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RESEARCH ARTICLE

Testing for hybridisation of the Critically Endangered *Iguana delicatissima* on Anguilla to inform conservation efforts

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Abstract

The Caribbean Island of Anguilla in the north-eastern Lesser Antilles is home to one of the last populations of the Critically Endangered Lesser Antillean iguana *Iguana delicatissima*. This population is highly threatened primarily because of hybridisation with non-native *Iguana iguana*. This study assesses the degree of hybridisation between Anguilla's *Iguana* species firstly using morphological characteristics and then genetic analysis to validate the genetic integrity of morphologically identified *I. delicatissima*. We also examined the genetic diversity of Anguilla's *I. delicatissima* population, and that of a population on the nearby island of Îlet Fourchue, St Barthélemy. Forty-five iguanas were captured in Anguilla and 10 in St Barthélemy, and sequences from 3 nuclear and 1 mtDNA genes were obtained for each. Of the 45 iguanas captured in Anguilla, 22 were morphologically identified as *I. delicatissima*, 12 as *I. iguana* and the remainder were identified as hybrids. Morphological assignments were all confirmed by genetic analyses except for one *I. iguana* and one hybrid individual. These two individuals appeared likely to have originated following ancestral hybridisation events several generations ago. A significant paucity of genetic diversity was found within Anguillan and St Barthélemy *I. delicatissima* populations, with a single haplotype being identified for each of the three nuclear genes and the mtDNA sequence. This study highlights the urgency for immediate action to conserve Anguilla's remnant *I. delicatissima* population. Protection from hybridisation will require translocation to *I. iguana*-free offshore cays, with supplementary individuals being sourced from neighbouring islands to enhance the genetic diversity of the population.

Keywords Critically Endangered · DNA analysis · Lesser Antilles · *Iguana delicatissima* · Translocation

Introduction

West Indian iguanas are considered the most endangered lizards in the world (Alberts et al. 2004). Two genera of the Iguanidae, *Cyclura* and *Iguana*, are native to the West Indies: the genus *Cyclura* is restricted to the Bahamas and Greater Antilles, while the genus *Iguana* is native to the

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Lesser Antilles and parts of Central and South America. Two species of *Iguana* are currently recognised: the Critically Endangered Lesser Antillean iguana *Iguana delicatissima* (van den Burg et al. 2018), the green or common iguana *Iguana iguana* (classified by IUCN as Least Concern Bock 2018). The latter includes several native subspecies, including *Iguana iguana insularis* (first described and evaluated as Vulnerable by Breuil et al. 2019) and *Iguana iguana sanctaluciae* (described and evaluated as Critically Endangered, Breuil et al. 2019).

Historically, *I. delicatissima* is believed to have existed throughout the northern Lesser Antilles, from Anguilla to Martinique, having been recorded from Anguilla, Saint-Martin/Sint Maarten, St Barthélemy, St Eustatius, St Kitts and Nevis, Antigua and Barbuda, Guadeloupe, Dominica, and Martinique (Fig. 1). However, its current range is much reduced and the conservation status of *I. delicatissima* has recently been elevated on the IUCN Red List to Critically Endangered due to its restricted and severely fragmented distribution. These factors, along with reports from 2017 of the first sightings of *I. iguana* on the island of Dominica, which was considered one of the most secure strongholds of *I. delicatissima*, have highlighted the extreme vulnerability of this species (van den Burg et al. 2018).

Iguana delicatissima is threatened across its range by several factors including habitat loss and fragmentation, particularly in recent years by coastal development for tourism (Debrot et al. 2013) and extensive over-browsing by livestock that causes a shift in plant species composition and habitat structure (Breuil 2002). Predation by introduced invasive species (rats, cats and mongooses) is also a major threat (Barun et al. 2011; Medina et al. 2011). For example, on islands where Asian mongoose *Herpestes auropunctatus* have been introduced *I. delicatissima* is either extinct or highly threatened (Breuil 2002). However, the most widely reported threat facing *I. delicatissima* is competition and hybridisation with *I. iguana* (Vuillaume et al. 2015). In recent years *I. iguana* has been expanding its range northwards through the Lesser Antilles and northern Caribbean (Brisbane 2018; Lopez-Torres et al. 2010; van den Burg et al. 2019), with invasions of this species being assisted by tropical storms (Censky et al. 1998), hurricanes, ship traffic between islands (which have inadequate biosecurity measures) and the pet trade (van den Burg et al. 2018). Hybridisation between *I. delicatissima* and *I. iguana* has been confirmed through both molecular and morphometric analyses from Guadeloupe and all other main islands across their range (Day and Thorpe 1996; Vuillaume et al. 2015; van den Burg et al. 2018, 2019; Brisbane 2018). Post-invasion displacement of *I. delicatissima* is rapid and population extirpations due primarily to the presence of *I. iguana* have been recorded from several islands in the French West Indies (van den Burg et al. 2018; Breuil 2013).

