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Linking local movement and molecular analysis to explore philopatry and population connectivity of the southern stingray *Hypanus americanus*

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Abstract

Limited data pertaining to life history and population connectivity of the data-deficient southern stingray (*Hypanus americanus*) are available. To determine potential vulnerabilities of their populations, this study aimed to analyse their movement patterns and genetic variability. A population of southern stingrays encompassing nine sites around Cape Eleuthera, the Bahamas, has been monitored using mark-recapture, spanning a 2.5 year period. Out of 200 individual stingrays, more than a third were encountered again. The home range of the females appears to be restricted, which supports the notion of high site residency. As resident populations of stingrays could suffer from a lack of population connectivity and be predestined for genetic isolation and local extirpation, this study further investigated the genetic connectivity of four sample sites in the central and western Bahamas. A haplotype analysis from the mitochondrial D-loop region showed that no distinct population structure strictly correlated with the sample site. These findings were complemented by five microsatellite loci that revealed high degrees in genotypic variability and little population differentiation. The results suggest gene flow mediated by both males and females.

KEYWORDS

Bahamas, batoid, gene flow, mark-recapture, microsatellites, sex-biased dispersal

1 | INTRODUCTION

More than 1000 extant species of elasmobranchs (sharks and rays) occupy a variety of aquatic habitats, populating coastal and continental shelf areas, pelagic and deep-sea environments and fresh and estuarine waters (Carrier *et al*., 2004; Dulvy *et al*., 2014; Martin, 2005). As most elasmobranchs have long life spans and mature late, their populations are vulnerable to overexploitation. In fact, severe population declines have been attributed to targeted fishing and by-catch (Stevens *et al*., 2000). Small populations are at risk from potential inbreeding, which consequently can lead to a loss of genetic variability and adaptive potential, compromising chances to adapt to changing environments (Johri *et al*., 2019). For isolated populations, a small amount of gene flow maintaining diversity supports long-term persistence (Mills & Allendorf, 1996).

The extent and forms of movement and residency that animals display can have an impact on their population structure and connectivity (Flowers *et al*., 2016). Population (or reproductive) connectivity

describes the degree of reproductive exchange, which consequently is a significant factor in evolutionary as well as ecological contexts. High reproductive exchange maintains connectivity between subpopulations and leads to a large metapopulation with high levels of genetic diversity. A lack of genetic admixture can lead to population fragmentation and isolation, which might promote processes like inbreeding and loss of genetic variability (Frankham *et al*., 2010). Meanwhile, strong site fidelity and natal homing (or breeding philopatry) can reinforce reproductive isolation of populations, as all generations stay at or return to the same site for reproduction (Secor, 2015). Philopatric tendencies might be expressed in only one sex, as has been shown for elasmobranch species (Day *et al*., 2019; Pardini *et al*., 2001; Portnoy *et al*., 2015; Roycroft *et al*., 2019). For estimating the vulnerability of elasmobranch populations and implementing effective management, an assessment of genetic diversity, as well as species-specific movement and reproductive connectivity, is needed (Johri *et al*., 2019; Le Port *et al*., 2012).

One of the most threatened elasmobranch families is the whiptail stingrays Dasyatidae (Dulvy *et al*., 2014). In coastal and continental shelf areas, dasyatid rays are found in a variety of habitats, including sand flats, mangroves and coral reefs. Many species are epibenthic mesopredators that forage for infaunal invertebrates and consequently act as agents of disturbance through bioturbation in their environment (O'Shea *et al*., 2012). In several parts of the world, dasyatid rays are targeted by artisanal and industrial fisheries and are also taken as by-catch (Last *et al*., 2016; Oliver *et al*., 2015). As most by-catch reports lack species-specific information, the estimation of population losses is virtually impossible, and 30% of dasyatid species assessed by the IUCN are categorized as data deficient as their population trends are unknown (Stevens *et al*., 2000, IUCN 2019). The Caribbean Sea belongs to the regions with the most data-deficient elasmobranch species (Dulvy *et al*., 2014). One dasyatid ray that is ubiquitous within this region is the southern stingray (*Hypanus americanus*, Hildebrand & Schroeder, 1928), which is distributed throughout the coastal areas of the Caribbean Sea, the Gulf of Mexico and the northeast coast of South America. They are a valuable subject of ecotourism activities and attract more than a million visitors annually to the Stingray City Sandbar, Grand Cayman (Vaudo *et al*., 2018). Meanwhile, fisheries in the southern Gulf of Mexico targeting the southern stingray are most likely responsible for a decrease in abundance (Shepherd & Myers, 2005). In addition, these animals have been reported as frequent by-catch in Cuban shrimp trawl fisheries (Briones *et al*., 2017; Ramirez Mosqueda *et al*., 2012). In the USA, southern stingray populations have been documented as healthy, whereas the global assessment lists the species as data deficient due to a lack of information on overfishing and in the other parts of its range (IUCN 2019). Little information is available concerning their movement ecology and its impact on the species genetic diversity. A limited activity space of less than a square kilometre in short-term tracking suggested site fidelity (Corcoran *et al*., 2013; Tilley *et al*., 2013). An investigation of historical connectivity of populations of southern stingrays across the northern part of the Caribbean Sea showed differentiated clades in a mitochondrial DNA marker with slightly inconsistent correlation to geographical locations (Richards *et al*., 2019). Interestingly, genetic analyses of other benthic-batoids like *Urobatis halleri*, *Dipturus oxyrinchus* and *Hypanus sabinus* revealed population structure or isolation on small geographic scales (Bernard *et al*., 2015; Griffiths *et al*., 2011; Plank *et al*., 2010).

To gain a deeper understanding of population structure across the distribution of stingrays, an analysis of fine scale movement and population connectivity in this species is critical. In this study, the site affinity and genetic connectivity of a population of wild southern stingrays residing within the coastal and nearshore waters of Cape Eleuthera, in the central Bahamas and the Bimini Islands in the western Bahamas, were investigated. At Cape Eleuthera, capture and recapture data of 200 tagged animals spanning 2.5 years were analysed to gain insights into philopatric tendencies of this population. Limited movement indicating high site fidelity that would confirm previous short-term observations was expected. Special attention was paid to sex-specific movement patterns, as behavioural differences between males and females had been observed (O'Shea, pers. comm.). To evaluate the impact of movement on the gene flow between populations in the Bahamas, the genetic connectivity and population differentiation between Eleuthera and other Bahamian sites were investigated with mitochondrial as well as nuclear microsatellite markers. Regarding previous findings in Richards *et al.* (2019), it was expected that open water would pose as a possible barrier to gene flow. Further, a high level of site fidelity could result in genetic differentiation between populations, possibly with sex-biased gene flow ratio.

2 | MATERIALS AND METHODS

All sampling was performed under research permits issued by the Department of Marine Resources of the Commonwealth of The Bahamas. All animals were treated in accordance with regulations of the Institutional Animal Care and Use Committees.

2.1 | Mark-recapture

The Elasmobranch Research Group of the Cape Eleuthera Institute captured southern stingrays around the island of Eleuthera, the Bahamas. Habitats like sand flats, cays and creeks were opportunistically scanned for stingrays from either boat or shore. The southern stingray can be distinguished from similar species in the area (chupare stingray, *Styracura schmardae*; Atlantic stingray, *H. sabinus*) by the shape of the body, rostrum and size. Sampling sessions of 3–8 h were conducted, at least once a month (on average five times a month), from January 2015 to May 2017 at one of nine different sites in proximity of Cape Eleuthera (Figure 1). Sampling success depended on the presence of sunshine and calm sea surface to spot the animals. Sites were sampled whenever the conditions were suitable for them and therefore irregularly with different amounts of effort taking place at each. Southern stingrays were encircled and captured in a dip-net (Ward *et al*., 2019).

