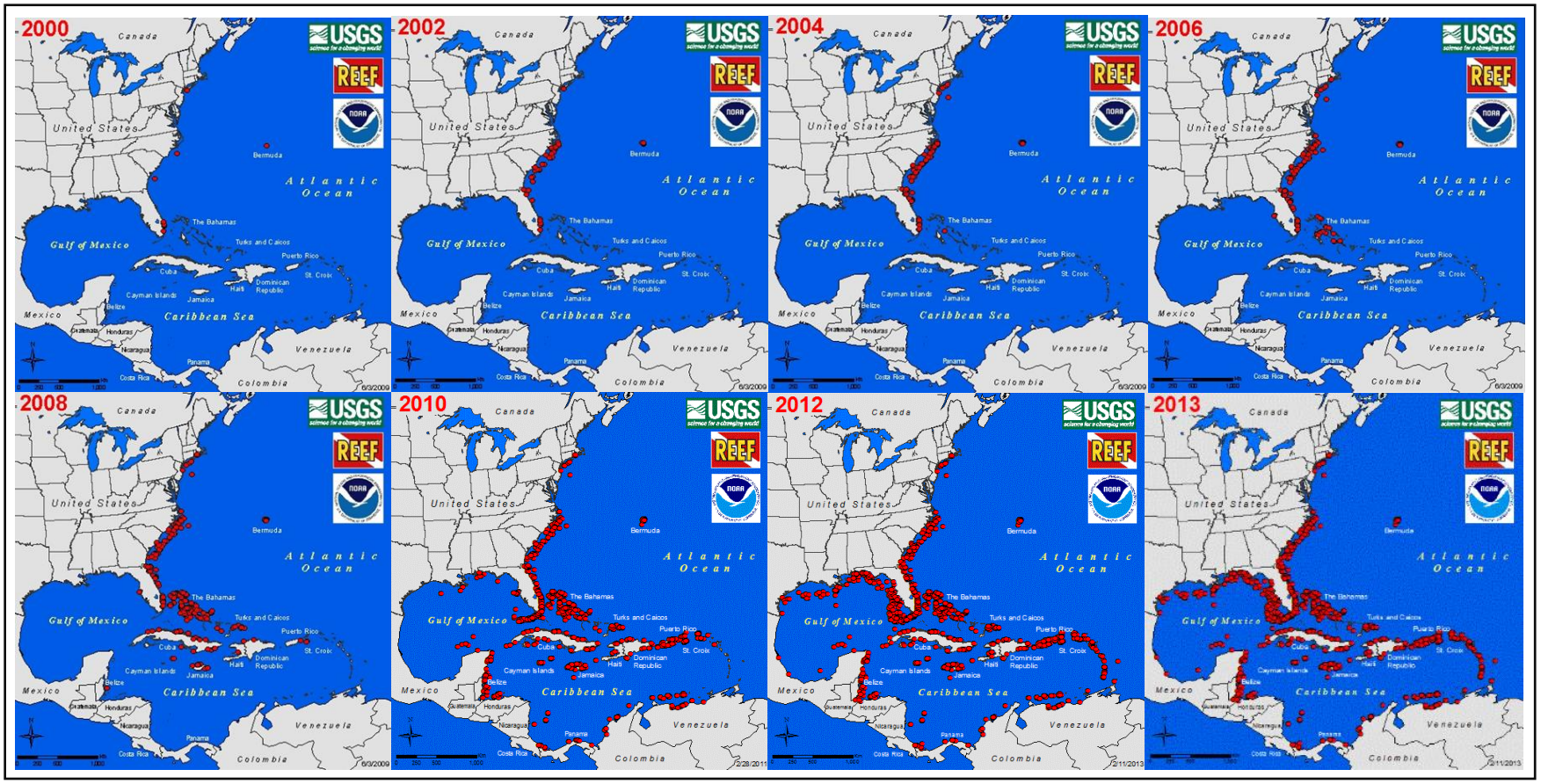


Figure 3. Chronological occurrences of lionfish (*Pterois volitans* and *P. miles*) in the Western Central Atlantic from 2000 - 2013 (Schofield et al. 2013)

1.2 The Lionfish Threat

The progress and scope of lionfish invasions has garnered an appropriate response investigating the biology and ecology of the two species (Bariche, Torres, and Azzurro 2013). Various studies have documented the negative impacts of the invasion on the diversity and function of native communities as lionfish have become a high-risk threat both ecologically and economically in the Wider Caribbean region. The Green et al. (2012) study in the Bahamas documented an increase in lionfish abundance that coincided with a 65% reduction in the biomass of small-bodied fishes (42 fish species) over a time frame of just two years.

Lionfish are considered to represent a significant threat to the coral-reef ecosystems of the Caribbean by decreasing survival of native reef animals not only through predation but also competition, with unconfirmed estimates suggesting that invasive lionfish may consume as much as half their body weight daily. This feeding behaviour of lionfish may result in high competition and predation efficiency and ultimately large ecological effects on prey species and on potential competitors when comparing its native range to the invaded system (Albins and Hixon 2008). One major ecological effect being the alteration of food webs leading to ecosystem degradation, as according to Lesser and Slattery (2011) these voracious predators, through their predation on herbivorous reef fish especially, may reduce herbivore populations to such an extent that a phase shift to an algal dominated coral community may result. Furthermore, not only are lionfish voracious predators that overpopulate reefs, they also display aggressive tendencies and force native species into waters which may be sub-optimum habitats (Whitfield *et al*. 2007).

Furthermore, lionfish envenomation constitutes a serious health emergency for humans, requiring immediate treatment, and therefore posing an additional social burden as the invasion has spread across the region. Badillo et al. (2012) reported that in humans, lionfish venom has been found to have many effects ranging from mild reactions, including swelling, dizziness, local numbness and sweatiness to rare, but more serious symptoms including nausea, vomiting, extreme abdominal pain, temporary paralysis of the limbs, loss of consciousness, heart complications and even death. However, according to Morris and Green (2012), the severity of the reaction to a sting depends on several factors including the amount of venom delivered, the immune system of the victim (very young children, the elderly, persons with a weak immune system or those allergic to the venom), and the location of the sting.

1.3 Genetics

Populations of marine fish species are normally considered to have high levels of gene flow and exhibit little population structure. However variations in dispersal capabilities of adults and early life-history stages will result in population differentiation within some species (Ravago-Gotanco and Juinio-Menez 2004). In the case of lionfish, genetic research has already become a powerful tool for assessing various dispersion pathways, divergence of sub-populations, expression of phenotypes that are driving invasiveness, and possibly detection of new introduction events (Morris and Green 2012).

Several genetic studies have analysed the United States east coast lionfish invasion. Haplotypes derived from a relatively conservative genetic marker (879 bp sequence from the cytochrome *b* locus of mtDNA) have been used to distinguish between the two morphologically indistinguishable lionfish species and have determined that both *P. miles* and *P. volitans* are involved in the invasion of the WCA region (Hamner, Freshwater, and Whitfield 2007;

Freshwater et al. 2009) with 93% of specimens from the east coast of the USA being identified as *P. volitans* and the remaining 7% as *P. miles* (Hamner, Freshwater, and Whitfield 2007; Freshwater et al. 2009). Additionally, cytochrome *b* (cyt *b*) sequences have revealed that the two species are characterized by low genetic diversity owing to a founder effect, with only three *P. volitans* haplotypes present in the invading population (n=158 samples) compared with 25 haplotypes (n=77 samples) found in *P. volitans* from their native range, and 1 haplotype (n=12 samples) found in *P. miles* compared with 12 (n=22 samples) from their native range (Hamner, Freshwater, and Whitfield 2007). A subsequent study has revealed that only *P. volitans* appears to have spread to the western (Cayman islands and San Andrés archipelago) and southern Caribbean (Santa Marta, Colombia) and that a secondary founder effect has occurred with their continued dispersal into the Caribbean (Betancur *et al*. 2011). However, no specimens from the Eastern Caribbean's Lesser Antilles island chain have yet been analysed, and therefore the species and genetic composition of lionfish in the Eastern Caribbean sub-region remains unknown, and the source population has not been determined.

Despite cyt *b* being an important marker for species determination, the barcodes based on the sequences of the cytochrome oxidase I (COI), are becoming a wider standard in species identification (Valdez-Moreno et al. 2012).

Although initial studies of the WCA lionfish invasion were based on sequence analysis of the mitochondrial cyt b gene (Hamner, Freshwater, and Whitfield 2007; Freshwater et al. 2009) to differentiate among lionfish species and examine the genetic basis for different phenotypes (Morris and Freshwater 2008), subsequent studies examining relatedness among invasive lionfish populations in the WCA have used a 680 bp sequence from the less conserved mitochondrial control region (the d-loop). These studies have revealed the presence of nine mtDNA d-loop sequence haplotypes in the WCA lionfish populations compared with 36 from native lionfish (Betancur et al. 2011).

As the lionfish invasion continues, genetic research will continue to be a powerful tool for assessing various dispersion pathways and divergence of sub-populations, and will allow the study of expression of phenotypes that are driving invasiveness, and possibly detection of new introduction events (Morris and Green 2012).

2 PURPOSE

Understanding patterns of marine connectivity and the implications for marine organisms is essential for the understanding population dynamics of all marine species (Betancur *et al*. 2011). The lionfish invasion has provided an opportunity to examine the pattern of marine connectivity within the WCA by observing how they are dispersing in their new ecosystem. Incorporating the order of events of the lionfish invasion using sightings with population genetics has provided not only an assessment of dispersal but also of marine connectivity across the phylogenetic breaks separating the USA east coast, the Bahamas, the northwest and southwest Caribbean (Betancur et al. 2011) but this has not been examined for the Eastern Caribbean biogeographic zone.

Genetic analyses of the invasive lionfish in Barbados to assess mtDNA d-loop haplotype diversity will aid in filling this gap in the understanding of the invasion and connectivity of

WCA biogeographic zones, by supporting a broader study of lionfish from several locations in the Eastern Caribbean ([2]Ximena Velez-Zuazo, pers. comm.).

3 RESEARCH AIM

This project aims to expand upon previous genetic studies of the invasive lionfish by adding data on the lionfish population now established in Barbados.

3.1 Objectives

This research will undertake a detailed examination of the lionfish invasion in Barbados to help determine the spatial and/or temporal distribution of successful settlers originating from one or more source populations. The specific objectives are to:

- Map the chronology of the lionfish invasion using reported sightings.
- Determine the lionfish species composition in Barbados using the mitochondrial control region (d-loop)
- Determine the mtDNA d-loop haplotype diversity of the first arrivals.
- Determine the mtDNA d-loop haplotype diversity of the lionfish population currently established in Barbados.
- Compare the mtDNA d-loop haplotype diversity of the first arrivals with lionfish currently established in Barbados.
- Compare the mtDNA d-loop haplotype diversity of lionfish from the west, south and east coasts of Barbados.
- Determine the likely source population (s) of the invasive lionfish using mtDNA d-loop haplotypes

4 STUDY DESIGN AND METHOD

4.1 Lionfish Sightings and Sample Collection

This study collected lionfish for DNA analysis from around the island of Barbados from the first reported sighting in November 2011 up to September 2013. For the first year of the lionfish invasion (November 24, 2011 to November 15, 2012) The Barbados Lionfish Project conducted by the Centre for Resource Management and Environmental Studies (CERMES) of the University of the West Indies, in collaboration with partners; the Coastal Zone Management Unit (CZMU) and the Fisheries Division (FD); Oxenford, Phillips, and Valles (2013) launched a 24 hour telephone hotline to handle reports of lionfish sightings as well as set up a Facebook page where persons could post reports of sightings and/or catches. All reports were recorded on standardised lionfish sightings forms (Appendix 2) and all collected specimens were stored at the Fisheries Division for later analysis. The first 40 lionfish collected were considered to represent the 'pioneer population' or 'first arrivals'.

During the second year of the invasion (November 20, 2012 to September 30, 2013) a few specimens were still being handed over to the Lionfish Project staff and stored frozen, although public interest in voluntary reporting and donation of samples had declined considerably. As

[2] Ximena Velez-Zuaz

such, a renewed sampling effort was initiated from June to September 2013 to ensure a sample size of approximately 50 specimens from each distinct coast (west, south and east). This second year sample was considered to represent the 'established population'. These lionfish were obtained directly from local fishers, recreational divers and dive operators, as well as from our own culling dives independently and in association with the CZMU. Specimens were collected using spearguns, small spears such as the 'Hawaiian sling' and a Zookeeper "Lionfish Containment Unit" or other home-made bucket device while using SCUBA gear or free diving (Figure 4). Whole samples were either stored on ice and subsequently frozen at –80°C until tissue sampling, or a small piece (approximately 1 cm^3) of muscle tissue from the caudal peduncle was dissected immediately and preserved in 15 mL Falcon™ Centrifuge Tubes containing 5 mL of 20% DESS buffer (DMSO/EDTA/NaCl).