Based on the range of threats, recent predictions suggest that within one generation, five of the eight remaining populations of *I. delicatissima* plus four of the currently invaded/hybridised locations will be extirpated (van den Burg et al. 2018). In addition, only 13% of the species' current estimated area of occupancy is predicted to remain three generations from now (van den Burg et al. 2018). Eradicating introduced and established *I. iguana* populations is probably unrealistic in most cases, both logistically and financially (Radford 2017). As such, recent conservation efforts have focused on protecting populations in sites that have not yet been colonised by *I. iguana*. These efforts include translocation from mainland sites to more easily protected offshore cays with no human or *I. iguana* populations. This conservation strategy has been successful in the French Overseas Territory of St Barthélemy where, in 2011, 14 individuals were translocated from the main island to augment the Îlet Fourchue population (reported as a few lone individuals) (Questel 2019). By 2019, at least 159 iguanas and 5 breeding areas were identified on Îlet Fourchue (Unpublished). A further translocation was implemented in 2011 to Îlet Frégate: surveys in 2018 reported a population of 41 iguanas, including 27 that had hatched on the island.

This study focuses on the most northerly distributed population of *I. delicatissima* located on Anguilla (18° 13′ 13.994″ N, 63° 4′ 7.014″ W) a UK Overseas Territory with a surface area of only 90 km^2. Day and Subin (1997) indicated that the *I. delicatissima* population had a restricted range on Anguilla, occurring in small pockets along the northern coast (Fig. 2). The study reported that Anguilla's *I. delicatissima* population was below the environmental carrying capacity and that intervention would be required to prevent the local extinction of the species (see also Gerber 1999). Adding further pressure to *I. delicatissima* on Anguilla, an unknown number of *I. iguana* (and/or *I. iguana* × *I. delicatissima* hybrids) unexpectedly appeared on mainland Anguilla following Hurricane Luis in September 1995, having been dispersed on flotsam (Censky et al. 1998). Questionnaire surveys of residents of Anguilla, conducted in January 2015, and subsequent captures and sightings *of I. iguana*, have revealed a rapid colonisation of *I. iguana* across Anguilla's mainland over the last 23 years, with this species now widespread over much of the island (Mukhida personal observation). With the increase and spread of the alien population of *I. iguana* it is plausible that any new offspring from the remnant declining population of *I. delicatissima* on Anguilla's mainland represent hybrids from recent matings with the invasive *I. iguana*.

Unique morphological characteristics have been defined for both *I. delicatissima* and *I. iguana* (Breuil 2013) and the use of several diverse characters is known to be advantageous when identifying hybrids within iguana populations (Vuillaume et al. 2015). While it has been alluded to (Martin et al.