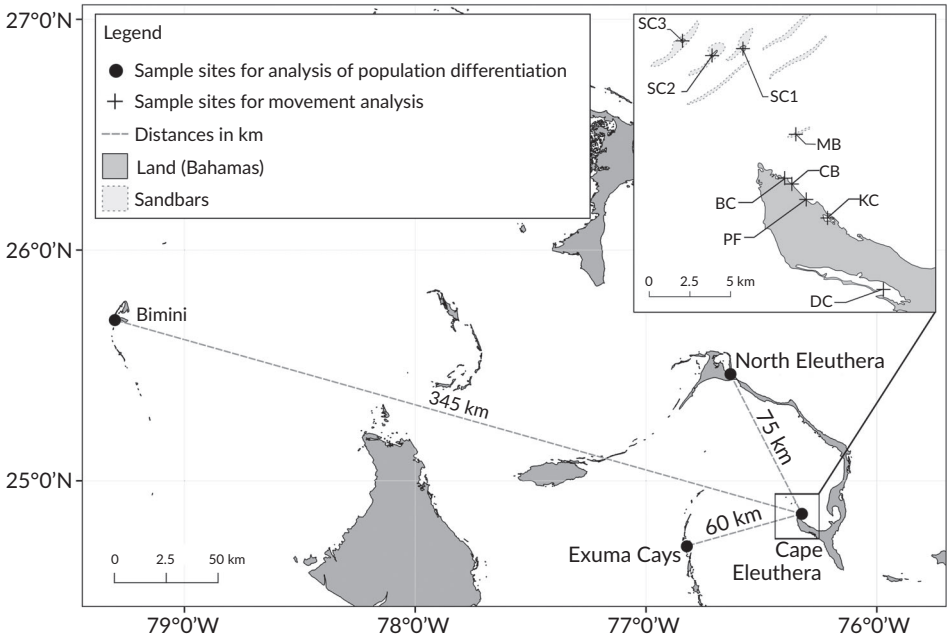

FIGURE 1 Capture locations for the analysis of population differentiation of *Hypanus americanus* individuals in 2015–2017; insert shows sample sites in Cape Eleuthera for mark-recapture analysis of southern stingrays. SC1: first Schooner Cay; SC2: second Schooner Cay; SC3: third Schooner Cay; MB: Markerbar; BC: Boathouse Cut; CB: CEI Beach; PF: Page Flat; KC: Kemp's Creek; DC: Deep Creek

The disc width, body length and total length were measured to the nearest millimetre. Sex was identified by the presence/absence of male reproductive organs, and maturity was confirmed by the disc width, with threshold being 750 mm for females and 470 mm for males (Ramirez Mosqueda *et al*., 2012; Vaudo *et al*., 2018). Neonates would be expected to measure 200–340 mm disc width (Henningsen, 2000). On first capture, all stingrays received an external dart tag (Hall Print Fish tags) positioned in the left pectoral fin, which was then used for identification on subsequent captures (Latour, 2005). When encountering tagged animals, they were captured with the individual number of the tag noted and the same measurements recorded as before. In every capture event, GPS coordinates were recorded in the vicinity of capture. Animals without tags were scanned for scarred tissue or marks that suggested previous capture and therefore tag loss, in which case they were retagged but not included in the analysis. All animals were immediately released after completing the sampling procedure.

2.2 | Tissue sampling

For the analysis of population connectivity, animals from Cape Eleuthera were considered as one population and complemented by samples from three more distinct locations in the west and central Bahamas (Figure 1). Capture and data collection was performed as stated earlier. For tissue sampling, tonic immobility was induced in the ray, which is an effective anaesthetic but has the benefit of immediate recovery and no side effects of chemical injections and reduced overall handling time (Kessel & Hussey, 2015). A fin clip was taken from the right pelvic fin. The tissue was stored in 100% ethanol or 20% DMSO (dimethyl sulphoxide) at −20°C. Tissue samples of 244 individuals were used for molecular analysis: 140 individuals from Cape Eleuthera, 13 from Exuma Cays, 11 from North Eleuthera and 80 from South Bimini.

2.3 | DNA extraction and genotyping

DNA was extracted from tissue samples of ~3 mm^2 using Chelex according to Altschmied *et al*. (1997).

A mitochondrial marker was amplified and sequenced for a subset of 64 individuals (Bimini n = 21, Cape Eleuthera n = 21, Exuma Cays n = 11, North Eleuthera n = 11). With primers published in Le Port and Lavery (2012), fragments of the mitochondrial D-loop were amplified according to their protocol. Because of irregular and insufficient success, new primers, specifically for southern stingrays, were designed based on resulting fragments in Geneious V.11.1.5 (Kearse *et al*., 2012) using Primer3 V. 2.3.7 (Untergasser *et al*., 2012), HypamF (5'-TTTGCGCAAAGTTGGTCAGAATAT-3′) and HypamR (5'-CCCTGG AAATACTATGCCCGATTA-3′). PCR (15 μl) contained 1.5 μl of buffer (VWR) (×10), 1.5 μl of dNTP (deoxynucleotide triphosphate) mixture (2 mM each), 0.9 μl of $MgCl_2$, 0.3 μl of each primer, 0.15 μl of Taq polymerase (VWR) and 1.5 μl of genomic DNA. PCR amplification was conducted with an initial denaturation of 5:00 min at 95°C followed by 40 cycles of 95°C for 00:30 min, annealing with 58°C for 00:30 min and an extension at 72°C for 1 min followed by a final extension at 72°C for 30:00 min. PCR products were prepared by adding 1 μl of FastAP and 0.5 of ExoI to 11 μl of the product. The purification reaction was carried out for 15 min at 37°C and 25 min at 80°C. The products were sequenced by the Applied Biosystems 3130xl Genetic Analyser either at the Faculty of Chemistry and Biochemistry, Ruhr University Bochum, or at GATC Biotech Cologne.

In addition to the haplotype analysis, 244 samples were genotyped with nuclear microsatellites. Primer pairs of previously

published microsatellite markers for stingrays were tested (Anderson, 2017; Le Port *et al*., 2016). A fluorescent-dye labelled M13-tail (5'-CACGACGTTGTAAAACGA-3′) was added to either the forward or reverse primer sequence of each originally published primer pair. PCR (10 μl) contained 1 μl of VWR buffer (×10), 1 μl of dNTP mixture (2 mM each), 0.6 μl of $MgCl_2$, 0.05 μl of the tailed primer, 0.2 μl of the untailed primer, 0.2 μl of dyed M13 primers, 0.1 μl of VWR Taq polymerase, 0.5 μl of DMSO and 1 μl of genomic DNA. PCR amplification with the primers from Anderson (2017) was conducted with an initial denaturation of 5:00 min at 95°C, followed by 40 cycles of 95°C for 00:30 min, annealing on specific annealing temperature for 00:30 min and an extension at 72°C for 00:30 min followed by a final extension at 72°C for 10:00 min. The PCR amplification with other primers was conducted according to the given protocol (Le Port *et al*., 2016). The microsatellite fragments were genotyped through polyacrylamide gel electrophoresis on a LI-COR 4300 DNA Analyser using the SAGA^{GT} (LI-COR) software and visual confirmation. All ambiguous or faint data were omitted from the final data set.