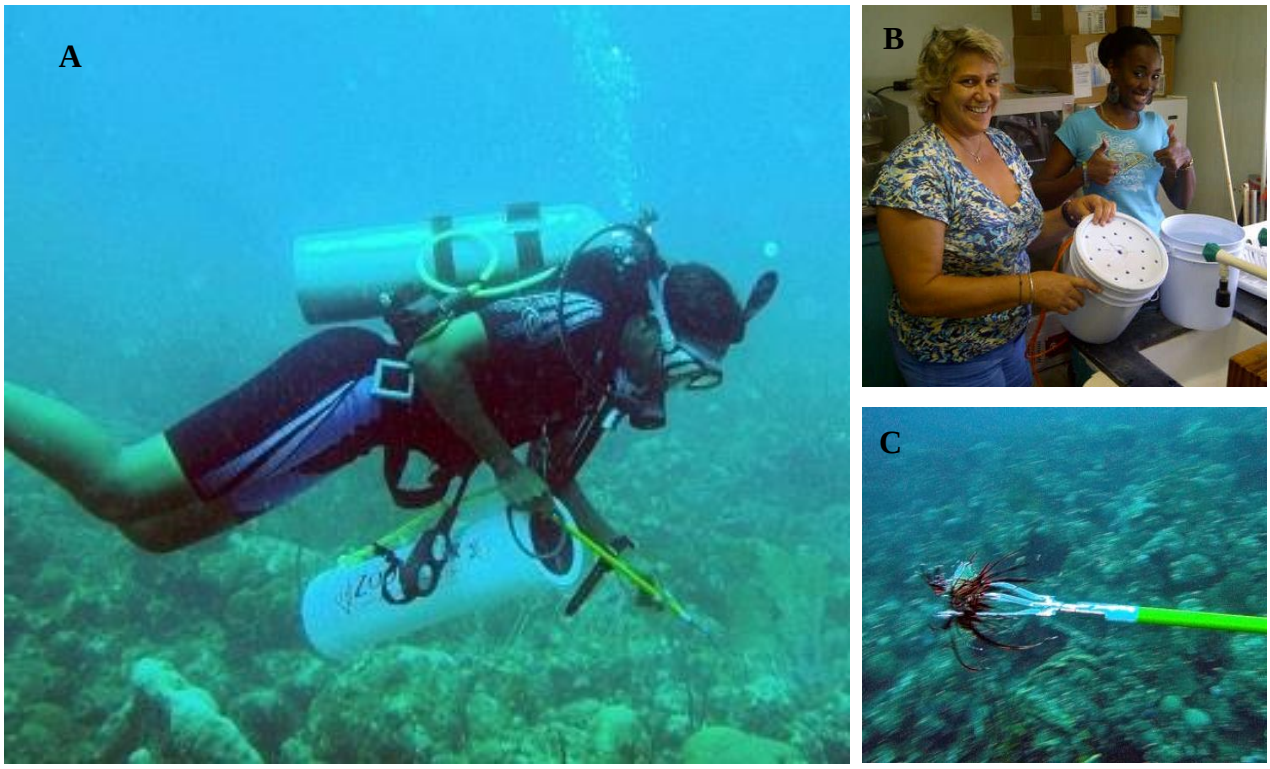


Figure 4.Photographs showing (a) author on a lionfish culling dive with a 'Hawaiian sling' and a Zookeeper "Lionfish Containment Unit" (b), home-made bucket device for containment of speared lionfish, and (c), speared lionfish on the reef

4.2 Biological Data Collection

Upon collection of whole specimens, size data were recorded for all pioneer and established population samples. This included taking total and standard length measurements to the nearest mm on a fish measuring board, wet weight to the nearest gram on a desktop balance or digital field balance, and gape height and width measurements to the nearest mm (Figure 5).

Figure 5. Collecting size data of the lionfish specimens caught in Barbados

A full dissection was performed by The Lionfish Project researchers on a sub-sample of the pioneer population to examine gender and maturity stage, following the detailed dissection guide of Green, Akins, and Morris (2012). All data were entered into a Microsoft Excel worksheet.

4.3 DNA Extraction

Total DNA was extracted from 0.05 – 0.1 g of fresh, frozen or DESS buffer-preserved muscle tissue. Approximately 40% of samples were processed using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol and the remaining 60% of samples were extracted using a standard phenol/chloroform extraction technique adapted from Bello, Francino, and Sánchez (2001). Samples were placed in 1.5-mL microcentrifuge (Eppendorf) tubes containing 500 µL of lysis buffer [(10 mM Tris-HCl, 125 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 and 2% sodium dodecyl sulfate (SDS)]. 20 µl of Ribonuclease A was added to each tube and the tissues were incubated at 37°C for 1 hour after which 50 µl of Proteinase K was added and lysis was performed at 55°C overnight. Following lysis, the samples were vigorously vortexed and centrifuged in an Eppendorf 5417C Micro Centrifuge at 14,000 rpm for one minute to remove undigested debris. The resulting supernatant was transferred to a clean 1.5 mL Eppendorf tube, and DNA was extracted twice with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1). The final aqueous layer was transferred to a fresh tube 1.5 mL Eppendorf tube and DNA was precipitated by adding 50 µL of 5M NaCl and 2 volumes of absolute ethanol followed by an overnight incubation at −20°C. DNA was recovered by centrifugation at 14,000 rpm for one minute, after which the supernatant was discarded. The pellet was washed briefly with 70% ethanol and centrifuged at 14,000 rpm for two minutes after which the ethanol was poured off. The DNA pellet was allowed to air dry and resuspended in 100 µL of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and stored at −20°C.

4.4 Polymerase Chain Reaction (PCR)

The d-loop region of lionfish DNA samples were amplified on a MJ Research PTC-225 Peltier Thermal Cycler DNA Engine by allele specific PCR with the following reaction conditions: 5x

GoTaq® Flexi buffer, 1.25mM $MgCl_2$, 0.25mM each dNTP, 2.5pmol each forward and reverse primers [LionA-H (5-CCA TCT TAA CAT CTT CAG TG-3) and LionB-L (5-CAT ATC AAT ATG ATC TCA GTAC-3)] (Betancur et al. 2011), 0.8 Units GoTaq® Flexi DNA Polymerase (Promega) and 10-50ng Template DNA in a final volume of 20 µL. The thermocycling protocol outlined in Freshwater et al. (2000) was modified and reactions were performed with an initial denaturation step at 94ºC for 10 minutes, followed by 35 cycles of 94ºC for 30 seconds, 53ºC for 30 seconds, ramp to 72ºC at 0.2ºC per second and a final extension at 72ºC for 5 minutes. 5 µL of each PCR product was visualized by electrophoresis on 1.5% agarose gels supplemented with SYBR Green (I) DNA Stain (Invitrogen) with 1× TAE buffer at an applied voltage of 10 V/cm for 30 minutes. Amplicons were visualized using a FBTIV-88 variable intensity transilluminator (FisherBiotech) (Figure 6).

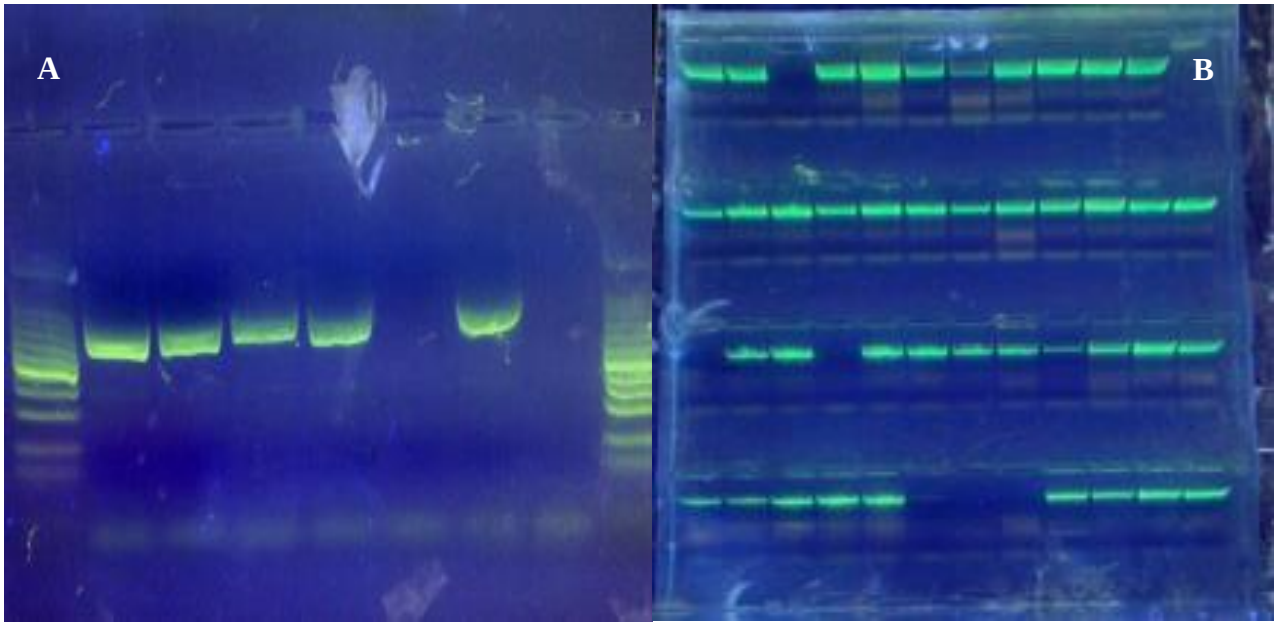


Figure 6. Amplified PCR products on 1.5% agarose gels using LionA-H and LionB-L primers. Photographs showing: (a) 1000 bp marker (lanes 1 and 9), fluorescent bands showing ~800 bp amplicons (lanes 2-5), negative control (lane 6) and positive control (lane 7), and (b) Fluorescent bands showing 800 bp amplicons. Blanks indicate samples in which DNA was not successfully amplified.

Following amplification, amplicons were cleaned of excess dNTPs and primer following the protocol of Werle et al. (1994). A 10 µL PCR amplicon was incubated with a mix of 20 Units Exonuclease I (Fermentas) and 2 Units FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) at 37ºC for 15 minutes followed by 85ºC for 15 minutes.

4.5 Sequence and Haplotype Analysis

Sequencing of all cleaned amplicons was performed by Molecular Cloning Laboratories (California, USA). Sequences for each specimen were compiled and aligned using a biological sequence alignment editor; BioEdit (Hall 1999) and matched against published mtDNA control region sequences in the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI). The identification of lionfish specimens as either *P. miles* or *P. volitans* was made based on species-specific differences observed in the alignment of sequences. Distinct d-loop haplotypes were identified by alignment with registered sequences. Haplotype diversity (Nei 1987 cited by Betancur et al. 2011), nucleotide diversity (π, Nei, 1987),

and sequence diversity (k, Tajima, 1989 cited by Betancur et al. 2011) were examined using Arlequin v. 3.5.1.3 (Excoffier and Lischer 2010). Haplotype frequencies between the pioneer and established population samples as well as among coastlines (west, south and east) were compared using an analysis of molecular variance (AMOVA).

4.6 Quality Control and Assessment

The risk of sample contamination or cross contamination from DNA extraction or PCR amplification was reduced by performing all tissue extractions in a separate space from where PCR reactions were set up, additionally, all surfaces were routinely wiped with 95% ethanol and DNA Away (Molecular BioProducts). All manipulations were carried out with sterile equipment and gloves were changed regularly. Following DNA extractions, the analysis of the quantity and quality of DNA was undertaken to determine the amount of DNA obtained and if the quality was high enough for PCR amplification. Gel electrophoresis was used to quantify the amount of DNA extracted in this study by comparing the intensity of the fluorescence of the DNA samples to that of a Lambda EcoRI/HindIII ladder. Additionally a positive control and a tube with no template DNA (a negative control) were always utilized during PCR reactions (Figure 6a).

5 THE INVASIVE LIONFISH IN BARBADOS

5.1 Number of Sightings

The first lionfish was reported and confirmed in Barbados on November 24th, 2011. Sporadic sightings and collections continued over the first year to November 15th, 2012 with only 6 specimens reported after the first six months (May 24th 2012) of the invasion. Eight months after the first reported and confirmed sighting, the rate of confirmed lionfish sightings around the island drastically increased from a total of 8 lionfish in July 2012 to 54 confirmed lionfish sightings by November 15th 2012 (Figure 7).

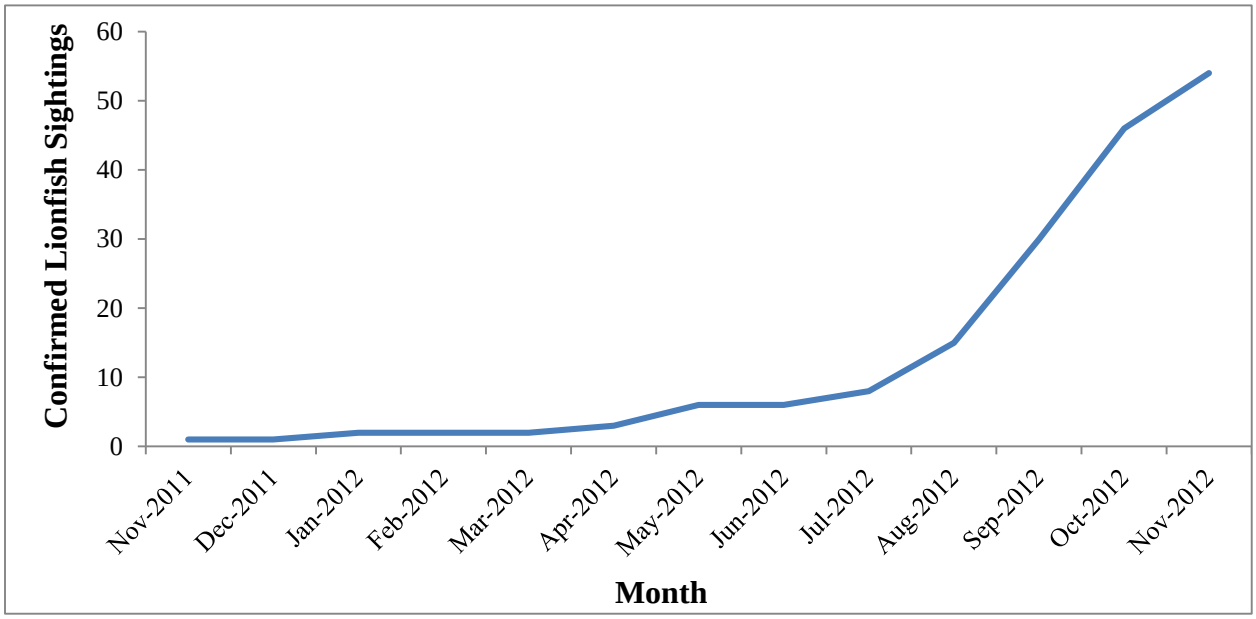


Figure 7. Lionfish arrivals in Barbados, shown as cumulative number of confirmed lionfish sightings per month over the first year (24 Nov 2011 – 15 Nov 2012)

Over the second year, official reported sightings to The Lionfish Project via the hotline declined as resource users became accustomed to seeing and catching the invasive fish. Anecdotal reports

and observations indicated frequent sightings, but the lionfish were either killed and left on the reef by divers and fishers, or were retained for consumption.