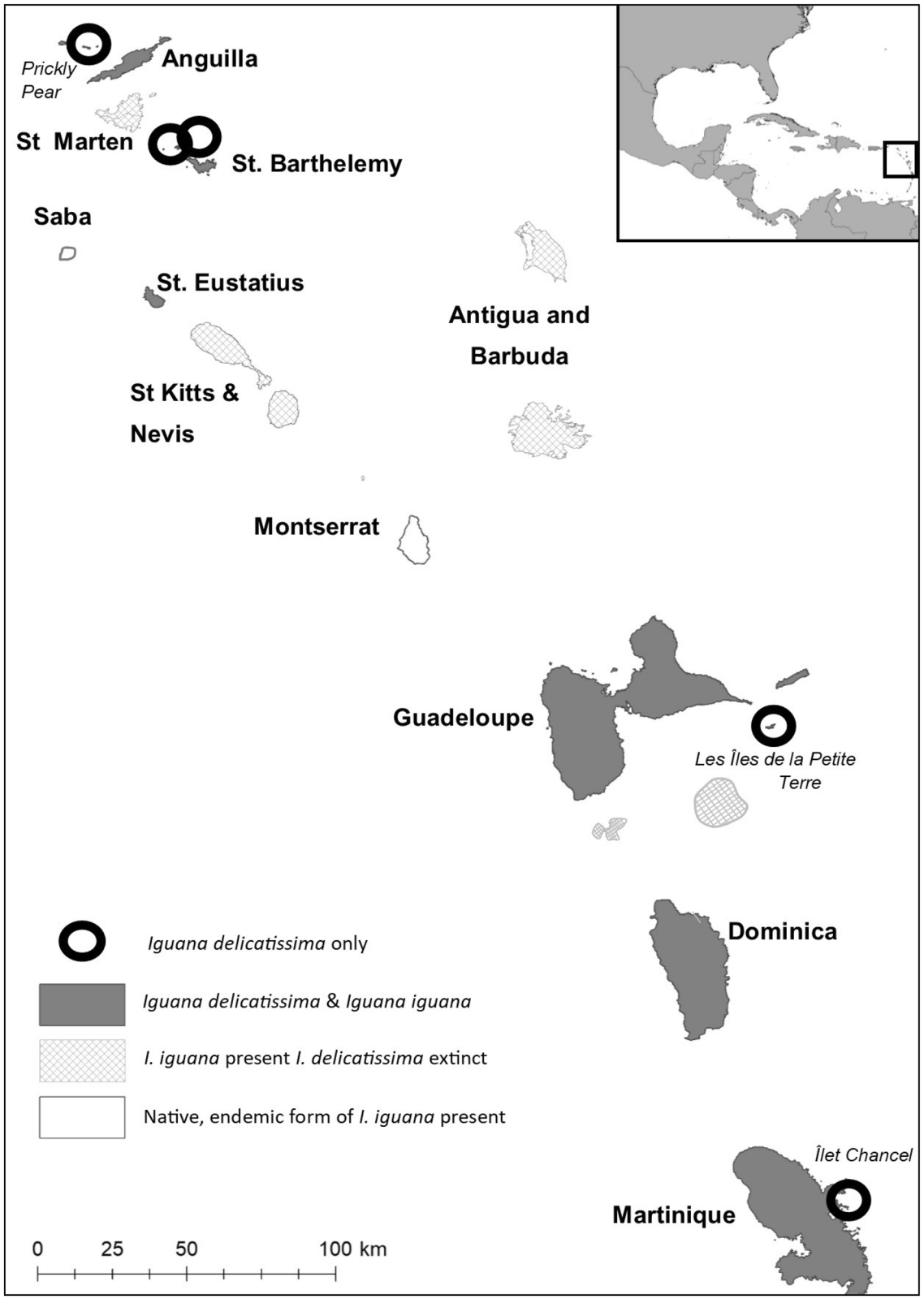

Fig. 1 Current and historic range of *Iguana delicatissima* in the Lesser Antilles. Only four uninhabited offshore cays are free of *Iguana iguana* and support *Iguana delicatissima* populations alone (highlighted by black circles). In addition, *I. delicatissima* has been translocated to the uninhabited offshore cay of Prickly Pear East, Anguilla. Inset map (square) shows the range of *I. delicatissima* on a wider continental scale

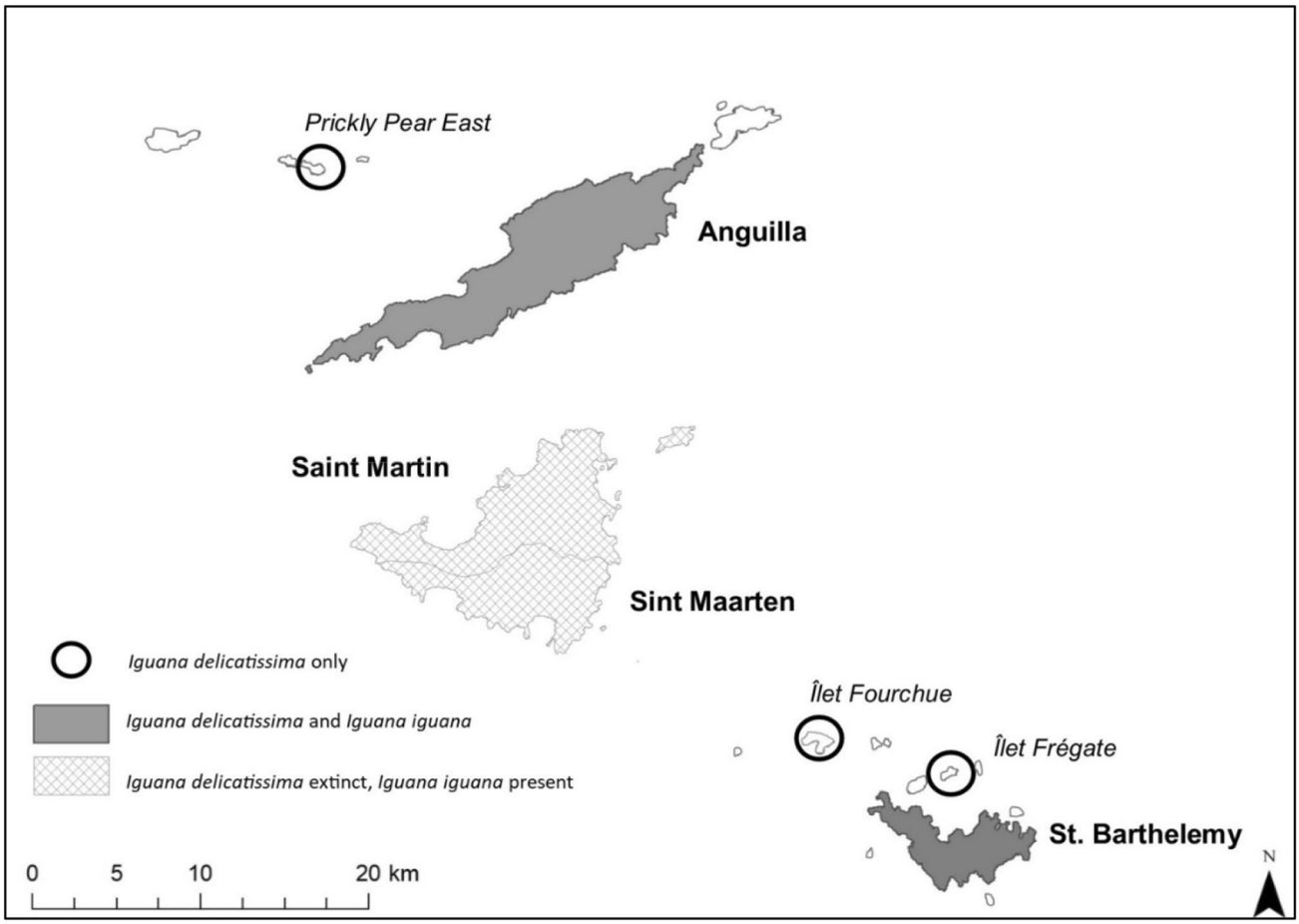

Fig. 2 The most northerly distributed populations of *Iguana delicatissima* located on Anguilla and St Barthélemy