2.4 | Molecular analysis

The D-loop sequences were trimmed and aligned using Geneious alignment in Geneious 11.1.5 (Kearse *et al*., 2012). The software DnaSP V. 6.12.01 (Librado & Rozas, 2009) detected haplotype and nuclear diversity. Using PopART version 1.7 (Leigh & Bryant, 2015), a minimum spanning network according to Bandelt *et al*. (1999) was created. Using Arlequin version 3.5 (Excoffier & Lischer, 2010), a hierarchical AMOVA was performed to estimate F_{ST} (Weir & Cockerham, 1984) pair-wise population differentiation for D-loop sequences by F_{ST} and their significance using 10,000 permutations.

A Bayesian phylogenetic reconstruction of the resulting haplotypes and 71 available sequences of the southern stingray from GenBank (Richards *et al*., 2019) and the pale-edged stingray *Telatrygon zugei* as out-group (Chen *et al*., 2013) was calculated using MrBayes version 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) under the GTR (general time-reversible) substitution model with gamma-distributed rate variation. Trees were sampled every 10,000 generations in an overall run of 1,000,000 generations. The resulting tree was converted to a graphic file using Figtree V 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Among other software, the microsatellite genotypes were analysed in RStudio version 1.1.442 (R Studio team 2015) with functions of the packages adegenet (Jombart, 2008; Jombart & Ahmed, 2011), pegas (Paradis, 2010), PopGenReport (Adamack & Gruber, 2014) and hierfstat (Goudet, 2005). Allelic richness, diversity and heterozygosity (observed and expected) were measured (adegenet), and deviation from Hardy–Weinberg equilibrium was calculated (pegas). Potential frequencies of null alleles (*r*) were estimated (Brookfield, 1996) (PopGenReport), so was linkage disequilibrium in Arlequin version 3.5 (Excoffier & Lischer, 2010). On the basis of allele frequencies and sample sizes per population, the statistical power of the microsatellite data set was analysed using POWSIM (Ryman & Palm, 2006). For effective population sizes of 100, 500 and 1500, simulations were run with 1000 replicates under a population differentiation of F_{ST} = 0.01, 0.05, 0.1 and 0.2. The frequency of replicates that detected significant differentiation was used as an indication of statistical power. Overall fixation index F_{ST} was calculated (hierfstat). Pairwise F_{ST} (Weir & Cockerham, 1984) was calculated using hierfstat and in Arlequin version 3.5 (Excoffier & Lischer, 2010) using 10,000 permutations. Isolation by distance was tested using a Mantel test for correlation between geographic and genetic distance with 1000 permutations (adegenet). A discriminant analysis of principal components (DAPC) was initially performed with groups defined by locality. In addition, a DAPC with group membership according to genetic clusters was carried out (Jombart *et al*., 2010) (adegenet). Genetic clustering was performed with the amount of clusters k determined by the Bayesian information criterion as it has been proven to be suitable in K-means clustering (Jombart *et al*., 2010).

The inbreeding coefficient F_{IS} in the overall population and individual likelihood estimation were calculated (adegenet). Using the linkage disequilibrium method in Ne Estimator (Do *et al*., 2014), effective population size (N_e) was calculated only with the immature animals as a single cohort, which actually estimates the number of breeders (N_b) in the parental generation (Waples *et al*., 2014). Mating was assumed to be random, and rare alleles were excluded, with threshold being 0.01.

To detect sex-biased dispersal, the analysis of pair-wise F_{ST} and haplotype network of the mitochondrial d-loop was repeated with only female samples, as a more distinct structure would be expected in the case of less-dispersing females. For the microsatellites, pairwise F_{ST} was calculated for both females and males to detect differences in their gene flow. Further, the mean assignment index (mAIc) and variance of the assignment index (vAIc) were calculated with the microsatellite data for each sex, and a test on significant differences was performed with 1000 permutations (Goudet *et al*., 2002) (hierfstat). This test was repeated for each sample site separately except for Exuma Cays as no male samples were available. Using both mitochondrial and nuclear markers, the male-specific differentiation as well as the ratio of male-to-female gene flow was calculated based on Equations 7a and 7b given in Hedrick *et al*. (2013).

3 | RESULTS

3.1 | Mark-recapture

From January 2015 to May 2017, 200 individuals of *H. americanus* were captured and tagged from nine sites around Cape Eleuthera, the Bahamas. Females dominated captures with 155 individuals (mean disc width = 717 mm), about 60% being immature, whereas 45 male animals (mean disc width = 524 mm) 9% were immature (Figure 2).

Thirty-one per cent of these rays were recaptured between 1 and 19 occasions in 148 recapture events. Whereas 36% of females were recaptured, only 13% of males were encountered on multiple

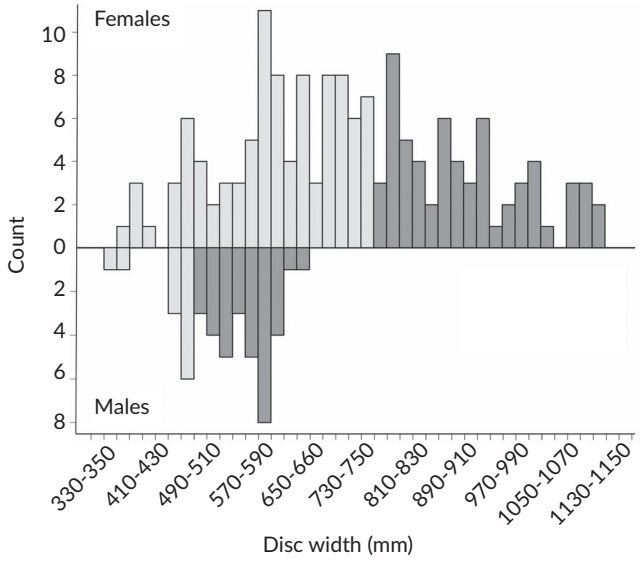

FIGURE 2 Size–frequency distribution of *Hypanus americanus* individuals captured between January 2015 and May 2017 at Cape Eleuthera, Bahamas (n = 200, females = 155, males = 45). Size threshold for maturity is ≥750 mm for females and ≥ 470 mm for males, indicated by grey scale according to the legend males (Ramirez Mosqueda *et al*., 2012; Vaudo *et al*., 2018). (□) Immature and (■) mature

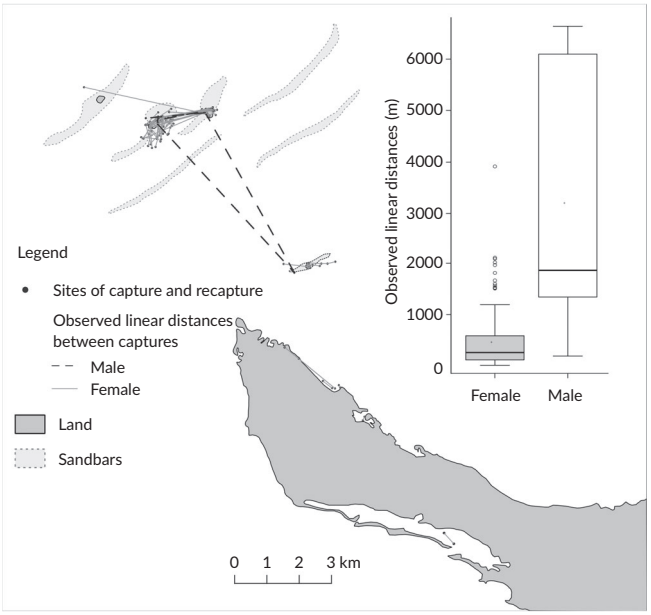

FIGURE 3 Observed linear distances between capture sites of *Hypanus americanus* individuals between January 2015 and May 2017 at Cape Eleuthera, Bahamas, separated by males (n = 5, dashed lines) and females (n = 103, non-dashed lines)

occasions. Both female and male animals of any size were found throughout the year; the monthly average number of stingrays caught per sampling session varied between 1.5 and 4, without peak in any season. Total days at liberty between first and last encounters ranged from 1 to 726 in females and 13 to 244 in males. The days at liberty derived from 141 recapture events of females ranged from 1 to 397; 75% of recaptures occurred after <100 days (median = 47). No seasonal pattern of presence/absence was noted in frequently captured stingrays. Most stingrays were recaptured within proximity of their previous capture site, observed linear distances in females (8–3887 m, median 257 m) being shorter than in males (186–6626 m, median 1857 m) (Figure 3). More than 95% of female recapture events took place <2000 m from previous capture.