5.2 Geographical Extent

The first reported lionfish was from the northern section of the west coast. The next four reports indicated a gradual spread down the sheltered leeward west coast and by the November 15th 2012, lionfish had been found along the entire west coast, the western part of the south coast and one had also been captured off the exposed windward east coast (Figure 8). However, there had been no reports from the Eastern section of the south coast up to the end of the first year.

Lionfish can now be found around the entire island, and the approximate locations of all those sampled from the established population for this research are shown in Figure 9.

5.3 Biological Characteristics

5.3.1 Maturity and Gender

The gonads of 28 of the first arrivals were examined. The majority of the specimens (64%) were immature, while 18% were in the early developing stage or were capable of spawning. According to Morris (2009), lionfish females mature around 180 mm total length (TL), while male lionfish mature at approximately 100 mm TL and the five specimens capable of spawning were all in excess of 180 mm and therefore confirmed this.

5.3.2 Size

5.3.2.1 First Arrivals

Total length (TL) of the first arrivals ranged from 59 mm to 285 mm (average length = 154 mm TL) and the average weight of the specimens was 64 g ranging from 2.1 g to 294.9 g (Figure 10). This pioneer population was clearly young, essentially comprising a single immature cohort and indicating the invasion was by the early life history stages and not adults.

5.3.2.2 Established Population

Lionfish specimens collected from the established population in the second year of the invasion ranged in size from 68 mm to 369 mm (average length = 212 mm TL) and from 4 g to 799 g (average weight = 188 g) (Figure 11). The established population comprised two clear cohorts, an immature group of small individuals (average length = 165 mm TL), and an adult group of larger individuals (average length = 283 mm TL), indicating a discrete annual recruitment.

The strong length-weight relationship for all lionfish samples to date is shown in Figure 12.

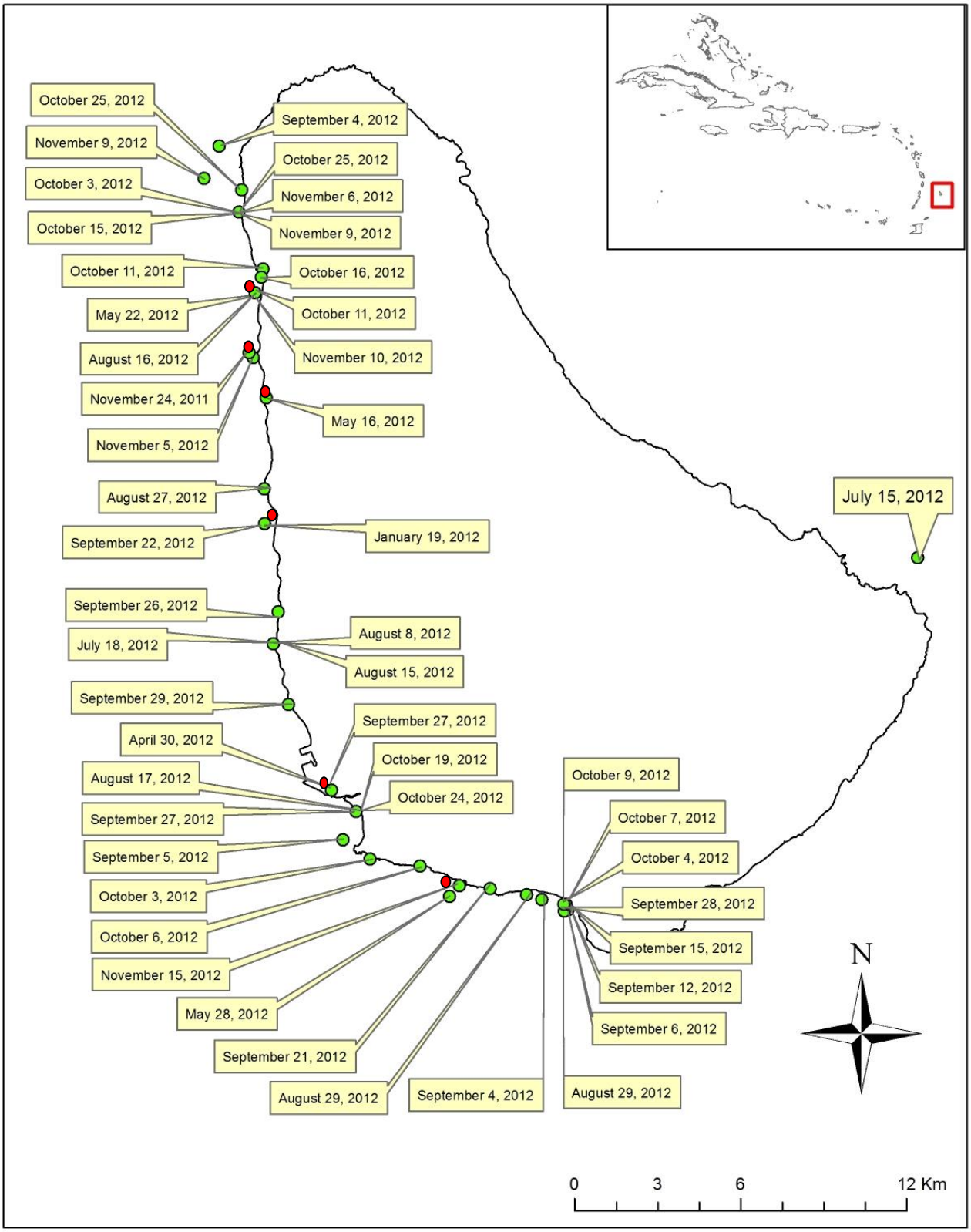


Figure 8. Dates and reported locations of all confirmed lionfish sightings (n = 54) over the first year of the invasion (24 Nov 2011 - 15 Nov 2012) in Barbados. Arrivals over the first six months are shown in red. Inset map shows the location of Barbados in the Eastern Caribbean

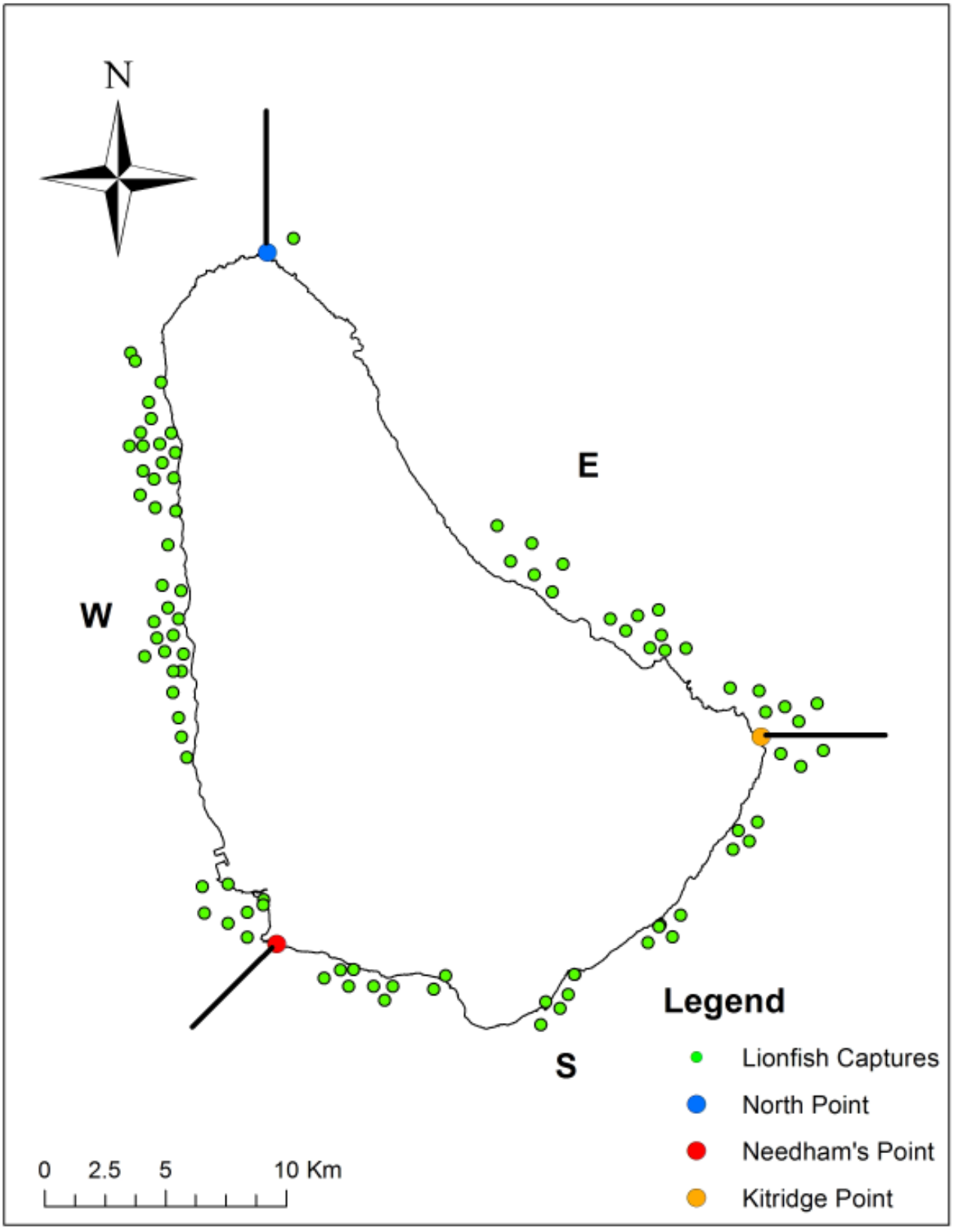
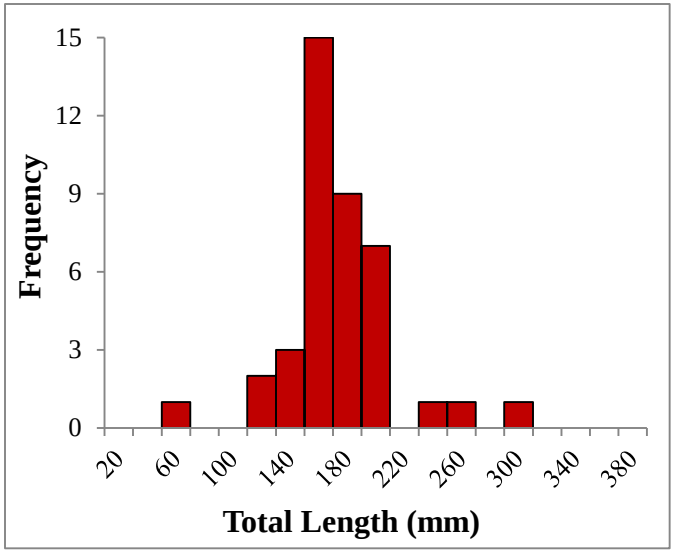


Figure 9. Approximate capture locations for all lionfish sampled from the established population (n = 163) during the second year of the invasion (20 Nov 2012 – 30 Sep 2013) in Barbados.



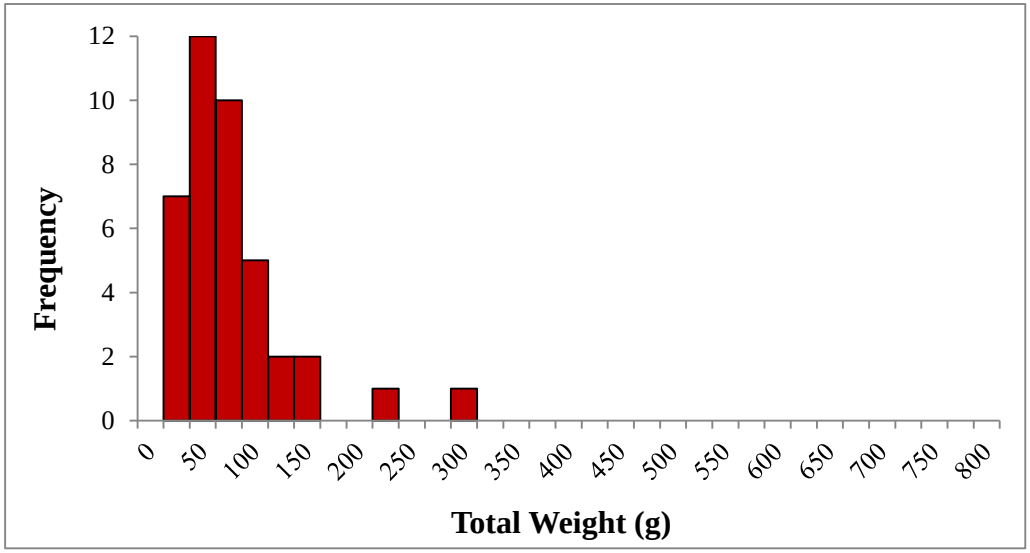
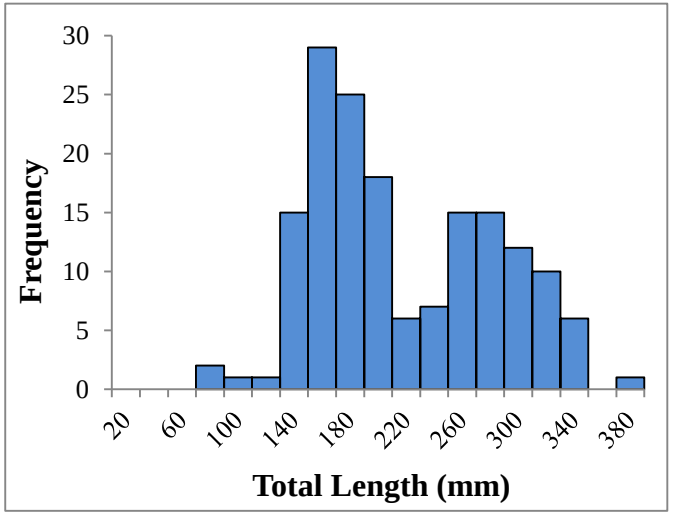


Figure 10. Size frequency distribution of captured first arrivals in Barbados (24 Nov 2011 - 15 Nov 2012)