2015; Vuillaume et al. 2015) it is unknown whether, when using Breuil (2013) detailed morphological descriptions for identification, "cryptic hybrids" may also be present in the population that bear the diagnostic characteristics of only one species. This study assesses the degree of hybridisation between Anguilla's *Iguana* species, firstly using morphological characteristics, and then using genetic analysis to test the genetic integrity of morphologically-identified *I. delicatissima*. This work was conducted with the aim of informing conservation strategies such as translocating *I. delicatissima* to *I. iguana*-free offshore cays. With this purpose in mind, we also analysed the genetic integrity of a growing population of previously translocated *I. delicatissima* individuals captured on the *I. iguana*-free island of Îlet Fourchue one of St Barthélemy uninhabited offshore cays, and the closest neighbouring *I. delicatissima* population to Anguilla.

Methods

Field methods

Between May 2015 and May 2018, comprehensive surveys for *I. delicatissima* were conducted along the northern coast of Anguilla (reflecting previously reported known distributions of this species (Gerber 1999) (Fig. 2). Surveyors attempted to capture all iguanas encountered using a noose or net or by hand. Captured animals were weighed,

morphological measurements and photographs were taken, and those individuals deemed to be *I. delicatissima* or those exhibiting any of the 12 defined *I. delicatissima* morphological characteristics (see Breuil 2013) were temporarily housed in a specifically designed captive facility. The presence of *I. delicatissima* or *I. iguana* states for each of the 12 morphological characters was recorded for each iguana using a checklist ("Appendix"). In addition to surveying the previously reported sites for *I. delicatissima*, field surveys were undertaken ad hoc in a range of sites across the island, and whenever a member of the public reported an iguana, field staff would visit to identify and attempt capture. In addition to the surveys undertaken on Anguilla, during a 2-day visit in June 2018, 10 iguanas morphologically identified as pure *I. delicatissima* were captured using a stick with a noose or directly by hand on Îlet Fourchue (17°57'23.59"N, 62°54'5.70"W), St Barthélemy (Fig. 2).

For all iguanas captured, the end of the tail was sanitised with 70% alcohol, and 1–2 cm was removed from the tip with scissors. This tissue was immediately scored with a sanitised blade and cut into smaller segments before being stored in a 3 ml cryovial containing DNAgard® (Sigma Aldrich, UK). Samples were shipped to the University of Liverpool, UK for genetic analysis with permission from the Government of Anguilla. Samples were analysed to test for the presence of *I. iguana* haplotypes circulating in this population and to compare the genetic diversity of populations in Anguilla and St Barthélemy.

Gene sequencing and analyses

Genomic DNA was extracted from approximately 10 mg iguana tail segments using the DNeasy Blood and Tissue Kit (Qiagen, UK) according to the manufacturer's instructions. PCR was used to amplify partial sequences as previously described for 3 nuclear genes and 1 mitochondrial gene: 572 bp oocyte maturation factor Mos (C-mos) (Noonan and Chippindale 2006), 498 bp Neurotrophin-3 segment (NT3) (NT3-F3 and NT3-R4: Noonan and Chippindale 2006), 569 bp polymerase alpha catalytic subunit (PAC) (PACs1 and PACs2: Pasachnik et al. 2009) and 801 bp NADH dehydrogenase subunit 4 (ND4) (a maternally inherited marker used to elucidate the direction of crosses Martin et al. 2015). PCR products were verified by gel electrophoresis and amplicons purified using Qiagen PCR purification kits (Qiagen, UK). DNA sequencing of the purified products was performed by GATC Biotech (GATC Biotech, Germany). Unique sequences were deposited in GenBank.

Nucleotide sequences from all specimens and available published Iguanidae sequences were aligned for all loci using MEGA7 (Kumar et al. 2016). Accession numbers for all sequences retrieved from GenBank are shown next to taxonomic names on the phylogenetic tree. Sequence identities were compared using Geneious 11.0.5 (http://www.geneious.com, Kearse et al. 2012). DNA substitution models were selected based on likelihoods, using the Akaike corrected information criterion in MEGA7. Maximum likelihood phylogenetic trees were generated using these models in MEGA7 (Kumar et al. 2016) with node support based on 10,000 bootstrap replicates (Felsenstein 1985). Further, haplotype networks were constructed using PopART version 1.7 (Leigh and Bryant 2015) using the TCS method (Clement et al. 2002), in order to visualise instances of hybridisation. Finally, the ability for the genetic markers employed in this study to categorise species and hybrids within a population, was ascertained by comparing our data to species specific loci identified from currently published sequences.