3.2 | Haplotype analysis

The 64 sequences of the partial D-loop were aligned and trimmed, resulting in 358 bp fragments for analysis. Sample sizes for each population and sex are listed in Table 1. The sequences are accessible on GenBank (accession numbers MN544314–MN544377). In this alignment, 22 variable nucleotide positions and 12 parsimony informative sites were present, showing 12 transitions and 1 deletion. Thirteen haplotypes were detected; overall haplotype diversity was h = 0.756. The haplotype diversity differed only slightly between sites, Exuma Cays showing the highest and North Eleuthera the lowest diversity (see Table 1).

AMOVA showed a non-significant overall genetic differentiation (F_{ST} = 0.042, P = 0.098). Most pair-wise differentiation was relatively low (F_{ST} < 0.1) and non-significant (Table 3), whereas significant population differentiation could be detected between North Eleuthera and Cape Eleuthera (F_{ST} = 0.106, P < 0.05) and North Eleuthera and Exuma Cays (F_{ST} = 0.293, P < 0.05).

In the minimum spanning network with both sexes (n = 64), no distinct correlation was found between locations and haplogroups (Figure 4a). Overall, haplotype IV was the most common haplotype (28 individuals). It was present in all four field sites. The second most common was haplotype III, which, like the similar haplotypes I, VI, IX and XI, was absent in North Eleuthera.

Haplotype II, which was found only in a sample from Bimini, poses an outlier in this alignment with 14 mutational steps to its closest haplotype. To verify that this sample is indeed a southern stingray and not a misidentified or cryptic species, the sequence was aligned and phylogenetically analysed using further sequences of the southern stingray and the pale-edged stingray *T. zugei* as an out-group (Chen *et al*., 2013; Richards *et al*., 2019). The resulting tree revealed the presence of three haplogroups. Although most haplotypes found in this study clustered together in a clade with samples from Antigua and the U.S. Virgin Island (east of the Bahamas), two haplotypes were found in a second clade together with samples mostly from Grand Cayman. The outlier of the data set, haplotype II, grouped into a further distinct clade with samples predominantly from the U.S. coast and Belize (Figure 4b).

3.3 | Microsatellite analysis

Ten microsatellite loci could be amplified for southern stingrays; half of them, however, were monomorphic. Except for minor dropouts, the samples were genotyped with the remaining five polymorphic loci.

TABLE 1 Summary statistics from mitochondrial haplotype analysis for each population of *Hypanus americanus* across the Bahamas

	Overall	Bimini	Cape Eleuthera	Exuma Cays	North Eleuthera
Samples	64	21	21	11	11
Females	48	18	14	11	5
Males	16	3	7	0	6
Number of haplotypes	13	8	7	5	4
Haplotype diversity	0.756	0.776	0.733	0.818	0.673
Nucleotide diversity	0.01053	0.01411	0.00894	0.00886	0.00611

TABLE 2 Characteristics of 10 microsatellites amplified for 242 individuals of *Hypanus americanus* sampled across the Bahamas

						Allele diversity and (private alleles)				
Locus	***n***	**Allele size (bp)**	**Null**	H_O	H_E	**Overall**	**Bimini**	**Cape E.**	**Exuma Cays**	**North E.**
DAM5[a,f]	242	223–279	0.055	0.6530*	0.7438	15	9 (1)	13 (5)	7	8 (1)
DAM20[a,f]	240	182–218	0.035	0.7	0.7602	9	7	9 (2)	6	6
DAM26[a,r]	235	207–239	0.042	0.7575	0.831	14	13 (1)	13	8	8
Dbr142[b,f]	233	264–294	0.067	0.6481*	0.7587	11	6	11 (5)	5	5
Dbr264[b,f]	240	201–213	0.014	0.2917*	0.3101	5	4	4 (1)	4	2
Dbr091[b,f]	21	255		Monomorphic no tests done						
Dbr206[b,f]	21	240								
Dbr285[b,f]	21	190								
Dbr332[b,f]	21	130								
Dbr370[b,f]	21	175								

Note. Null: estimated potential frequency of null alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; * $P < 0.01$. Cape E.: Cape Eleuthera; North E.: North Eleuthera.
[a]Anderson (2017).
[b]Le Port *et al*. (2016).
[f]Forward primer tailed with M13.
[r]Reverse primer tailed with M13.

Across all samples, 242 unique genotypes were identified. One clone pair was found in Bimini and one in Cape Eleuthera. As the two affected animals in each pair were of the same size and sex, one individual of a pair was excluded from further analysis to rule out accidental double sampling. The final set included 68 females (20 immature) and 11 males (4 immature) from Bimini, 98 females (34 immature) and 41 males (1 immature) from Cape Eleuthera, 13 females (10 immature) from Exuma Cays and 5 females (4 immature) and 6 males (3 immature) from North Eleuthera.

No linkage was detected between loci. Frequency estimates of null alleles (*r*) ranged from 0.014 to 0.067, which according to Chapuis and Estoup (2007) can be classified as negligible ($r < 0.05$) to just moderate ($0.05 < r < 0.2$), with the latter classification possibly biasing F_{ST} estimations moderately. Consequently, the loci are considered to be adequate for analysis, as have other studies which kept loci with moderate null allele frequencies, for example, $r < 0.13$ (Villemey *et al*., 2016), $r < 0.2$ (Lawson Handley *et al*., 2007), $r < 0.1$ (Spear & Storfer, 2010). There were significant deviations of Hardy–Weinberg equilibrium in the loci DAM5, Dbr142 and Dbr264. All were an excess of homozygotes, which could have been caused by, for example, overlapping generations or a deviation of panmixia like inbreeding or population structure (Waples, 2015). As deviations were relatively low with discrepancies between 0.02 and 0.11 between H_E and H_O, all loci were kept for analysis. The details on microsatellite characteristics are shown in Table 2. Allelic diversity per population ranged between 2 and 13 and was highest in Cape Eleuthera. Mean allelic richness was similar in all populations, ranging from 5.034 in North Eleuthera to 5.47 in Bimini. The test for statistical power of the microsatellite loci in POWSIM revealed that in a minimum 99.7% of runs the data set had sufficient ability to detect significant population differentiation on all tested levels, with F_{ST} as high as 0.2.

The overall fixation index F_{ST} for the whole population, including both sexes, was $F_{ST} = 0.0230$. The pair-wise genetic differentiation values between the four sample sites showed negligible differences between calculation methods (Table 3). No significant correlation between genetic and geographic distances was found (Mantel test, $P = 0.46$).