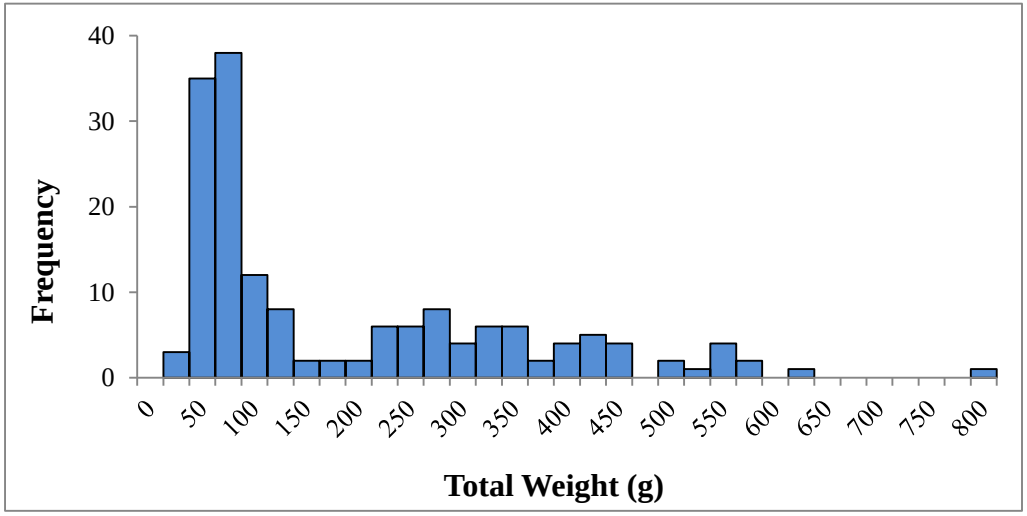


Figure 11. Size frequency distribution of captured lionfish of the established population (20 Nov 2012 - 30 Sep 2013)

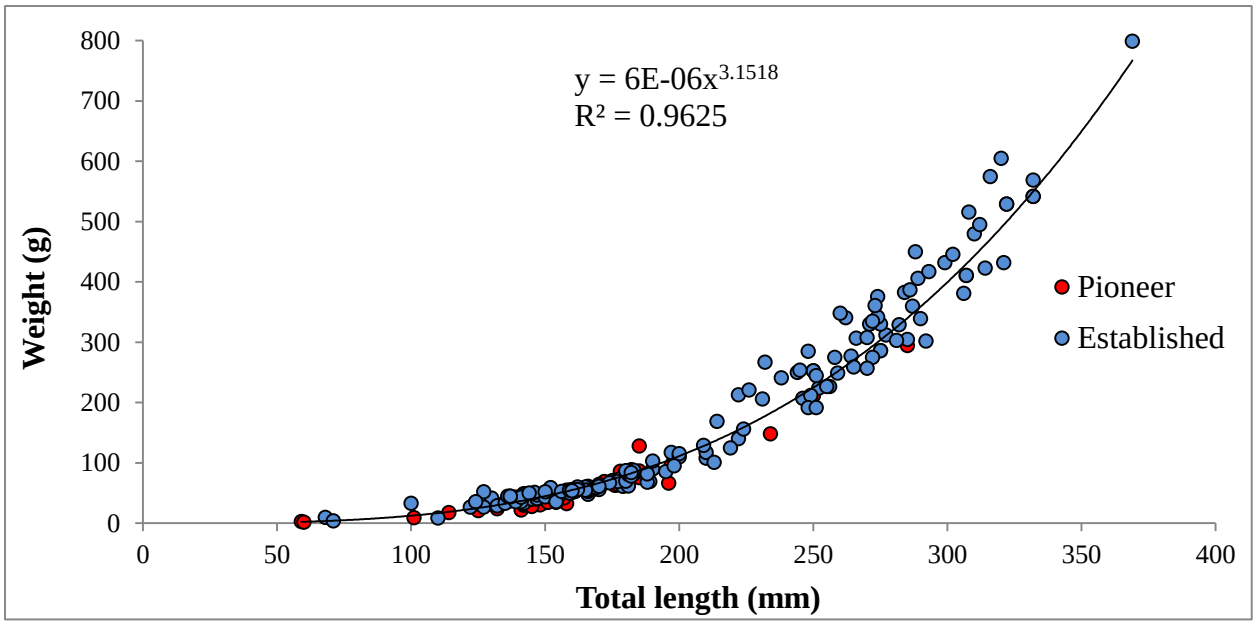


Figure 12. Length - weight relationship for all captured lionfish in Barbados (Nov 2011 - Sep 2013) showing individuals from the pioneer and established populations separately.

6 DNA CHARACTERIZATIONS OF THE INVASIVE LIONFISH IN BARBADOS

6.1 Species Identification

This study analysed 680 base pairs of the mitochondrial d-loop region in 178 invasive Barbados lionfish specimens. Sequences complemented existing mtDNA datasets for the red lionfish *P. volitans* previously obtained from the Bahamas by Freshwater et al. (2009) with over 99% similarity, thus indicating that all individuals from Barbados are *P. volitans*. In the 178 *P. volitans* specimens, ten polymorphic sites were detected, yielding six haplotypes (Figure 13; Appendix 3).

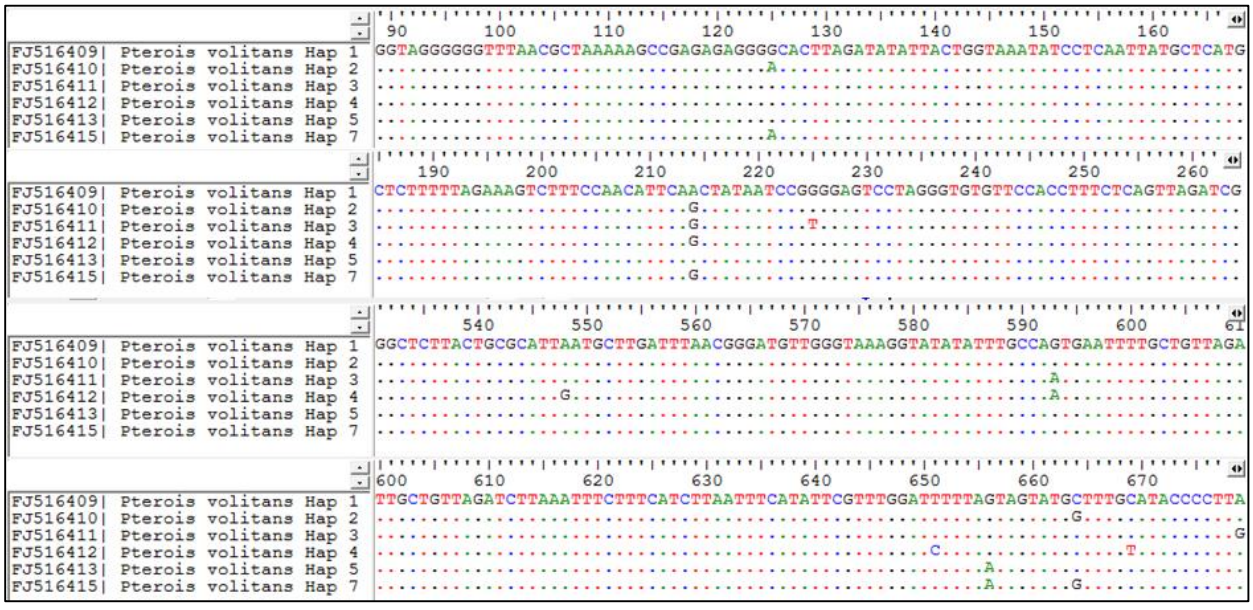


Figure 13. Screenshot of polymorphic sites for each of the six lionfish haplotypes identified in BioEdit

6.2 Haplotype Analysis

Six lionfish haplotypes (H01, H02, H03, H04, H05 and H07) registered by Freshwater et al. (2009) (680 bp; GenBank accession numbers FJ516409–FJ516413 and FJ516415) were identified in the 178 Barbados *P. volitans* specimens. The majority of the invasive lionfish exhibited one of the two dominant Western Central Atlantic (WCA) haplotypes (H01 = 30.3%, H02 = 65.7%) as shown in Table 1.

Table 1. *P. volitans* haplotypic composition summary for Barbados based on d-loop sequences

Haplotypes	H01	H02	H03	H04	H05	H07	Total
Number of lionfish	54	117	1	4	1	1	178
Haplotype percentage	30.3	65.7	0.6	2.2	0.6	0.6	100

Reported mtDNA d-loop haplotypes for the *P. volitans* populations in the WCA were compared (Table 2; Appendix 5) and the measure of genetic variations calculated (haplotype, nucleotide and sequence diversity) were lower than those calculated for the native *P. volitans* population from Western Indonesia.

Table 2. Summary of reported genetic diversity indices for *Pterois volitans,* populations in the WA

Source	Sample size	Number of haplotypes		Haplotype diversity	Nucleotide diversity
North Carolina[1,2]	264	8	(H01-07, H09)	0.704	0.0038
Bermuda[3]	45	5	(H01-03, H06-07)	0.627	0.0030
Bahamas[2]	127	8	(H01-08)	0.648	0.0033
Grand Cayman[3]	79	4	(H01-04)	0.432	0.0021
San Andréa Islands[3]	50	3	(H01-02, 04)	0.541	0.0029
Santa Marta[3]	169	3	(H01-02, 04)	0.524	0.0031
Puerto Rico[4]	118	4	(H01-04)	0.4492	0.0021
Barbados[5]	178	6	(H01-05, H07)	0.4780	0.0023

[1](Hamner, Freshwater, and Whitfield 2007); [2] (Freshwater et al. 2009); [3] (Betancur et al. 2011); [4] (Vélez-Zuazo et al. 2011) unpublished; [5] this study

6.2.1 Pioneer versus Established Population

The d-loop haplotypes for the pioneer and established population are shown in Figure 14 and Appendix 4. The pioneer population carried three haplotypes (H01, H02 and H04), whereas the established population, has an additional three haplotypes (H03, H05 and H07).

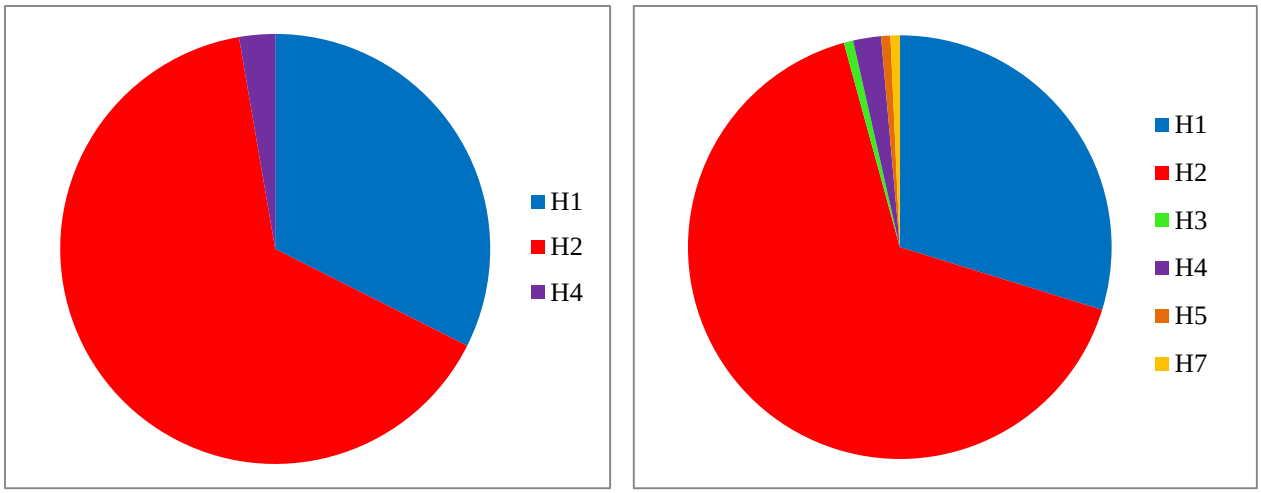


Figure 14. Haplotypic compositions of the pioneer population and the established population of the lionfish *P. volitans* in Barbados

The calculated measures of genetic variation (haplotype, nucleotide and sequence diversity values) for the invasive populations of *P. volitans* in Barbados were lower (h = 0.48 – 0.49, π = 0.002 and k = 1.56 – 1.60) than those reported by Freshwater et al. (2009) for the Bahamas and Western Indonesia populations of *P. volitans* (h = 0.65 and 0.96 respectively) (Table 3).

Table 3. Comparison of measures of genetic variation in the invasive populations of *P. volitans* from Barbados and the Bahamas and the native population from Western Indonesia

Sample	Sample size	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	Sequence diversity (k)
Pioneer Population[1]	37	3	0.4865 ± 0.0591	0.0024 ± 0.0016	1.6036 ± 0.9728
Established Population[1]	141	6	0.4790 ± 0.0333	0.0023 ± 0.0015	1.5601 ± 0.9371
Barbados [1]	178	6	0.4780 ± 0.0289	0.0023 ± 0.0015	1.5606 ± 0.9362
Bahamas[2]	127	8	0.648 ± 0.028	0.0033 ± 0.002	2.23 ± 1.24
Western Indonesia[2]	42	26	0.962 ± 0.017	0.0132 ± 0.0069	8.92 ± 4.20

[1] This study; [2] (Freshwater et al. 2009)

Despite the observed differences in the number of haplotypes present, a hierarchical AMOVA was performed with the specimens divided into two "groups": Barbados pioneer population /established *P. volitans* population and Bahamas *P. volitans* population. The largest portion of variation (90.04%) occurred within populations using hierarchical arrangement (Table 4). The 'among populations within groups' component, which is composed of variation between the pioneer and established population, was the only component found not to be significantly different (p = 0.91007).

Table 4. AMOVA for three sampled populations divided into two "groups" (Barbados Pioneer/ Established *P. volitans* populations and Bahamas *P. volitans* population)

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P*-value
Among groups	1	16.339	0.11652	11.42	0.00000
Among populations within groups	1	0.042	-0.01496	-1.47	0.91007
Within populations	302	277.409	0.91857	90.04	0.00000
TOTAL	304	293.790	1.02014		

The AMOVA contrasting the two "groups" of Barbados Pioneer/ Established *P. volitans* population and the Bahamas *P. volitans* population found the greatest genetic variation at the within population (90.04%) and among groups (11.42%) components, with significant fixation indices for both. The total variance among the groups included in the analysis (ϕ_{ST} = 0.09956, p = 0.00000) indicated that overall divergences among the two groups were significant as they were likely separate populations and the variance within populations was also significant (ϕ_{CT} = 0.11422, p = 0.00000). The variance within populations indicated that overall divergences among the pioneer and established population were not significant (ϕ_{SC} = -0.01655, p = 0.91007).