A Bayesian analysis of population structure (BAPS) v.6.0 (Corander et al. 2006, 2008; Cheng et al. 2011) was employed to identify existing genetic groups among the individuals sampled for each of the four genes utilised and in order to identify putative hybrids. The haplotypes for 55 unclassified [Anguilla (n = 45) and St Barthélemy (n = 10)] and two reference individuals were analysed with no priori assumption. An alignment of all published sequences of each Iguana species was used to identify all species specific sites in order to generate a consensus for each reference individual. Our test population was assumed to be comprised of two species, therefore the number of clusters was set to two (K = 2). An admixture analysis was performed after the mixture analysis to calculate the genetic mixture of each individual from the genetic groups found (Corander et al. 2006, 2008). The analysis was run with 1000 iterations.

Results

Sampling and identification

On Anguilla, approximately 600 h of survey effort over the previously reported range of *I. delicatissima* (Fig. 2) resulted in the capture of 26 individuals (a further 8 individuals with morphological characteristics of *I. delicatissima* were sighted but evaded capture). Of those captured, 22 individuals exhibited all morphological characteristics of *I. delicatissima*, three exhibited morphological characteristics of both *I. delicatissima* and *I. iguana*, and 1 appeared morphologically to be *I. iguana* (Table 1). In addition, 12 individuals that were morphologically diagnosed as *I. iguana*, and 7 that exhibited at least 1 characteristic of both *I. delicatissima* and *I. iguana* (Table 1) were caught from a range of locations distributed widely across the main island of Anguilla (Fig. 3). Based on the 12 morphological features (Table 1), 9 of the 10 morphologically identified hybrids appeared most similar to *I. iguana* and 1 most similar to *I. delicatissima*.

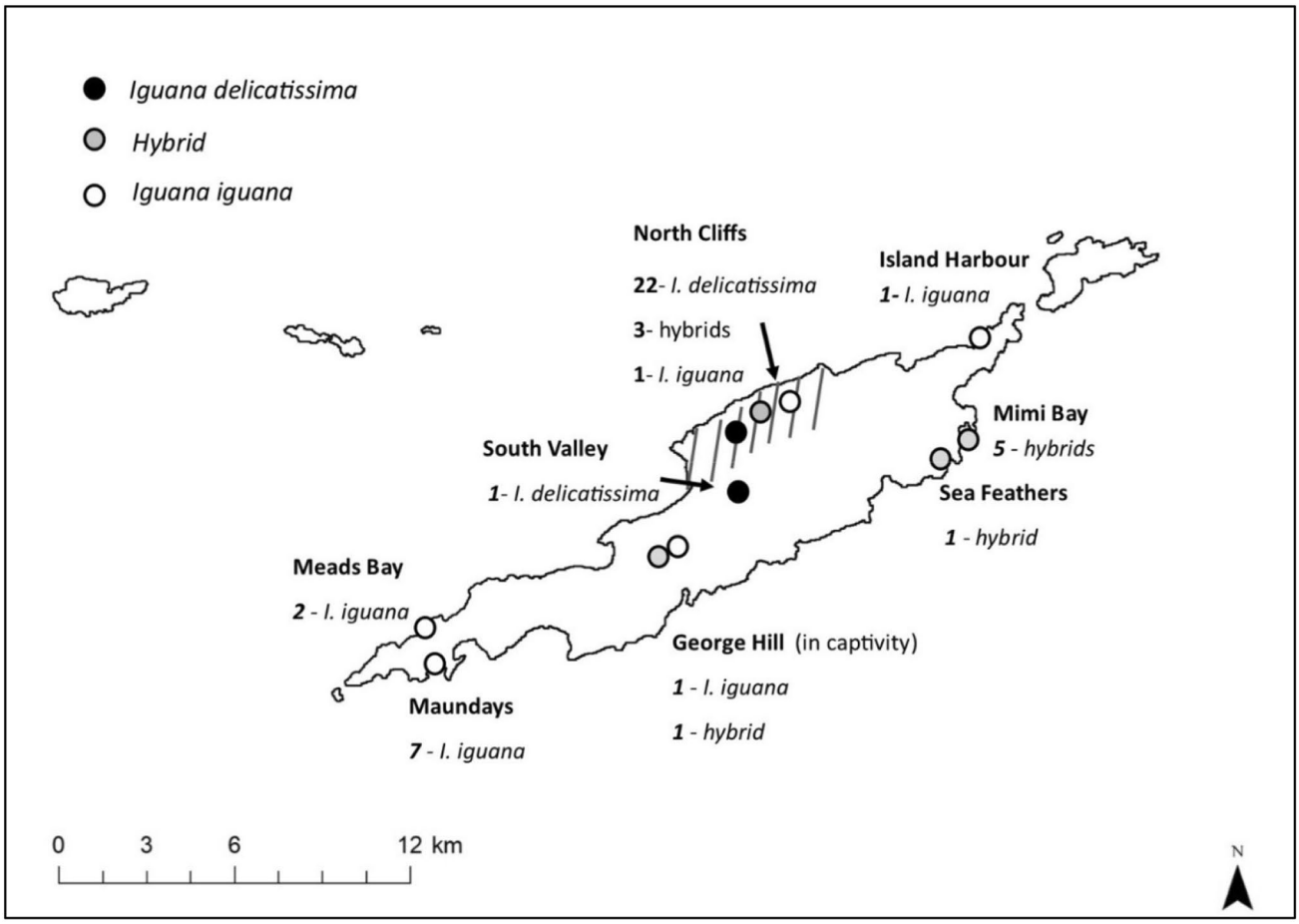

Fig. 3 The locations of captured *Iguana delicatissima*, *Iguana iguana* and hybrids on mainland Anguilla. Hatched area represents the previously reported range of *I. delicatissima* on Anguilla's mainland by the mid 1990s (Day and Subin 1997)