For the DAPCs with group membership defined by sample site, 20 principal components and 2 discriminant functions were retained, resulting in one cluster without a distinct structure (Figure 5a). Although samples of Bimini, Cape Eleuthera and Exuma Cays are found on either side of the *x*- and *y*-axes, North Eleuthera is restricted by the *x*-axis. The samples of Cape Eleuthera and Exuma Cays are overlapping for the

TABLE 3 Pair-wise fixation indices calculated for the haplotypes and microsatellites in four populations of *Hypanus americanus*

		Bimini	Cape Eleuthera	Exuma Cays	North Eleuthera
D-loop	Bimini	0	0.88367 ± 0.0032	0.45194 ± 0.0050	0.05623 ± 0.0024
Arlequin	Cape Eleuthera	−0.03020	0	0.30680 ± 0.004	0.04920 ± 0.0022
AMOVA F_{ST}	Exuma Cays	−0.01147	0.00593	0	0.00693 ± 0.0007
	North Eleuthera	0.08382	0.10553*	0.29302*	0
D-loop	Bimini	0	0. 85,645 ± 0.0029	0.45015 ± 0.0043	0.53519 ± 0.0047
Arlequin	Cape Eleuthera	−0.04060	0	0.37194 ± 0.0055	0.26799 ± 0.0040
AMOVA F_{ST}	Exuma Cays	−0.01182	−0.00902	0	0.09702 ± 0.0028
Females only	North Eleuthera	−0.02084	0.00855	0.1748	0
Microsatellites	Bimini	0	0.00000 ± 0.0000	0.00198 ± 0.0004	0.00010 ± 0.0001
Arlequin F_{ST}	Cape Eleuthera	0.02065*	0	0.31155 ± 0.0048	0.00129 ± 0.0003
	Exuma Cays	0.03258*	0.0049	0	0.01614 ± 0.0011
	North Eleuthera	0.04917*	0.0386*	0.0443*	0
Microsatellites	Bimini	0			
hierfstat F_{ST}	Cape Eleuthera	0.021243	0		
Weir & Cockerham, 1984	Exuma Cays	0.030628	0.002276	0	
	North Eleuthera	0.047745	0.035966	0.037717	0
Microsatellites	Bimini	0	0.00000 ± 0.0000	0.00287 ± 0.0005	0.01614 ± 0.0013
Arlequin F_{ST}	Cape Eleuthera	0.01786*	0	0.20364 ± 0.0042	0.01396 ± 0.0012
Females only	Exuma Cays	0.03194*	0.00816	0	0.14028 ± 0.0035
	North Eleuthera	0.05561*	0.05162*	0.04194	0
Microsatellites	Bimini	0	0.03465 ± 0.0017	NA	0.01505 ± 0.0013
Arlequin F_{ST}	Cape Eleuthera	0.02986*	0	NA	0.07643 ± 0.0028
Males only	North Eleuthera	0.06315*	0.03417	NA	0

Note. Left column lists the marker and calculation method. Matrix below diagonal shows the pair-wise F_{ST} values between sample sites; matrix above the diagonal shows the *P*-values if available. *$P < 0.05$.

most part, whereas samples from Bimini spread out along the *x*-axis and several from North Eleuthera along the *y*-axis.

Group membership derived from *k*-means clustering resulted in 13 clusters. The DAPC, retaining 20 principal components and 5 discriminant functions, showed only very weak differentiation between clusters (Figure 5b), and the clusters did not correlate with field sites.

3.4 | Inbreeding and number of breeders

The inbreeding coefficient F_{IS} in the whole population was low (F_{IS} = 0.0943). The mean average inbreeding showed values lower than 0.4 in 83% of the whole population. Calculated estimates of the number of breeders were especially low in North Eleuthera (N_b = 28), whereas Exuma Cays (N_b = 99) and Cape Eleuthera (N_b = 200) showed higher numbers, and Bimini gave an infinite estimation.

3.5 | Sex-biased dispersal

The mitochondrial haplotype network of only females did not show a distinct correlation to location (Supporting information Figure S1). The AMOVA analysis including only females (n = 48) produced all non-significant values of population differentiation. As only 16 sequences were available for males, no test was attempted. Pair-wise F_{ST} of microsatellites between populations showed similar patterns when comparing females, males and all sexes (Table 3), though Exuma Cays could not be included in the male calculation as no samples were available. The overall F_{ST} values for maternal and bi-parentally inherited markers did not differ evidently (0.042 and 0.023, respectively). This similarity consequently resulted in a slightly negative value close to zero for the estimated gene flow ratio of males to females (m_m/m_f = −0.069). No evidence for sex-biased dispersal was found in the mean assignment indices neither for the whole data set (Table 4 and Figure 6) nor for each single population separately (Supporting information Figure S2).

4 | DISCUSSION

The population of southern stingrays at Cape Eleuthera has been monitored for 2.5 years and shows indications for high site fidelity and long-term use of this area. Despite the apparent limited movement and discrepancies in female and male observation, the genetic