An AMOVA contrasting the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Bahamas suggested that genetic variation is partitioned mainly within populations (92.7%) rather than among populations (7.3%; Table 5). The fixation index F_{ST} was significant in the AMOVA comparison (p = 0.00098), indicating that overall divergences among the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Bahamas were significant.

Table 5. Results of analysis of molecular variance (AMOVA) invasive populations of *Pterois volitans*

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P*-value
Barbados pioneer population versus Bahamas					
Among populations	1	5.754	0.08229	7.34	0.00098
Within populations	162	168.203	1.03829	92.66	
TOTAL	163	173.957	1.12058		
Barbados pioneer population versus Santa Marta					
Among populations	1	1.066	0.00093	0.09	0.32356
Within populations	204	205.847	1.00905	99.91	
TOTAL	205	206.913	1.00998		

An AMOVA contrasting the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Santa Marta and found the greatest genetic variation occurred within populations (99.9%; Table 5). The fixation index F_{ST} supported the AMOVA partition (ϕ_{ST} = 0.00092, p =

0.32356), indicating that overall divergences among the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Santa Marta were not significant and they were likely the same population.

An AMOVA contrasting the *P. volitans* established population of Barbados and the *P. volitans* population of Bahamas suggested that genetic variation is partitioned mainly within populations (89.8%; Table 6) rather than among populations (10.2%). The fixation index F_{ST} was significant in the AMOVA comparison (p = 0.00000), indicating that overall divergences among the *P. volitans* established population of Barbados and the *P. volitans* population of Bahamas were significant.

Table 6. Results of analysis of molecular variance (AMOVA) invasive populations of *Pterois volitans*

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P*-value
Barbados established population versus Bahamas					
Among populations	1	15.094	0.10596	10.18	0.00000
Within populations	266	248.544	0.93438	89.82	
TOTAL	267	263.638	1.04033		
Barbados established population versus Santa Marta					
Among populations	1	2.844	0.01246	1.32	0.04008
Within populations	308	286.188	0.92918	98.68	
TOTAL	309	289.032	0.94164		

An AMOVA contrasting the *P. volitans* established population of Barbados and the *P. volitans* population of Santa Marta and found the greatest genetic variation occurred within populations (98.7%; Table 6) rather than among populations (1.3%). The fixation index F_{ST} supported the AMOVA partition (ϕ_{ST} = 0.01323, p = 0.04008), indicating that overall divergences among the *P. volitans* established population of Barbados and the *P. volitans* population of Santa Marta were significant and they were likely different populations.

A hierarchical AMOVA was performed with the specimens divided into two "groups" Bahamas/Santa Marta and the established population of Barbados. The largest proportion of variation (94.9%) occurred within populations rather than among populations within groups (10.9%) using this hierarchical arrangement (Table 7). The among populations within group component, which is composed of variation between the Bahamas and Santa Marta was significant (p = 0.00000) and the among groups component was the only component found not to be significantly different (p = 1.00000).

Table 7. Results of analysis of molecular variance (AMOVA) for three sampled populations divided into two "groups" (Bahamas/Santa Marta *P. volitans* populations and Barbados Established *P. volitans* population)

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P*-value
Among groups	1	5.691	-0.06033	-5.84	1.00000
Among populations within groups	1	17.304	0.11256	10.90	0.00000
Within populations	434	425.527	0.98048	94.94	0.00000
TOTAL	304	293.790	1.02014		

6.2.2 Comparison among coasts

The d-loop haplotypes for specimens sampled in year two taken from the west, south and east coasts were compared by regions (Table 8; Figure 15).

Table 8. Haplotypic composition of the invasive population of *P. volitans* on the west, south and east coasts

Haplotypes	H01	H02	H03	H04	H05	H07
West coast	12 (26%)	31 (67%)	1 (2%)	1 (2%)	0 (0%)	1 (2%)
South coast	12 (30%)	28 (70%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
East coast	14 (32%)	27 (61%)	0 (0%)	2 (5%)	1 (2%)	0 (0%)

Two haplotypes (H01 and H02) were dominant in all regions and the other haplotypes were all found at low frequencies (less than or equal to 5% of the total sample size). However, despite similar sample sizes, there were differences in the haplotype frequencies observed: the south coast population had no other haplotypes; the west coast shared one other rare haplotype (H04) with the east coast and had two rare unique haplotypes (H03 and H07); and the east coast had a rare unique haplotype (H05) (Figure 15). Measures of genetic variation (haplotype, nucleotide and sequence diversity values) for the coastal populations of *P. volitans* in Barbados were calculated (Table 9).

Table 9. Measures of genetic variation of the invasive population of *P. volitans* by coasts

Coast	Sample size	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	Sequence diversity (k)
West	46	5	0.4870 ± 0.0668	0.0024 ± 0.0016	1.6058 ± 0.9694
South	40	2	0.4308 ± 0.0599	0.0019 ± 0.0014	1.2923 ± 0.8269
East	44	4	0.5317 ± 0.0562	0.0027 ± 0.0018	1.8309 ± 1.0730

As shown in Table 9, the calculated measures of genetic variation (haplotype, nucleotide and sequence diversity values,) for the invasive coastal populations of *P. volitans* in Barbados were once again low in comparison to those calculated in previous studies. Despite these differences in the number of haplotypes present in the different coastal populations, as with the pioneer vs

established population comparison, the analysis of molecular variance (AMOVA) of samples by coastal region attributed all the molecular variance to within population variation (Table 10) and the fixation index value was not significant (ϕ_{ST} = -0.01184, p = 0.69208), indicating there was no significant population structure.

Table 10. An AMOVA for the west (n=46), south (n=40) and east coast (n=44) populations of *P. volitans*

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P*-value
Among populations	2	0.738	-0.00928	-1.18	0.69208
Within populations	127	100.694	0.79287	101.18	
TOTAL	129	101.477	0.78359		

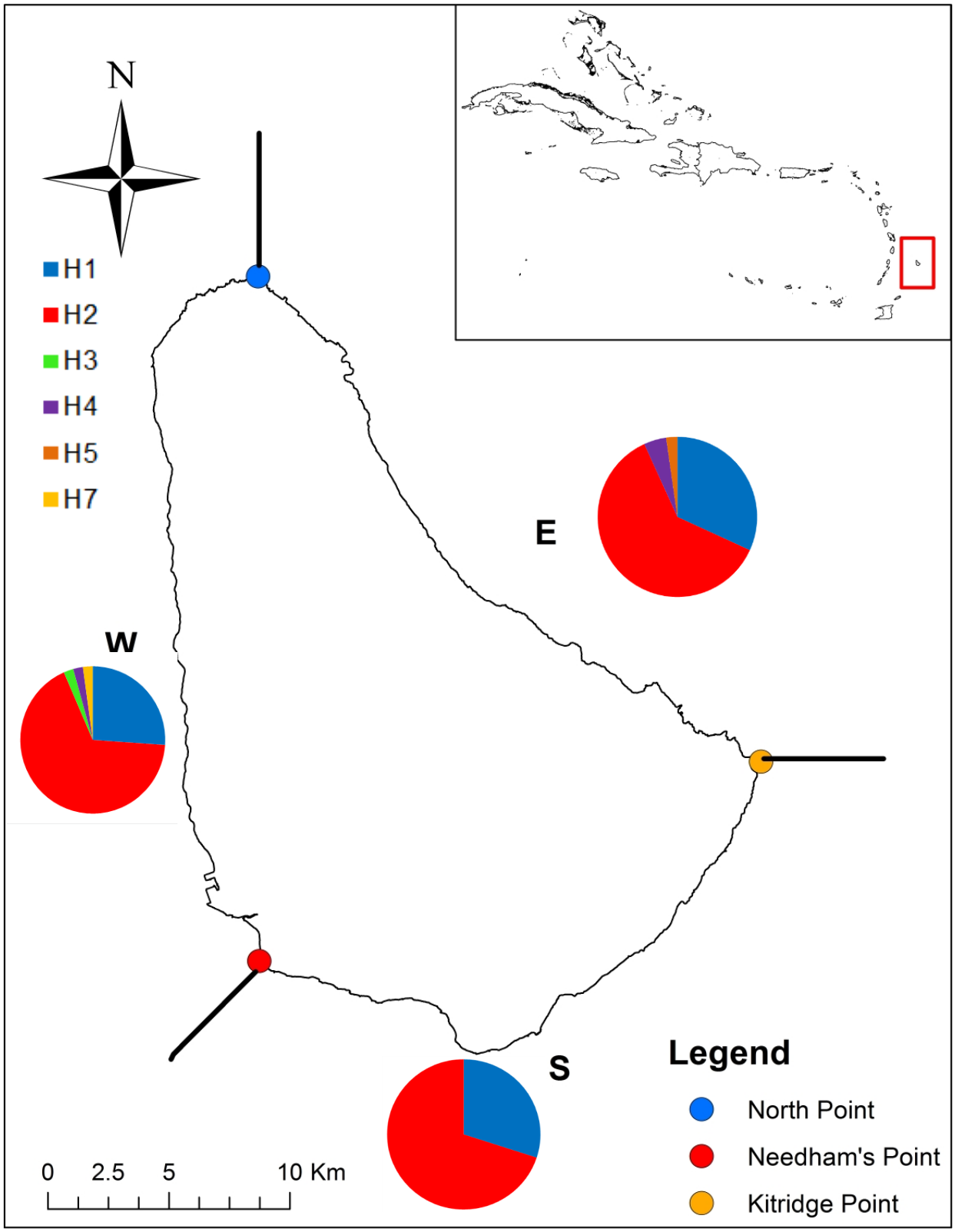


Figure 15. Red lionfish (*P. volitans*) coastal haplotypic compositions in Barbados based on the 680 bp d-loop fragment sequences. Inset map shows the location of Barbados in the Eastern Caribbean

7 DISCUSSION

This is the first report of the application of DNA sequencing to determine the species and haplotypic composition of the invasive lionfish in Barbados and is the first analysis from the Eastern Caribbean. The analysis of the 680 bp fragment of the mitochondrial d-loop region of 178 invasive lionfish confirmed that only one of the two species of lionfish known to have successfully established invasive populations in the Western Central Atlantic (WCA) (Hamner, Freshwater, and Whitfield 2007) is currently present in Barbados, i.e. the red lionfish, *Pterois volitans*. This is consistent with findings to date that have reported *P. volitans* across all the WCA sites examined, but have only found *P. miles* in North Carolina and Bermuda (Betancur et al. 2011).

The six haplotypes found in the Barbados lionfish population are defined by ten polymorphic sites (Figure 13; Appendix 3). These polymorphic sites support the theory proposed by Betancur et al. (2011) that haplotypes H05 and H07, the least abundant haplotypes, originated in the WCA population of *P. volitans* by a single nucleotide polymorphism from the more abundant H01 and H02 haplotypes, respectively. The transition (point mutation) in H01 and H02 both occur at the 656 base pair and result in a change from Guanine (G) to Adenine (A) to produce haplotypes H05 and H07, respectively (Appendix 3).

Sightings data from the US Geological Survey Non-Indigenous Aquatic Species Database (Schofield et al. 2013) indicate a gradual spread of lionfish southwards down the Lesser Antilles island chain. The results of this study suggest that the Barbados lionfish invasion was not a single event, which would have required the arrival of a minimum of six females with different maternal haplotypes, but that the invasion has been continuous over the two year period since their first arrival, with the appearance of three haplotypes in the pioneer group (year 1: 24 Nov 2011 – 15 Nov 2012) and an additional three haplotypes found in the established population (year 2: 20 Nov 2012 – 30 Sep 2013; Figure 14). With 65.7% of Barbados *P. volitans* specimens sharing haplotype H02, it suggests that a greater number of the females with this haplotype were involved in the Barbados invasion. Genetic analyses of lionfish populations from the other islands in the chain should help in determining the expansion route of lionfish to Barbados. The presence of six haplotypes (Appendix 3) in Barbados raises the interesting question of the possible distribution route. Given the estimated pelagic larval duration (PLD) of lionfish collected in the Bahamas which ranged from 20 to 35 days, with a modal PLD of 25 days by Ahrenholz and Morris (2010), the likelihood of dispersal via the North Atlantic gyre seems remote. Likewise, dispersal from the east coast of the United States of America to Barbados via ballast water also seems improbable, given that most vessels would be taking cargo to Barbados and would not be carrying ballast there.

According to Betancur et al. (2011), successive waves of dispersal from the north may lead to the genetic homogenization of the WCA populations. Very low levels of genetic diversity (haplotype, nucleotide and sequence diversity) were found in both the Barbados pioneer and established "populations" of *P. volitans* ($h = 0.49$ and 0.48 respectively) as shown in Table 3 when compared to a previous study conducted by Freshwater et al. (2009) of the Bahamas and Western Indonesia *P. volitans* populations. Additionally, the overall diversities for Barbados were low ($h = 0.4780 \pm 0.0289$, $\pi = 0.0023 \pm 0.0015$, $k = 1.5606 \pm 0.9362$). In the early stages of an introduction, low genetic diversity values are typical for invasive species according to (Hamner, Freshwater, and Whitfield 2007; Freshwater et al. 2009) and the low genetic diversity

is a result of strong founder effects. Comparing the haplotypes registered by Freshwater et al. (2009) for the Bahamas *P. volitans* with those found in Barbados suggests that *P. volitans* populations ultimately trace back to the same geographical origin of introduction (USA) and its expansion in the WCA according to Betancur et al. (2011) is likely to be the result of the initial invading population dispersing throughout the region.