Only 1 of the 12 morphological characteristics that were diagnostic for *I. iguana* was present in all hybrids: the presence of black bands on the tail (Table 1). In contrast, no single diagnostic feature of *I. delicatissima* was consistently observed in all of the hybrids, although presence of a bumpy head (but not necessarily the presence of occipital bumps) was a common *I. delicatissima* feature observed in these individuals and recorded in 9 of the 10 morphologically identified hybrids (Table 1, "Appendix").

DNA analyses

BAPS demonstrated a clear pattern of grouping all tested individuals into two distinct genetic clusters with high probability ($p < 0.0001$ for all). Animals morphologically assigned as *I. delicatissima* clustered together, as did all individuals designated *I. iguana*. Clustering differences between genes were only evident within individuals classified as hybrids (Fig. 4).

C-mos

All sequences that originated from the 22 individuals with *I. delicatissima* morphologies were identical and represent a currently unreported haplotype IdANG (MK605403). The haplotype showed 99.7% similarity to that previously reported for Anguilla, and neighbouring islands of Dominica and St Eustatius (HM352538; Stephen et al. 2013). Hybrid D1 was notable for solid bands on the tail, a feature commonly reserved for *I. iguana*, provided identical (homozygous) sequences clustering along with the previously reported Anguillian *I. delicatissima* (Fig. 5a; Table 2; Supplementary Fig. 1a). All individuals morphologically diagnosed as *I. iguana* were most similar (99.7%) to previously identified *I. iguana C-mos* sequences from individuals from Belize, Brazil, Curacao, El Salvador, Honduras, Mexico, St Lucia and Suriname (Stephen et al. 2013). Three individuals that were diagnosed as hybrids (GR9, GR11, GR19) were heterozygous at two interspecific sites located across the partial sequences, demonstrating the presence of haplotypes of both *I. iguana* and *I. delicatissima* (Table 2). The remaining

Table 1 Characteristics of the morphologically identified hybrids captured on the main island of Anguilla

Morphological characteristic	*Iguana iguana* characteristic	*Iguana delicatissima* characteristic	*GR6*	*GR9*	*GR10*	*GR11*	*GR15*	*GR16*	*GR17*	*GR18*	*GR19*	*GR22*	*D1*
1	Subtympanic plate present	Subtympanic plate absent	I	I	I	I	I	I	I	I	D	I	D
2	Lower sub-labial scales forming a mosaic	Sublabial row of scale parallel to labial scale	I	I	I	D	I	I	I	I	I	I	D
3	Flat sub-labial scales	Rounded scales + row of oval scales between the labials and the sub-labials	D	D	I	ID	I	I	I	I	I	I	D
4	Dewlap edges forming a right angle	Rounded dewlap edge	I	D	I	D	I	I	I	I	D	I	D
5	Gular spikes extending into the lower half of the dewlap	Gular spikes on the straight upper edge of the dewlap	I	D	I	D	I	I	I	I	I	I	D
6	> 10 Gular spikes	< 7–8 Gular spikes	I	D	I	I	I	I	I	I	I	I	D
7	Triangular gular spikes	Conical long curved gular spikes	I	I	I	ID	I	I	I	I	I	I	D
8	Top of head flat	Top of head bumpy + occipital bumps	I	D	I	D	D	D	D	D	D	D	D
9	Chestnut brown eye	Grey eye	I	I	I	I	I	I	I	I	I	I	D
10	Nuchal tubercles	Lack of Nuchal tubercles	I	I	I	I	I	I	I	I	I	I	D
11	Body colour greenish-grey	Body colour brownish grey	I	I	I	I	I	I	I	I	I	I	D
12	Bands on tail	No bands on tail	I	I	I	I	I	I	I	I	I	I	I

Hybrids with an *Iguana iguana* morphological characteristics are labelled as 'I' while those with *Iguana delicatissima* morphological characteristics are labelled as 'D'. If the morphological feature in question was a combination of both *I. iguana* and *I. delicatissima* morphology this is labelled as 'ID'. Morphological identification followed the methods of Breuil (2013)