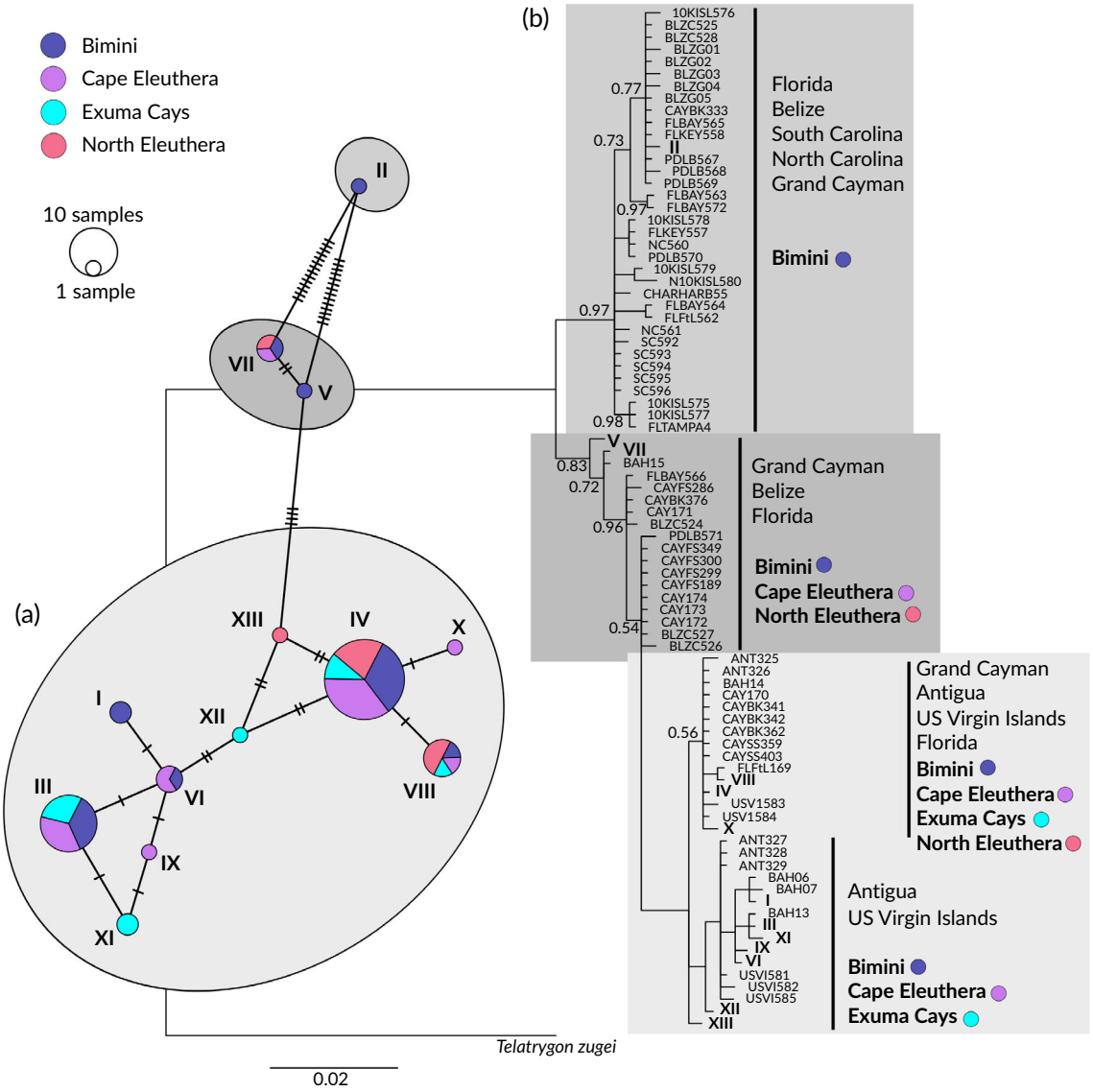

FIGURE 4 (a) A minimum spanning network including d-loop sequences of 64 *Hypanus americanus* individuals. Roman numbers indicate haplotype, sample sites are colour-coded according to legend and hatch marks indicate mutations. (b) Bayesian tree of 84 D-loop sequences of southern stingrays. In bold are the 13 haplotype sequences from this study; 71 sequences are from GenBank (Richards *et al*. 2014); pale-edged stingray as out-group, calculated using the GTR + G substitution model. ANT: Antigua; BAH: Bahamas (Bimini); BLZC: Cay Caulker, Belize; BLZG: Glover's reef, Belize; CAY(…): Grand Cayman; CHARHAB: Charlotte Harbor, Florida; FLBAY: Florida Bay, Florida; FLFtL: Fort Lauderdale, Florida; FLKEY: Middle Keys, Florida; FLTAMPA: Tampa Bay, Florida; NC: North Carolina; PDLB: Ponce de Leon, Florida; SC: South Carolina North Inlet; USV: U.S. Virgin Islands

analysis showed gene flow between the four sampled Bahamian sites in both mitochondrial and nuclear markers, mediated most likely by both female and male stingrays.

4.1 | Spatial analysis

Clear sex differences were found in capture and recapture rates, with females dominant (3:1, females:males). Nonetheless, surveys were conducted in shallow, sand-bottom habitats; thus, sexual segregation through sex-specific habitat choice could have affected male stingray capture rates. Shifted sex ratios of southern stingrays in specific habitats were evident in Cuba as well as Belize, where females were found in ratios up to 3:1 (Briones *et al*., 2017; Tilley & Strindberg, 2013). A specific habitat choice of females, possibly dependent on abiotic factors, may relate to parturition and mating (Tilley & Strindberg, 2013). Mating attempts of the prevalent mature males found have been observed on the sand flats sampled in this study, similar to previous documentation (Chapman *et al*., 2003). Female Atlantic stingrays (*H. sabinus*) appear to prefer higher temperatures especially when pregnant, which could be evident in southern stingrays as well (Wallman & Bennett, 2006). Nonetheless, because almost half of the captured females were immature, but not neonates, this habitat might not solely be chosen for reproduction purposes.

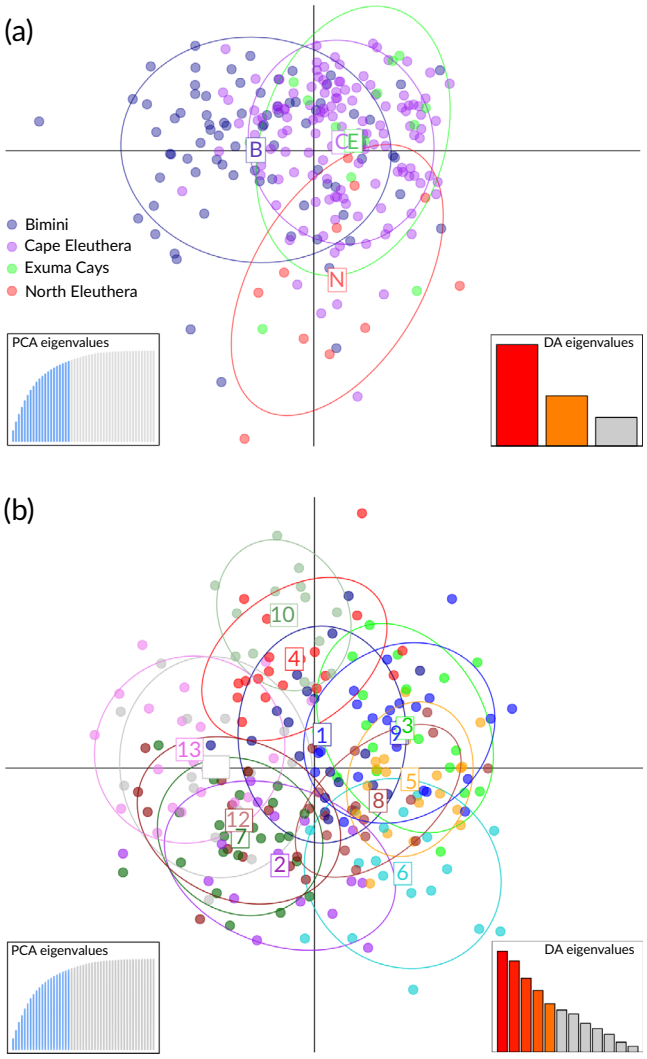

FIGURE 5 Population structure of 242 *Hypanus americanus* individuals based on microsatellites. (a) Discriminant analysis of principal component (DAPC) with group membership defined by locality; 20 principal components and 2 discriminant functions were retained, as signalled by coloured bar graphs. (b) DAPC with groups defined by *k*-means, *k* = 13; 20 principal components and 5 discriminant functions were retained, as signalled by coloured bar graphs

TABLE 4 Tests for sex bias in the F_{ST}, the mean and variance of the assignment index (vAIc) for *Hypanus americanus*, conducted using hierfstat

	Males	Females	*P*-value
F_{ST}	0.029906247	0.020017891	0.564
mAIc	0.009834	−0.0031	0.967
vAIc	6.859830	7.722924	0.473

The habitat could also provide shelter where females aggregate to avoid mating pursuits as only few males appear there, as has been suggested for other small-bodied coastal elasmobranchs, for example, the leopard shark (*Triakis semifasciata*) (Nosal *et al*., 2013). Considering

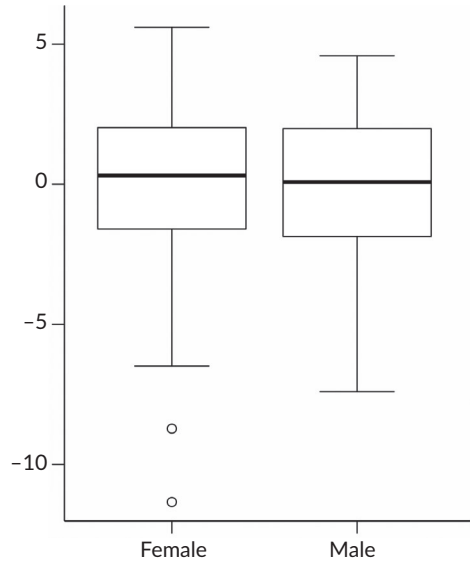

FIGURE 6 Corrected assignment index for 242 *Hypanus americanus* individuals, calculated based on the microsatellite data, conducted using hierfstat

that the females are bigger than the males, a competitive exclusion in terms of resource partitioning might be more likely, as has been suggested before (Corcoran *et al*., 2013).