A hierarchical AMOVA was performed with the specimens divided into two groups: Barbados' pioneer population /established population and Bahamas. The AMOVA indicated that the greatest genetic variation at the within-population (90.04%) and among-groups (11.42%) (Table 4) and the among-populations within-groups component, which is composed of variation between the pioneer and established population, was the only component found not to be significantly different. The fixation indices supported the AMOVA, partitions; F_{ST} = 0.09956, p = 0.00000 indicated that the two groups are two different populations and F_{SC} = -0.01655, p = 0.91007 indicated that there was no significant divergence between the pioneer and established populations, indicating that even though three new haplotypes had been introduced, the populations had not changed significantly. Considering the low genetic diversity of the Barbados lionfish population, in line with other WCA populations the expected founder effect of an alien species arrival (Table 2), it is not surprising that I did not detect any significant differences among the populations sampled from the different coasts, despite different presence of rare haplotypes. This was also the case in a similar unpublished study in Puerto Rico (Appendix 7).

Another interesting feature of the Barbados lionfish population is the size structure (Figures 10 and 11). Figure 10 shows a single immature cohort, supporting the expectation that it was the early life history stages (pelagic eggs and larvae) that were first arriving in Barbados, such that the newly settled population was likely to be dominated by young, sexually immature individuals. The strong bimodal size structure indicates two distinct cohorts in the established population during the second year of the invasion (Figure 11) and suggests non-continuous recruitment to the population. Given that in North Carolina and The Bahamas, female lionfish are likely to spawn approximately every 4 days during the summer months and less during the colder months (Morris 2009), this bimodal size structure could suggest a different reproductive behaviour in Barbados, or a second strong pulse of the invasive immigrants in year two.

Two haplotypes (H01 and H02) were shared among all three populations and were the dominant haplotypes in each, with H02 being the most common (>60%) in all populations (Table 8). Only two haplotypes (H01 and H02) were found in the south coast population, the west coast had two rare and unique haplotypes (H03 and H07, respectively) of its own, shared one haplotype (H04) with the east coast and the east coast had its own rare haplotype (H05) (Table 8; Figure 15). The different haplotypic composition of the three coasts, suggest that they were populated separately by potentially separate introduction events by a continued immigration of invasive lionfish recruits from one or more external populations. The presence of haplotypes H03 and H07 being found only on the west coast and H05 being found only the east coast suggests these haplotypes were never introduced to or spread to the other coasts. The levels of genetic (haplotype, nucleotide and sequence) diversity found in the different coastal populations are once again small (Table 9), and as with the pioneer vs established population comparison, the AMOVA analysis showed that the genetic variation was totally attributed to variance within and not among populations (Table 10) and therefore there was no significant population structuring among coasts (p = 0.69208), indicating that all local lionfish belong to a single population spread throughout the entire coastal region. The DNA analysis has revealed the presence of six

haplotypes in the Barbados population (H01 – H05 and H07), matching those found in other invasive populations of the WCA. The majority exhibited one of the two dominant WCA haplotypes (H02 = 65.7%), H01 = 30.3%) and this dominance of haplotype H02 (see Table 1; Appendix 4) was also observed by (Freshwater et al. 2009; Betancur et al. 2011; Vélez-Zuazo et al. 2011) in the invasive *P. volitans* populations of Bermuda, Puerto Rico, Grand Cayman, San Andre´s Islands and Santa Marta (Appendix 7). However, the presence of as many as six haplotypes in the Barbados lionfish population was unexpected due barriers to marine connectivity, especially as Barbados and Tobago were the last two countries in the Wider Caribbean to be invaded (November 2011 and February 2012 respectively; Figure 3), with over two decades of time lag from the establishment of the US east coast population, the original source population of the Bahamas lionfish invasion from 2004 (Freshwater et al. 2009) and subsequently of the Caribbean (Betancur et al. 2011).

Native *P. volitans* have at least 25 cytochrome *b* and 36 d-loop haplotypes, whilst the invasive populations in the WCA carry only three cyt b and 9 d-loop haplotypes (Table 2 and Appendix 5 for summary of Western Central Atlantic d-loop haplotypes), indicating an expected strong founder effect (Hamner, Freshwater, and Whitfield 2007; Betancur et al. 2011). Furthermore, Betancur et al. (2011) reported significant differentiation of lionfish population genetic structure between the Caribbean (Cayman Islands, San Andrés Island and Santa Marta) and the more northerly group (Bahamas, US east coast and Bermuda) (Appendix 6). Since nine haplotypes (H01-H09) have been reported for the more northerly group and only four d-loop haplotypes (H01-H04) were reported in Caribbean populations, there was greater genetic diversity in the more northerly group. This provided evidence of a secondary founder effect as the lionfish spread through the region across biogeographic breaks (barriers to marine connectivity) according to Cowen, Paris, and Srinivasan (2006). This finding was also supported by recent preliminary results from (Vélez-Zuazo et al. 2011) who found only the same four haplotypes (H01-H04) in lionfish from Puerto Rico (Table 2; Appendix 5; Appendix 7). The second founder effect in the Caribbean could be explained by a decrease in genetic diversity (see Table 2) associated with the dispersal of the lionfish out of the epicentre of its introduction in Florida (Betancur et al. 2011). The second founder effect is also supported by the progression of lionfish sightings in the region, where according to Schofield (2009), lionfish were frequently sighted in North Carolina and first in Bermuda in 2000, after which they appeared in the Bahamas (2004), Puerto Rico and Grand Cayman (2008), San Andrés and Santa Marta (2009) and Barbados (2011), demonstrating a temporal lag in their arrival into the Caribbean (Figure 3).

Barbados' location in the WCA provides a unique ability to assess marine connectivity of the invasive lionfish. The current state of knowledge suggests that the invasion of the Eastern Caribbean progressed southerly from the Bahamas. However, AMOVA analysis of Barbados' pioneer population versus the Bahamas population suggested that 92.7% of the genetic variation occurred within populations rather than among populations (7.3%; Table 5). The fixation index FST was significant (ϕ_{ST} = 0.07343, p = 0.00098), indicating that the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Bahamas are significantly different and therefore could not be the source of the invasion.

On the other hand a similar comparison between the pioneer population of Barbados with the population of Santa Marta, it was found that the greatest genetic variation occurred within the *P. volitans* pioneer population of Barbados and the *P. volitans* population of Santa Marta (99.9%; Table 5). The fixation index F_{ST} (ϕ_{ST} = 0.00092), indicated that overall divergences among the *P.*

volitans pioneer population of Barbados and the *P. volitans* population of Santa Marta were not significant ($p = 0.32356$) and were likely the same population. Additionally, the pioneer population carried only three haplotypes (H01, H02 and H04), which were also the only haplotypes found in Santa Marta (Appendix 5) and their compositions were similar with H02 being the dominant haplotype followed by H01 and H04.

The overall haplotypic composition of the established population though could not have been totally seeded from the Caribbean group simply because of the presence of haplotypes H03, H05 and H07 (Figure 16). These haplotypes through barriers to connectivity had so far been limited to only the northern groups. These results therefore suggest that the Barbados lionfish population is composed of recruits from both the northern group as well as the Caribbean group (Figure 16; Figure 17), with an initial wave of the lionfish invasion was from the south (Santa Marta) and subsequent waves from both the north (The Bahamas) and south (Santa Marta). Further evidence is seen in the AMOVA analysis of the established populations of Barbados being significantly different from both the Bahamas and Santa Marta populations, $p = 0.00000$ and $p = 0.04008$ respectively (Table 6), indicating that neither population individually served as the sole invasion pool, but the AMOVA analysis of the established Barbados population versus the grouped populations of Bahamas and Santa Marta showing no significant difference ($p = 1.00000$) among the two groups (Table 7). These data, along with evidence of the Barbados pioneer population arriving via the Santa Marta route support the conclusion that the established Barbados population could not have been derived from a single source. Had a single source been the case, it would be expected that either Barbados would only possess haplotypes H01, H02 and H04 from Santa Marta or that AMOVA analysis of the Barbados established population versus the Bahamian population would show no significant difference and/or composition of the two major haplotypes would be similar. Instead the Barbados establish population appears to be a combined population derived from both source locations, consistent with the Bahamas/ Santa Marta versus Barbados AMOVA result ($p = 0.91007$; Table 5; Figure 16).

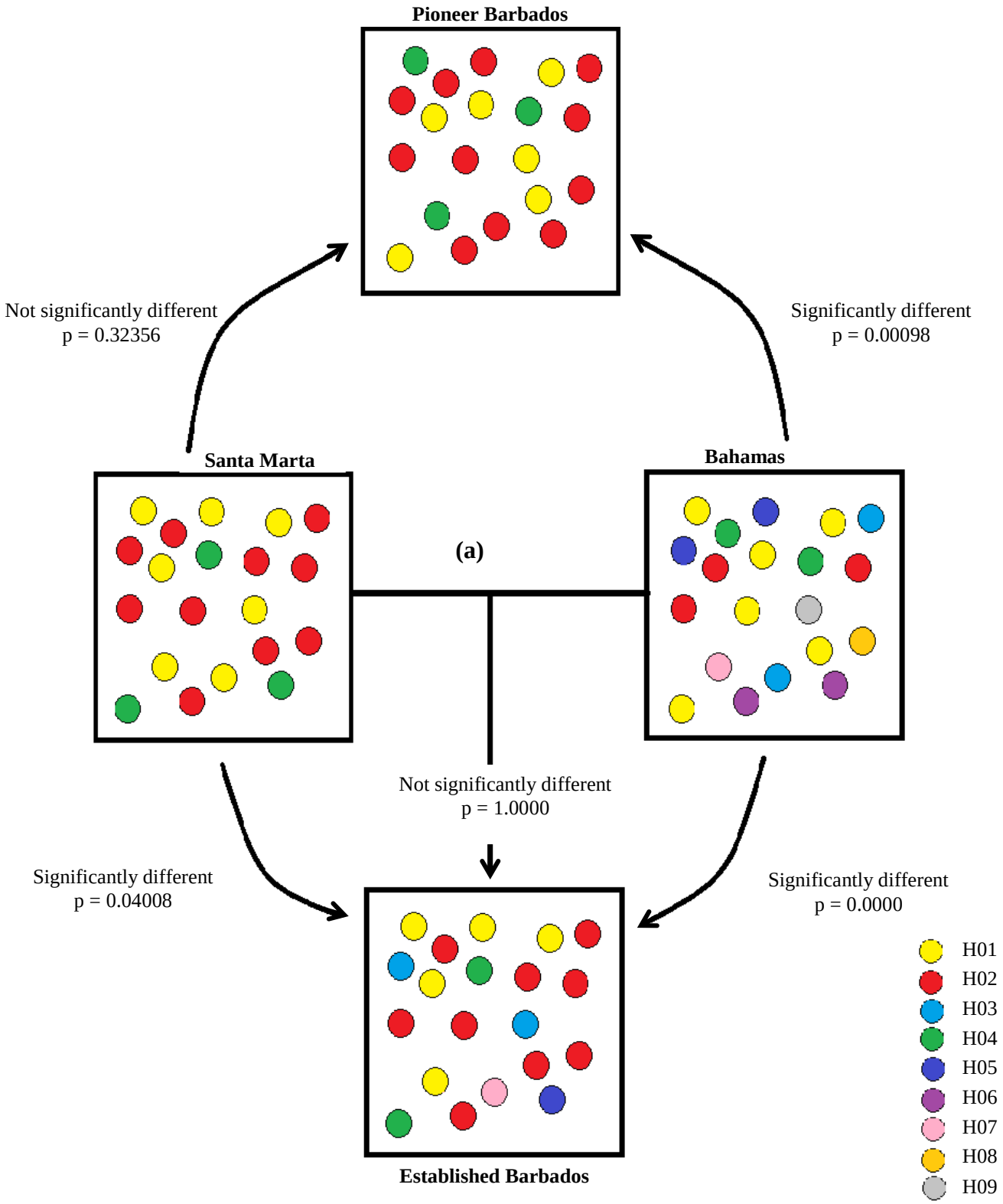


Figure 16. Simple schematic of Barbados' lionfish invasion: Both Santa Marta and Bahamian populations are significantly different from the established Barbados population, but when combined (a) show no significant difference, indicating both served as source populations.

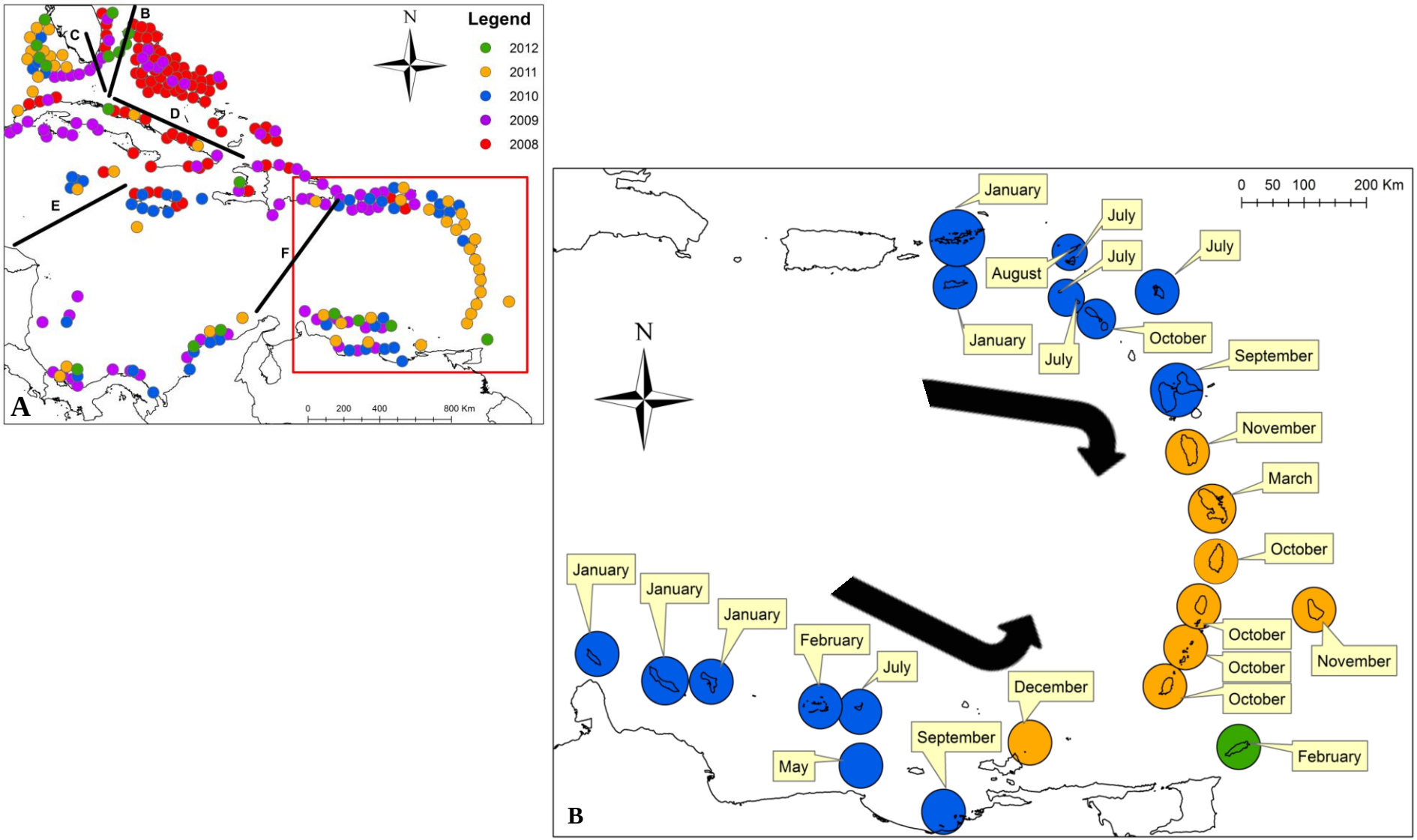


Figure 17. (a) Chronology of the lionfish (*Pterois volitans* and *P. miles)* in the Western Central Atlantic from 2008 – 2012 with phylogenetic breaks (black lines) for Greater Caribbean reef organisms and (b) an exert map of the lionfish (*Pterois volitans*) invasion chronology in the Lesser Antilles from 2010 – 2012. Black arrows indicate directions of invasion.