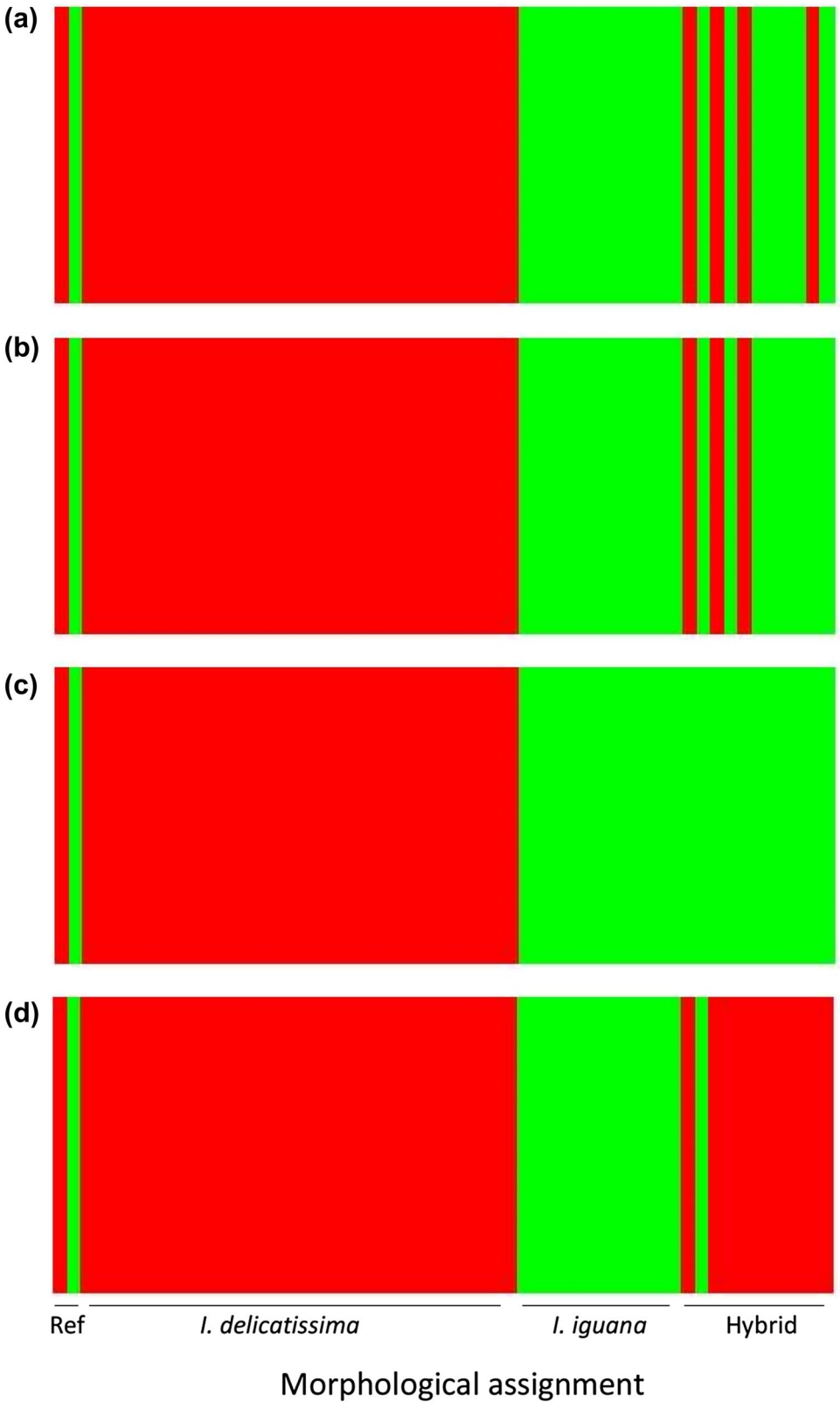

Fig. 4 Bayesian analysis of population structure (BAPS) clustering for each of the partial nuclear genes, **a** C-mos, **b** NT3, **c** PAC, and the mitochondrial gene, **d** ND4. Analyses were conducted on 57 *Iguana* individuals (55 unclassified and 2 reference) under the assumption of 2 genetic clusters (K = 2) present within the population (*I iguana and I. delicatissima*). Vertical bars represent the genotypes that have been apportioned to cluster 1 (*I. delicatissima*) and cluster 2 (*I. iguana*), red and green respectively. Hybrid order—D1, GR6, GR9, GR10, GR11, GR15, GR16, GR17, GR18, GR19, GR22

six morphological hybrids exhibited no heterozygosity and clustered with other *I. iguana* samples from Anguilla, suggesting that these individuals are the result of cross-species matings more than one generation ago (Fig. 5a; Table 2; Supplementary Fig. 1a).