Not only more than a third of females were recaptured, but most individuals were recaptured in proximity to their original capture site. The whole population showed a high recapture rate of 31%, which exceeded the 22% found for a population of wild southern stingrays at Grand Cayman (Corcoran *et al*., 2013) and indicates that site affinity is a common trait in this species. Collective seasonal movement, as exhibited by other elasmobranchs (Flowers *et al*., 2016), can most likely be ruled out for females, because neither seasonal discrepancies in population density nor clear indications for seasonal patterns in recaptured stingrays were found. The monitoring around Cape Eleuthera showed that female southern stingrays exhibit residency with restricted movement even between habitats in proximity. In contrast to female individuals, the male counterparts were recaptured rarely and at further distances. As the sample sizes were biased with only five data points for males, no test on significance was performed. Despite the lower sample size in males, this pattern does not seem random. As male activity space did not differ from that of females in previous studies, the movement might not be related to an extended home range but instead may correlate either to habitat preferences as stated earlier or to mating behaviour. It has been observed in this study and by Chapman *et al*. (2003) that occasionally several males try to mate with one female. This might cause males to move further distances to avoid mating competition. Because southern stingrays studied in an aquarium exhibited multiple paternity (Anderson, 2017), the males might benefit from locations with less competitors present. Nonetheless, the possibility remains that males benefit from each other's presence as they might get easier access to the larger females when they simultaneously grasp the females' pectoral fins orally (Chapman *et al*., 2003).

Movement between populations is needed to enable a reproductive exchange. Strong residency bears the threat of genetic isolation and consequently can result in a loss of genetic diversity and therefore a reduced capability to adapt to a changing environment (Johri *et al*., 2019). Although the monitored females showed strong site fidelity and limited movement in this and previous studies (Corcoran *et al*., 2013; Tilley *et al*., 2013), strategies like juvenile or sex-biased dispersal could maintain a genetic connectivity, as many elasmobranchs have been proven to consist of resident females and migrating males (*e.g*., Chin *et al*., 2013; Daly-Engel *et al*., 2012; Portnoy *et al*., 2015).

4.2 | Population connectivity

Genetic variability and connectivity were investigated using mitochondrial and nuclear markers in the southern stingray, sampled at four sites in the west and central Bahamas. The D-loop of the mitochondrial DNA was used for a haplotype analysis.

The overall haplotype diversity h = 0.756 was comparable to those of other elasmobranch studies where a marker in the mitochondrial control region/D-loop was used, such as h = 0.78 found for short-tail stingray *Bathytoshia brevicaudata* (n = 176), h = 0.672 for the sharpnose skate *D. oxyrinchus* (n = 28) and average h = 0.719 for the coastal living bonnethead shark *Sphyrna tiburo* in North Carolina (n = 23) (Griffiths *et al*., 2011; Le Port & Lavery, 2012; Portnoy *et al*., 2015). Southern stingrays previously showed a haplotype diversity of h = 0.948 in a longer fragment from the same mitochondrial region (Richards *et al*., 2019). The analysis of the D-loop revealed similar diversities across the different sample sites and showed no distinct population structure correlating to location, which suggests an admixture between the populations. The only striking structure was the absence of six haplotypes from North Eleuthera, which could indicate a lack of recruitment; nonetheless, a possible sampling bias due to the small sample size of North Eleuthera (n = 11) needs to be considered.

The study of Richards *et al*. (2019) investigating mitochondrial divergence in the southern stingray, which also included samples from Bimini, unveiled three clades that did show restricted gene flow between the mainland coast (USA, Belize) and the northern and eastern Caribbean islands (Bimini, U.S. Virgin Islands, Antigua), suggesting a connectivity promoted by coastlines and shallow water bodies as well as a possible historical phylogeographic break caused by the Straits of Florida. The samples from this study were represented in all three clades, though dominated by the clade found in the northeast of the Caribbean, revealing that also the majority of southern stingrays from the central Bahamas group together with those of the eastern Caribbean. Similar to the previous study, samples from Bimini showed several haplotypes associated with the USA, Belize and Grand Cayman, implying possibly a stronger connection of this site to the mainland of North and Central America in comparison to the sites of the central Bahamas (Richards *et al*., 2019). Though mitochondrial markers are of use in explaining part of the evolutionary history of populations, they are subject to several limitations when analysed exclusively to study population structure, as it, for example, reflects only the matrilineal history or might be subject to recurrent substitutions and therefore should in best case be studied in combination with nuclear markers (Liu & Cordes, 2004; Moritz *et al*., 1987; Zink & Barrowclough, 2008).

To add to the haplotype analysis of the three sites in the central and western Bahamas, the results were complemented by a microsatellite data set of 242 individuals. These data allowed the first analysis of populations of the southern stingray on the basis of individual genotypes. The diversity of alleles differed between sites, which partially can be linked to sample sizes as Exuma Cays and North Eleuthera showed fewer alleles than Bimini and Cape Eleuthera in four out of five loci. The overall inbreeding coefficient F_{IS} was quite low with 0.0943; however, the estimated number of breeders N_b showed differences between the sample sites. Particularly, North Eleuthera with estimated 28 breeders varied strongly from the others. This variation can be linked to the low genetic variability as well as the slightly less-genetic admixture found in the mitochondrial markers, as several haplotypes were absent from this site. In correspondence to this, North Eleuthera was significantly differentiated from Cape Eleuthera (F_{ST} = 0.11) and Exuma Cays (F_{ST} = 0.29) in the mitochondrial marker. Nonetheless, because of discrepancies in sample sizes, especially in North Eleuthera (n = 11), the results should be interpreted carefully.

The nuclear markers revealed detectable but little population differentiation and support the general pattern of the mitochondrial haplotypes as North Eleuthera is differentiated the most from other sites. Significant pair-wise differentiation values were found in the microsatellites between most populations, with low differentiation between Exuma Cays and Cape Eleuthera (F_{ST} < 0.01, non-significant) and higher differentiation between North Eleuthera and Bimini (F_{ST} = 0.049, significant). As the POWSIM analysis suggests a possible differentiation as high as F_{ST} = 0.2, the differentiation seems to be quite low despite being significant. This is confirmed in the DAPC analysis as it showed no strong structure between the four sample sites. The DAPC also supported the especially low differentiation between Exuma Cays and Cape Eleuthera, as their samples were overlapping for the most part. Similarly, the DAPC of genetic clusters showed no distinct structure or outlying clusters. This suggests a fair amount of gene flow to be present between most subpopulations in the central and western Bahamas, preventing a distinct isolation. The close relation between Cape Eleuthera and Exuma Cays suggested a correlation between differentiation and distance; however, the Mantel test did not detect a significant isolation by distance. This is also in accordance with the highest differentiation found between North Eleuthera and Exuma Cays, which are closer than other sites. To link the microsatellite data set directly back to the movement patterns found in the mark-recapture analysis and avoid a bias by the chosen geographical scale, the DAPC cluster analysis was performed for Cape Eleuthera as well, again not showing any distinct correlation between genetic clusters and sample site (Supporting information Figure S3).

Though similar patterns have been found in both marker systems, it must be considered that five microsatellite loci and the smaller sample sizes in North Eleuthera and Exuma Cays might have a limited

capability to detect a population structure. Nonetheless, earlier studies identified a structure with only a few microsatellite loci (four to six) (*e.g.*, Feldheim *et al*., 2001; Hoarau *et al*., 2002; Hoelzel *et al*., 1998; Schrey & Heist, 2003), and limited sample sizes are not uncommon in studies pertaining to similar species, which most likely is due to the difficult nature of sampling highly mobile aquatic animals (*e.g.*, Frodella *et al*., 2016; Hoelzel *et al*., 1998; Momigliano *et al*., 2017; Richards *et al*., 2019; Roycroft *et al*., 2019; Schrey & Heist, 2003). According to the POWSIM analysis, the statistical power of the sample size as well as marker resolution was sufficient to detect potential genetic structure in the sample set. Nonetheless, more loci would offer a stronger base for interpretation, as would a more balanced sample size.