When incorporated with population genetics, the progression of the lionfish invasion using sightings (Figure 3) provides a means of testing various hypotheses on marine connectivity across the phylogenetic breaks in the Greater Caribbean. Appendix 8 summarizes six major scenarios suggested by previous studies ((Betancur et al. 2011) applicable to the lionfish invasion. Assuming that a temporal lag in the arrival into specific areas in the Caribbean is an indication of phylogenetic breaks, and that coinciding reports at neighbouring locations are indications of marine connectivity, then the sequence of events of *P. volitans*' progression (Figure 3) generally support the scenarios (Appendix 8).

The presence of *P. miles* only in North Carolina and Bermuda (Freshwater et al. 2009) validates connectivity between the US east coast and Bermuda as well as the break between the Bahamas and the US east coast. Betancur et al. (2011) reported that an absence of genetic sampling in the Gulf of Mexico and the Lesser Antilles impedes conclusions on population structure for a break between the Gulf of Mexico and the US east coast in addition to an Eastern Caribbean break (Appendix 8). According to Betancur et al. (2011) the break between the Bahamas and the Turks and Caicos and the rest of the Caribbean was supported due to significant differentiation between those locations, while the north-western Caribbean break was deduced from biophysical modelling done by Cowen, Paris, and Srinivasan (2006) rather than by using the sequence of events of the lionfish invasion nor by the genetic assessments, it was instead Prior to this study, although the Eastern Caribbean break was supported by the chronology of the progression of *P. volitans*, it could not be validated by analysis of genetic structure. This research has shown that although scenario F (The Eastern Caribbean break) has been supported using the lionfish progression, using genetic structure analyses, there is no significant difference (ϕ_{ST} = 0.00092, p = 0.32356) between the *P. volitans* pioneer population of Barbados and the *P. volitans* population Santa Marta, and as such this break is not supported.

8 CONCLUSION

This research is the first report of the application of DNA to determine the species and haplotypic composition for the invasive lionfish in the Eastern Caribbean and provides the first comprehensive assessment of the invasive lionfish (*P. volitans*) in Barbados. The haplotype composition shows that the Barbadian population of *P. volitans* originated from both the northern and Caribbean groups. The calculated measures of genetic variation (haplotype, nucleotide and sequence diversity values) for the 178 Barbados *P. volitans* were low (h = 0.4780 ± 0.0289, π = 0.0023 ± 0.0015 and k = 1.5606 ± 0.9362). Additionally, this study does not support the presence of the Eastern Caribbean phylogeographic break. A more extensive genetic analysis using samples from more locations will be required to better resolve the route of invasion and relatedness of island populations in the Eastern Caribbean and continued sampling of the Barbados population, lionfish ecology, spawning behaviour, prey species analysis and regional ecological impact studies will contribute to advancing our knowledge of the spread of the voracious tertiary level predator.

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10 APPENDICES

10.1 Appendix 1 Major scenarios of connectivity (white double arrows) and phylogeographical breaks (white dashed lines) for Greater Caribbean reef organisms

Source: (Betancur et al. 2011).

10.2 Appendix 2 Lionfish sighting form

LIONFISH SIGHTING FORM

The red lionfish (*Pterois volitans*). Image courtesy NOAA

Coastal Zone Management Unit
Bay Street, St. Michael.
Tel: 2285950/ 1/ 2
Fax: 2285956
info@coastal.gov.bb

Fisheries Division,
Ministry of Agriculture, Food,
Fisheries, Industry and
Small Business Development
Bridgetown
Tel: 4263745/ 4265973/ 4278480
Fax: 4369068
fishbarbados@caribsurf.com

RECORD NO (*Official use only*): _____

Data Recorder: _____ ***Contact #***: _____

Observer _____
(if not the same as Recorder): ***Contact #***: _____

Date of sighting: (dd/mm/yy) ___/___/___ ***Time of sighting:*** _____

Sighting Location:
Site Name: _____ Landmarks: _____

Latitude: _____ Longitude: _____

Depth: _____

Habitat Type:
☐ Sand ☐ Rubble ☐ Patch Reef ☐ Bank Reef ☐ Fringe Reef ☐ Sea grass
☐ Other (Specify) _____

Activity of the observer:
☐ Diving ☐ Fishing ☐ Swimming ☐ Other (Specify) _____

Number of lionfish observed: _____

Approximate size (or size range if more than 1 fish) _____ (cm/ inches)

What was the lionfish doing?
☐ Hiding ☐ Hovering ☐ Swimming ☐ Feeding ☐ Other (Specify) _____

Was fish caught? If yes what gear was used? _____

PLEASE SUBMIT COMPLETED FORM TO THE FISHERIES DIVISION OR COASTAL ZONE MANAGEMENT UNIT. PLEASE CALL IF YOU NEED THE FORM COLLECTED.

10.3 Appendix 3 Summary of polymorphic sites

Base Number	Haplotypes					
	H01	**H02**	**H03**	**H04**	**H05**	**H07**
125	G	A	G	G	G	A
214	A	G	G	G	A	G
225	G	G	T	G	G	G
548	A	A	A	G	A	A
593	G	G	A	A	G	G
651	T	T	T	C	T	T
656	G	G	G	G	A	A
664	C	G	C	C	C	G
669	C	C	C	T	C	C
679	A	A	G	A	A	A

10.4 Appendix 4 Haplotypes of the 178 *Pterois volitans* found in Barbados

Sample ID	Specimen Voucher	Accession #	Haplotype	Sample ID	Specimen Voucher	Accession #	Haplotype
EC 01	MS-70	FJ516410.1	2	EC 23	MS-70	FJ516410.1	2
EC 02	MS-50	FJ516409.1	1	EC 24	MS-70	FJ516410.1	2
EC 03	MS-50	FJ516409.1	1	EC 25	MS-70	FJ516410.1	2
EC 04	MS-70	FJ516410.1	2	EC 26	MS-70	FJ516410.1	2
EC 05	MS-50	FJ516409.1	1	EC 27	MS-70	FJ516410.1	2
EC 06	MS-70	FJ516410.1	2	EC 28	MS-70	FJ516410.1	2
EC 07	MS-70	FJ516410.1	2	EC 29	MS-50	FJ516409.1	1
EC 08	MS-70	FJ516410.1	2	EC 30	MS-70	FJ516410.1	2
EC 09	MS-70	FJ516410.1	2	EC 31	MS-70	FJ516410.1	2
EC 10	MS-70	FJ516410.1	2	EC 32	MS-50	FJ516409.1	1
EC 11	MS-105	FJ516412.1	4	EC 33	MS-70	FJ516410.1	2
EC 12	MS-70	FJ516410.1	2	EC 34	MS-50	FJ516409.1	1
EC 13	MS-70	FJ516410.1	2	EC 35	MS-70	FJ516410.1	2
EC 14	MS-70	FJ516410.1	2	EC 36	MS-50	FJ516409.1	1
EC 15	MS-70	FJ516410.1	2	EC 37	MS-70	FJ516410.1	2
EC 16	MS-70	FJ516410.1	2	EC 38	MS-70	FJ516410.1	2
EC 17	MS-70	FJ516410.1	2	EC 39	MS-50	FJ516409.1	1
EC 18	MS-50	FJ516409.1	1	EC 40	MS-70	FJ516410.1	2
EC 19	MS-70	FJ516410.1	2	EC 41	FR-63	FJ516413.1	5
EC 20	MS-50	FJ516409.1	1	EC 42	MS-50	FJ516409.1	1
EC 21	MS-50	FJ516409.1	1	EC 43	MS-50	FJ516409.1	1
EC 22	MS-105	FJ516412.1	4	EC 44	MS-50	FJ516409.1	1

Sample ID	Specimen Voucher	Accession #	Haplotype	Sample ID	Specimen Voucher	Accession #	Haplotype
SC 01	MS-50	FJ516410.1	2	SC 35	MS-70	FJ516410.1	2
SC 02	MS-70	FJ516410.1	2	SC 36	MS-70	FJ516410.1	2
SC 03	MS-70	FJ516410.1	2	SC 37	MS-70	FJ516410.1	2
SC 04	MS-70	FJ516409.1	1	SC 38	MS-50	FJ516409.1	1
SC 05	MS-50	FJ516409.1	1	SC 39	MS-70	FJ516410.1	2
SC 07	MS-70	FJ516410.1	2	SC 40	MS-70	FJ516410.1	2
SC 08	MS-50	FJ516409.1	1	SC 41	MS-70	FJ516410.1	2
SC 09	MS-70	FJ516410.1	2	SC 42	MS-50	FJ516409.1	1
SC 10	MS-70	FJ516410.1	2	SC 43	MS-50	FJ516409.1	1
SC 11	MS-70	FJ516410.1	2	SC 44	MS-70	FJ516410.1	2
SC 12	MS-50	FJ516409.1	1	SC 45	MS-70	FJ516410.1	2
SC 21	MS-50	FJ516409.1	1	SC 46	MS-70	FJ516410.1	2
SC 22	MS-50	FJ516409.1	1	SC 47	MS-70	FJ516410.1	2
SC 24	MS-50	FJ516409.1	1	SC 48	MS-50	FJ516409.1	1
SC 25	MS-50	FJ516409.1	1	SC 49	MS-70	FJ516410.1	2
SC 26	MS-50	FJ516409.1	1	SC 50	MS-70	FJ516410.1	2
SC 27	MS-50	FJ516409.1	1	WC 01	MS-105	FJ516412.1	4
SC 28	MS-50	FJ516409.1	1	WC 02	MS-70	FJ516410.1	2
SC 29	MS-50	FJ516409.1	1	WC 04	MS-70	FJ516410.1	2
SC 30	MS-70	FJ516410.1	2	WC 05	MS-70	FJ516410.1	2
SC 31	MS-70	FJ516410.1	2	WC 06	MS-50	FJ516409.1	1
SC 32	MS-70	FJ516410.1	2	WC 07	MS-70	FJ516410.1	2
SC 33	MS-70	FJ516410.1	2	WC 08	MS-70	FJ516410.1	2
SC 34	MS-70	FJ516410.1	2	WC 09	MS-70	FJ516410.1	2

Sample ID	Specimen Voucher	Accession #	Haplotype	Sample ID	Specimen Voucher	Accession #	Haplotype
WC 10	MS-70	FJ516410.1	2	WC 36	MS-50	FJ516409.1	1
WC 11	MS-70	FJ516410.1	2	WC 37	MS-70	FJ516410.1	2
WC 12	MS-70	FJ516410.1	2	WC 38	MS-70	FJ516410.1	2
WC 13	MS-70	FJ516410.1	2	WC 39	MS-70	FJ516410.1	2
WC 14	MS-50	FJ516409.1	1	WC 40	MS-70	FJ516410.1	2
WC 15	MS-70	FJ516410.1	2	WC 41	MS-70	FJ516410.1	2
WC 16	MS-70	FJ516410.1	2	WC 42	MS-50	FJ516409.1	1
WC 17	MS-50	FJ516409.1	1	WC 43	MS-50	FJ516409.1	1
WC 18	MS-70	FJ516410.1	2	WC 45	FR-78	FJ516415.1	7
WC 20	MS-50	FJ516409.1	1	WC 46	MS-70	FJ516410.1	2
WC 21	MS-50	FJ516409.1	1	WC 47	MS-70	FJ516410.1	2
WC 23	MS-50	FJ516409.1	1	WC 48	MS-70	FJ516410.1	2
WC 24	MS-70	FJ516410.1	2	WC 49	MS-70	FJ516410.1	2
WC 25	PLO07-20	FJ516411.1	3	WC 50	MS-50	FJ516409.1	1
WC 26	MS-70	FJ516410.1	2	SCF 3	MS-70	FJ516410.1	2
WC 27	MS-70	FJ516410.1	2	WCF 4	MS-70	FJ516410.1	2
WC 28	MS-70	FJ516410.1	2	WCF 5	MS-50	FJ516409.1	1
WC 29	MS-50	FJ516409.1	1	ECF 6	MS-70	FJ516410.1	2
WC 30	MS-70	FJ516410.1	2	WCF 7	MS-50	FJ516409.1	1
WC 31	MS-70	FJ516410.1	2	WCF 8	MS-70	FJ516410.1	2
WC 32	MS-70	FJ516410.1	2	WCF 9	MS-70	FJ516410.1	2
WC 33	MS-70	FJ516410.1	2	WCF 10	MS-50	FJ516409.1	1
WC 34	MS-70	FJ516410.1	2	SCF 11	MS-70	FJ516410.1	2
WC 35	MS-50	FJ516409.1	1	SCF 12	MS-70	FJ516410.1	2

Sample ID	Specimen Voucher	Accession #	Haplotype	Sample ID	Specimen Voucher	Accession #	Haplotype
WCF 13	MS-105	FJ516412.1	4	WCF 41	MS-70	FJ516410.1	2
SCF 14	MS-70	FJ516410.1	2	WCF 45	MS-50	FJ516409.1	1
SCF 15	MS-70	FJ516410.1	2	WCF 46	MS-70	FJ516410.1	2
SCF 16	MS-50	FJ516409.1	1	WCF 48	MS-50	FJ516409.1	1
SCF 18	MS-70	FJ516410.1	2	WCF 49	MS-50	FJ516409.1	1
SCF 22	MS-70	FJ516410.1	2	WCF 51	MS-50	FJ516409.1	1
WCF 23	MS-70	FJ516410.1	2	WCF 52	MS-70	FJ516410.1	2
WCF 24	MS-50	FJ516409.1	1	SCF 53	MS-50	FJ516409.1	1
WCF 25	MS-50	FJ516409.1	1	WCF 54	MS-70	FJ516410.1	2
SCF 25a	MS-70	FJ516410.1	2	SCF 56	MS-70	FJ516410.1	2
SCF 25b	MS-70	FJ516410.1	2	SCF 57	MS-50	FJ516409.1	1
WCF 28	MS-70	FJ516410.1	2	SCF 59	MS-70	FJ516410.1	2
SCF 28	MS-70	FJ516410.1	2	SCF 60	MS-70	FJ516410.1	2
SCF 29	MS-70	FJ516410.1	2	WCF 64	MS-70	FJ516410.1	2
SCF 35	MS-70	FJ516410.1	2	SCF 65	MS-50	FJ516409.1	1
WCF 35	MS-70	FJ516410.1	2	WCF 66	MS-50	FJ516409.1	1
SCF 36	MS-50	FJ516409.1	1	ECF 67	MS-70	FJ516410.1	2
WCF 38	MS-70	FJ516410.1	2	UnF1	MS-50	FJ516409.1	1
WCF 39	MS-70	FJ516410.1	2	UnF2	MS-70	FJ516410.1	2

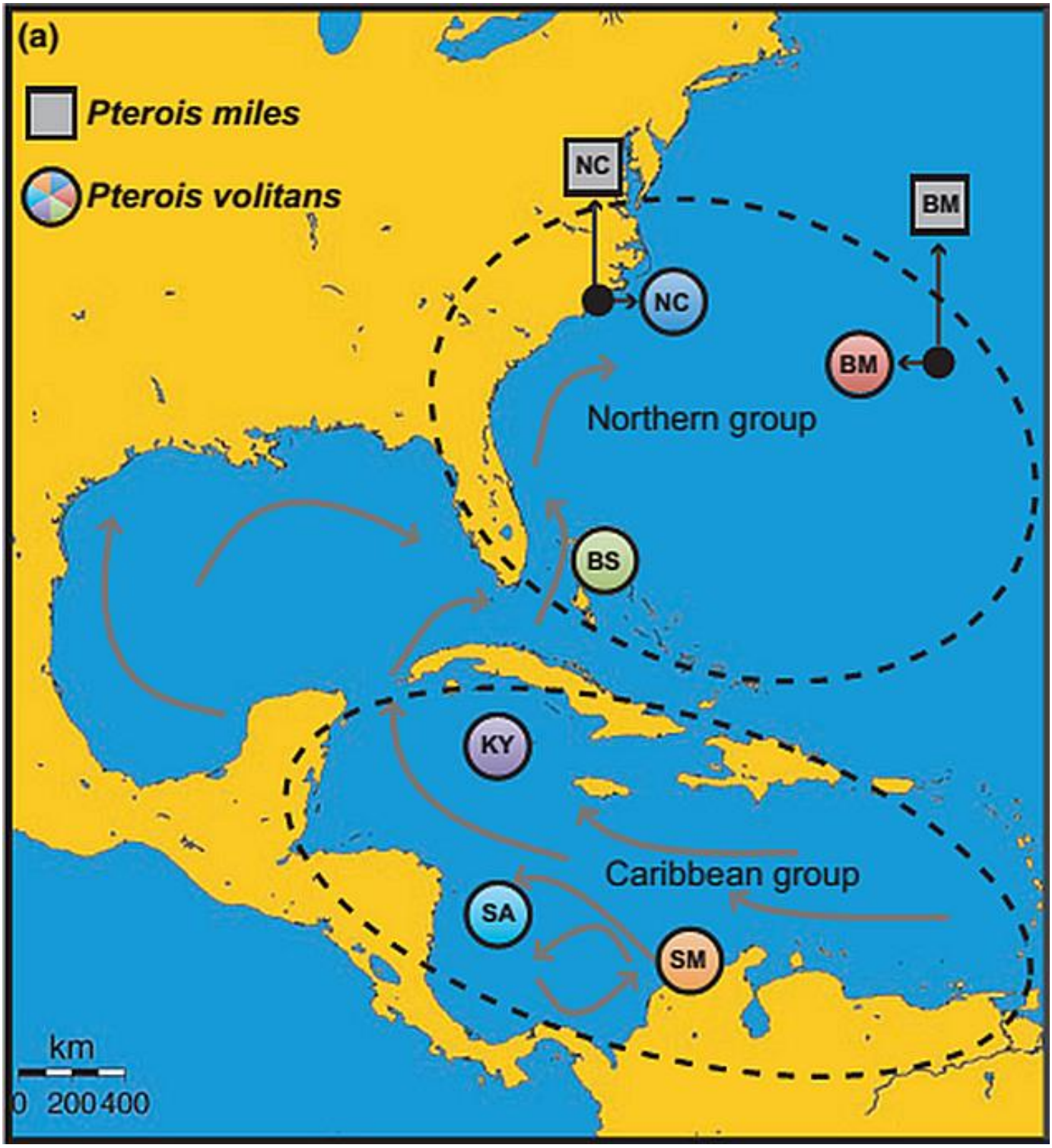
EC: East Coast; SC: South Coast; WC: West Coast; SCF: South Coast Fisheries; WCF: West coast Fisheries; ECF: East Coast Fisheries; UnF: Unknown Fisheries

10.5 Appendix 5 Summary of reported mtDNA d-loop haplotypes from lionfish, *Pterois volitans*, populations in the Western Central Atlantic

Location	n	HO1	HO2	HO3	HO4	HO5	HO6	HO7	HO8	HO9	Total Haplotypes	Haplotype Diversity
North Carolina	264	✓	✓	✓	✓	✓	✓	✓		✓	8	0.704
Bermuda	45	✓	✓	✓			✓	✓			5	0.627
Bahamas	127	✓	✓	✓	✓	✓	✓	✓	✓		8	0.648
Grand Cayman	79	✓	✓	✓	✓						4	0.432
San Andrés	50	✓	✓		✓						3	0.541
Santa Marta	169	✓	✓		✓						3	0.524
Puerto Rico[1]	118	✓	✓	✓	✓						4	0.4492
Barbados[2]	178	✓	✓	✓	✓	✓		✓			6	0.4780
Total Locations	1030	8	8	6	7	3	3	4	1	1	9	

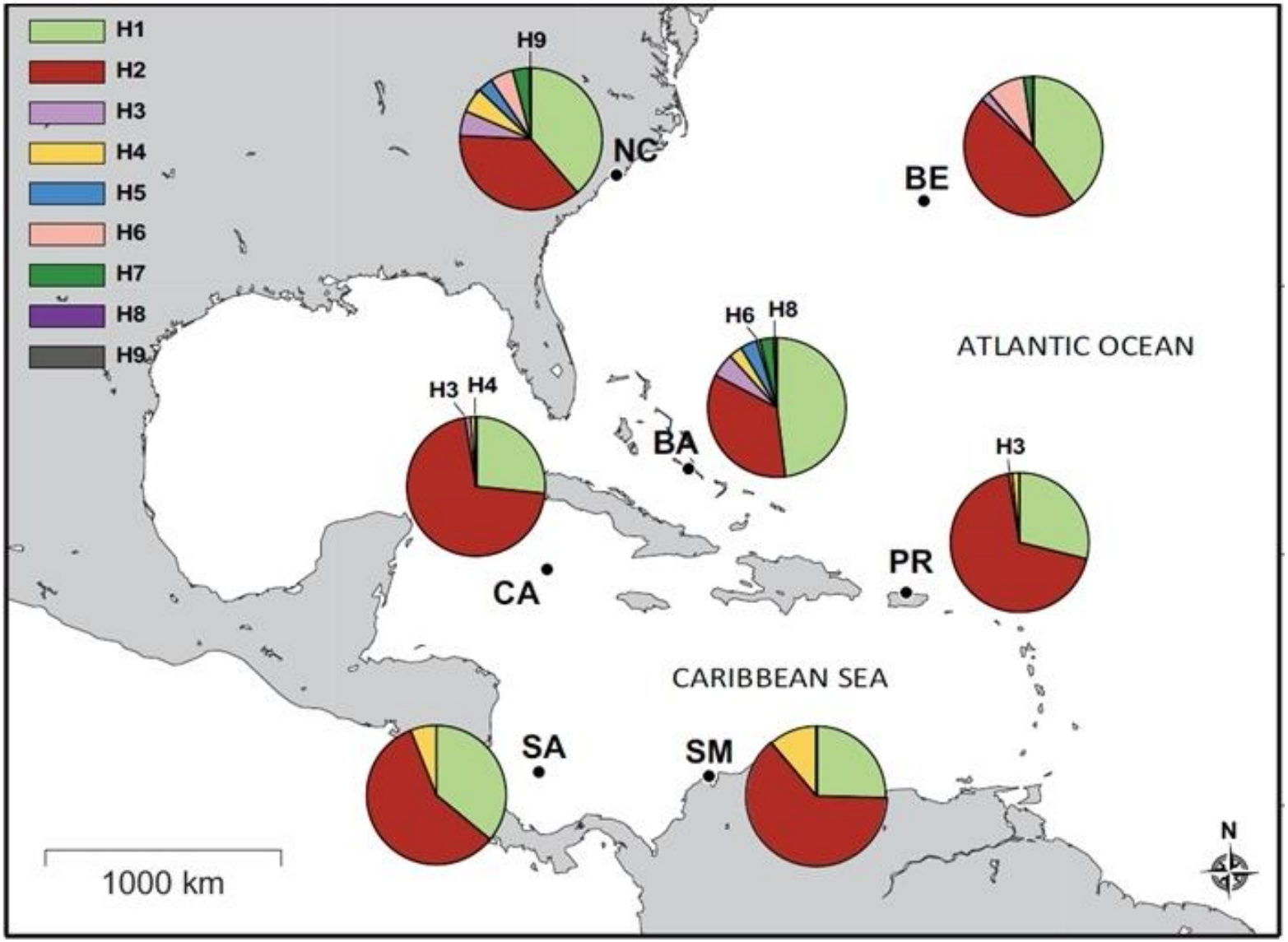
✓ = Present. Data are reported in Betancur et al. (2011), [1] Vélez-Zuazo et al. (2011) unpublished, and [2] this study.

10.6 Appendix 6 Sampling locations for invasive lionfish (*P. volitans* and *P. miles*) in the Western Central Atlantic.



Source: (Betancur et al. 2011). The estimated population groupings for *P. volitans* are delineated by dashed ellipses and the dark grey arrows represent oceanographic currents.

10.7 Appendix 7 Comparison of mtDNA control region haplotype proportions of *P. volitans* found across the Caribbean.



Source: Vélez-Zuazo et al. (2011). Poster presented in 2011.

10.8 Appendix 8 Utilization of the lionfish (*P. volitans* and *P. miles*) progression and genetics as tests for proposed scenarios of Greater Caribbean connectivity and phylogeographical breaks for reef organisms.

Code	Scenario	Reference	Support from the chronology of progression	Support from genetic structure
A	US east coast – Bermuda connection	1–2	Supported. Simultaneous arrival along the US coast and Bermuda by 2000 via the Gulf Stream. Also supported by *P. miles* invasion (only present at these locations)	Supported. No genetic differentiation*
B	US east coast – the Bahamas break	3–4	Supported. Temporal lag in the arrival in the Bahamas in 2004, four years after widely observed along the US east coast. Also supported by the absence of *P. miles* from the Bahamas	Not supported. No differentiation* (although unique haplotypes observed at both NC and BM locations)
C	US east coast – Gulf of Mexico break	5–8	Supported. Temporal lag in the arrival in the Gulf in 2006, six years after widely observed along the US east coast. Also, currently dispersing slowly into the Gulf	No data from locations in the Gulf of Mexico
D	The Bahamas, the Turks, and Caicos – Caribbean break	10	Supported. Temporal lag in the arrival in the Caribbean in 2007, three years after reported from the Bahamas.	Supported. Significant differentiation
E	North-western Caribbean break	10–11	Not supported. Simultaneous arrival at locations east and west of the break in 2008	Not supported. No genetic differentiation* between KY and SA/SM locations (although KY includes one haplotype absent from SA and SM samples)
F	Eastern Caribbean break (Mona; Guajira)	10, 12–15	Supported. Slowly dispersing eastwards from the break (Lesser Antilles)	No data from locations east of the break

[1]Schultz & Cowen (1994); [2]Hare *et al.* (2002); [3]Freshwater *et al.* (2009b); [4]Carlin *et al.* (2003); [5]Avise (1992); [6]Palumbi (1994); [7]Soltis *et al.* (2006); [8]Mobley *et al.* (2010); [10]Cowen *et al.* (2006); [11]Salas *et al.* (2010); [12]Taylor & Hellberg (2003); [13]Taylor & Hellberg (2006); [14]Baums *et al.* (2005); [15]Betancur-R. *et al.* (2010). *Lack of genetic differentiation may be a result of low variability of mitochondrial control region sequences.
BM, Bermuda; KY, Grand Cayman; NC, North Carolina, SA, San Andrés Archipelago; SM, Santa Marta.
Source: (Betancur et al. 2011)