All four sample sites exhibit genetic diversity and connectivity between them with no sign of distinct isolation or inbreeding, supported by both marker systems. This stipulates that despite the high site fidelity exhibited by the females, gene flow exists across the Bahamas. These results raise the question how reproductive connectivity between different sites is maintained.

As differences in the movement behaviour of male and female stingrays were observed, it was initially considered that existing gene flow could be mediated mainly by male southern stingrays, whereas females show philopatric behaviour with strong residency. Females of the short-tailed stingray, the great white shark *Carcharodon carcharias* and the bonnethead shark exhibit philopatry, whereas males mediate the crucial gene flow between populations (Pardini *et al*., 2001; Portnoy *et al*., 2015; Roycroft *et al*., 2019). The female/male gene flow ratio can provide an indication for sex-biased dispersal; for example, the gene flow caused by male short-tail stingrays is considered to be at least five times greater than that caused by females (m_m/m_f = 5.46) (Roycroft *et al*., 2019). In southern stingrays, the gene flow of males and females of the data set did not differ evidently (m_m/m_f = −0.069). Neither did the analysis of female haplotypes correlate with specific locations or reveal high differentiation, nor did the pair-wise F_{ST} and vAIc show a significant bias between sexes. This indicates that gene flow is likely to be caused by both sexes despite the shown site fidelity of females on a small regional scale, though these analyses of sex-biased dispersal can also be biased, for example, in terms of geographical scale. Mean and variance of AI on a smaller scale for Bimini, Cape Eleuthera and North Eleuthera separately also did not show a significant difference, though the limited sample sizes for each sex in North Eleuthera (n = 5/6) are unlikely to give a reliable result. Also several other ecological scenarios could result in similar female and male gene flow, whereas (sub-)adult females show strong site fidelity. As the mark-recapture study covered only just about 2.5 years, the site fidelity might be temporary. An obvious pattern of seasonal movement behaviour has not been observed in the recapture data; however, connectivity between sites could be induced by temporal movement behaviour which is not linked to season but to irregular environmental changes. Apart from classic annual cycles, the Caribbean experiences decadal variability in weather events as well as the influence of El Niño and La Niña, which can change cycles as well as intensify weather phenomena (Pulwarty *et al*., 2010). Tropical storms could be considered a driving force, as several coastal shark as well as bony fish species have reacted to them in the past with unusual movement (Heupel *et al*., 2003; Watterson *et al*., 1998). Abiotic changes can also cause indirect effects by influencing the prey density and therefore the biotic environment of elasmobranchs. Movement in cownose rays *Rhinoptera bonasus* likewise did not correlate to seasons but most likely was linked to prey accessibility and predator avoidance (Collins *et al*., 2007). Regarding the species in the current study, it has been shown that in a marine reserve in Belize southern stingrays seem to shift between habitats due to predator presence (Bond *et al*., 2019).

Other elasmobranch species have shown an ontogenetic shift in movement behaviour, which could also explain high gene flow in an apparently highly resident species when looking at only one life stage (Chin *et al*., 2013; Grubbs, 2010). This study included immature and mature animals (Figure 2); however, no neonates were investigated, assuming their disc width to be 200–340 mm (Henningsen, 2000). Several elasmobranch species use nursery areas as they can play a crucial role in the survival of offspring and from which they could disperse (Heupel *et al*., 2007; Martins *et al*., 2018). It remains unclear if southern stingrays use a specific habitat in their early life stage as no distinct nursery areas with neonates have been discovered yet (DeAngelis *et al*., 2008; Yokota & Lessa, 2006). Furthermore, other elasmobranchs, such as the flapper skate *Dipturus intermedius*, bull shark *Carcharias leucas* and blue shark *Prionace glauca*, exhibit different individual migration behaviours (partial migration), which could be the case for the southern stingray as well (Espinoza *et al*., 2016; Neat *et al*., 2015; Vandeperre *et al*., 2014).

Existing movement can additionally be shaped by barriers that limit dispersal. Le Port *et al*. (2012) assumed deep ocean basins to act as a major barrier to stingray dispersal and suggested connectivity facilitated by coastlines in the population of short-tail stingrays around New Zealand. Similar patterns were found for the southern stingray, although not consistently as some sites were well connected despite deeper waters between them (Richards *et al*., 2019). The Bahamas are characterized by shelf regions and islands separated by deep sea trenches. All sample sites of this study are located on the Great Bahama Bank, a carbonate platform which does not exceed 5 m water depth in 60% of its area (Harris *et al*., 2015). Considering coastal connectivity is an important factor for movement between subpopulations, the shallow region consisting of sand bars and cays connecting Cape Eleuthera and Exuma Cays is likely responsible for the close genetic connection found between the two sites. Because of the tongue of the ocean and the Northwest Providence Channel, the shortest path between Bimini and the central Bahamas crosses a deep sea trench. The gene flow between Bimini, however, suggests that connectivity is not limited by short distances of deep ocean. The higher values of differentiation found in North Eleuthera cannot directly be attributed to these assumptions, as the coastline of Eleuthera should connect it quite well. Nonetheless, the habitat sampled in North Eleuthera was a large semi-enclosed bay with openings to the North West Atlantic that measured 150–750 m. Shark species, including larger apex predators, have been observed to be frequent

visitors at one of these gaps (O'Shea, pers. comm.). The partial enclosure of the bay and presence of predators might affect the potential movement of southern stingrays to the outside. Future studies could explore the movement of the population in North Eleuthera further; additionally missing links of gene flow could be found through southern stingrays from Andros, Abaco and Current beach in northwest Eleuthera.

5 | CONCLUSION

Many elasmobranch species are vulnerable to extinction, and assessing movement patterns and genetic connectivity is essential to the management of threatened populations (Johri *et al*., 2019; Le Port *et al*., 2012; Stevens *et al*., 2000). The southern stingray is of high economic and ecological value to the Caribbean, but data on genotypic diversity, population sizes and migration rates are still lacking. This study detected high site fidelity in southern stingrays residing in the central Bahamas, which was feared to cause reproductive isolation and increase the risk of inbreeding, should the populations further decrease. Nonetheless, the population in the central and western Bahamas revealed fair levels of gene flow, genetic variability and little population structure. Although these are reassuring results for the current status of wild living southern stingrays in the Bahamas, these animals are still vulnerable to anthropogenic impacts in the Caribbean like fishing and habitat degradation, affecting these animals directly and indirectly. If populations decrease, genetic variability might get lost which could impair their ability to adapt to a changing environment. As of now, only the populations of southern stingrays in the USA have been estimated to be healthy by the IUCN. Because little connection between their populations and the Caribbean islands seems to be evident, the populations outside U.S. waters would benefit from an estimation of their trends as well. Furthermore, investigating ontogenetic shifts of spatial ecology as well as identifying sites of parturition would enhance the understanding of this species and its vulnerabilities.

Southern stingrays, as ecosystem engineers, food resources and an ecotourism commodity, are of high value throughout the Caribbean region, and investigating intimate details of their ecology is therefore of great interest when not only the conservation of threatened or vulnerable batoids throughout the region is considered but also the ecosystems that support them.

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