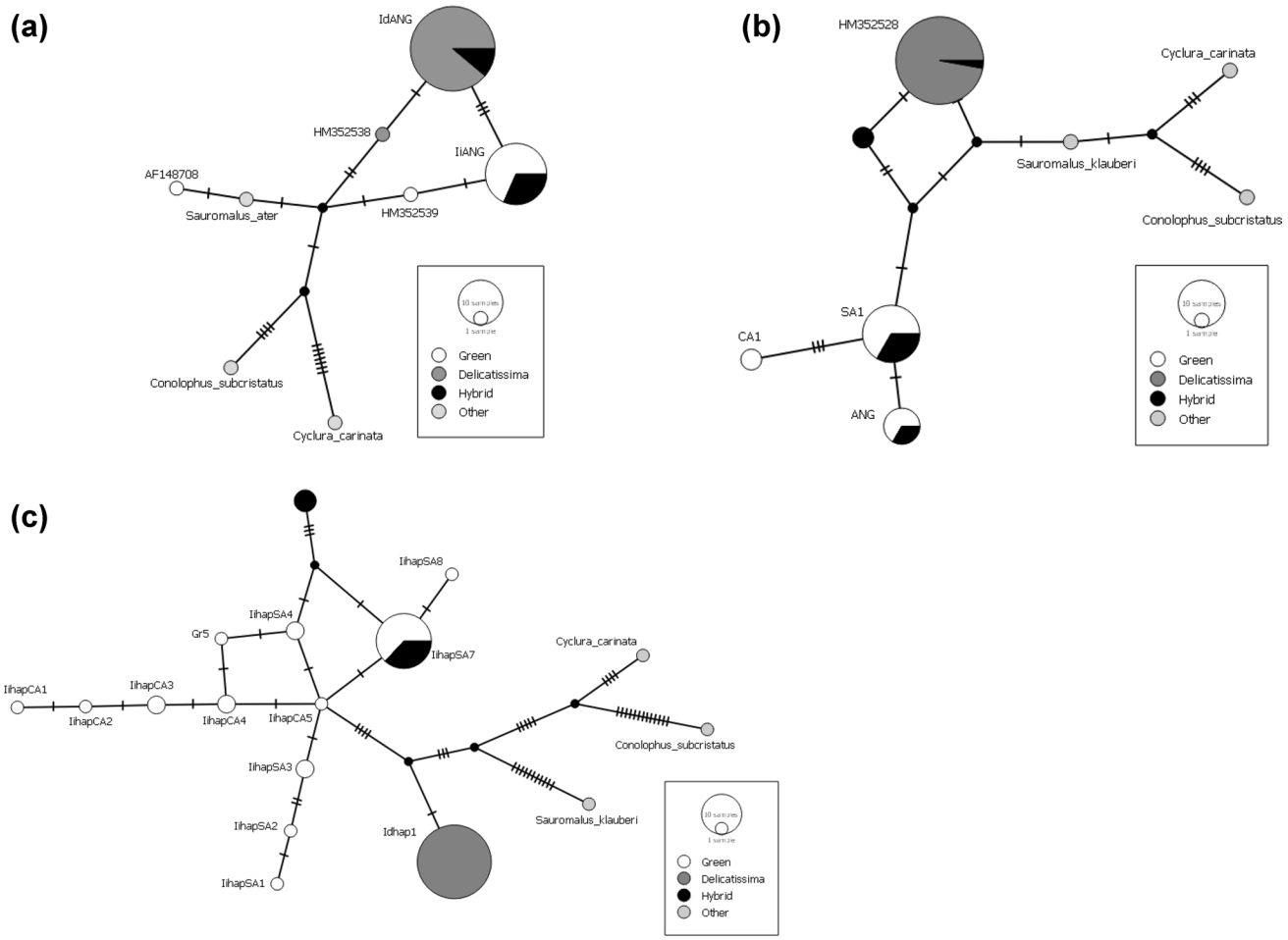

Fig. 5 TCS haplotype networks generated using PopART version 1.7 for the four partial genes isolated in this study, C-mos (**a**), NT3 (**b**), PAC (**c**), and ND4 (**d**). Each coloured circle represents an individual Iguana haplotype. The size of the circle indicates the relative frequency of sequences belonging to a particular haplotype (smallest circle = 1). Hatch marks along the network branches indicate the number of mutations. Small black circles represent unsampled intermediate haplotypes

NT3

All 22 morphologically assigned *I. delicatissima*, along with hybrid D1 were 100% identical across the partial NT3 gene, and identical to previously reported haplotypes (HM352528 and MF191675; Stephen et al. 2012 and van den Burg et al. 2018 respectively), isolated from individuals on Anguilla, Dominica and St Eustatius. None of the sequences obtained from these individuals showed any polymorphic sites when aligned together (Table 2). All sequences from morphologically identified *I. iguana* as well as eight of the hybrids clustered with previously reported *I. iguana* sequences from Mexico, Caribbean, as well as Central and South America (Stephen et al. 2012), with evidence suggesting the presence of at least three *I. iguana* NT3 haplotypes on Anguilla, CA1, SA1 and ANG reported here (HM352529, HM352530 and MK605401, respectively) (Fig. 5b; Supplementary Fig. 1b). Six of these individuals including hybrid GR6 and GR18 were heterozygous for two *I. iguana* NT3 haplotypes (SA1 and Ang) (Table 2). GR9 and GR11 were the only two hybrids identified genetically, clustering with *I. delicatissima* as a result of heterozygosity at three interspecific sites across the partial regions (Table 2).

PAC

As for other loci, all 22 partial gene sequences tested from morphologically assigned *I. delicatissima*, for the PAC region were 100% identical and matched reported *I. delicatissima* haplotypes Idhap1 and StE (JN811104 and MF191674; Stephen et al. 2012 and van den Burg et al. 2018 respectively), isolated from individuals on Anguilla, Dominica and St Eustatius. The hybrid D1 was homozygous yet differed to the Anguillan *I. delicatissima* in that it had five base substitutions and six base additions at *I. iguana*

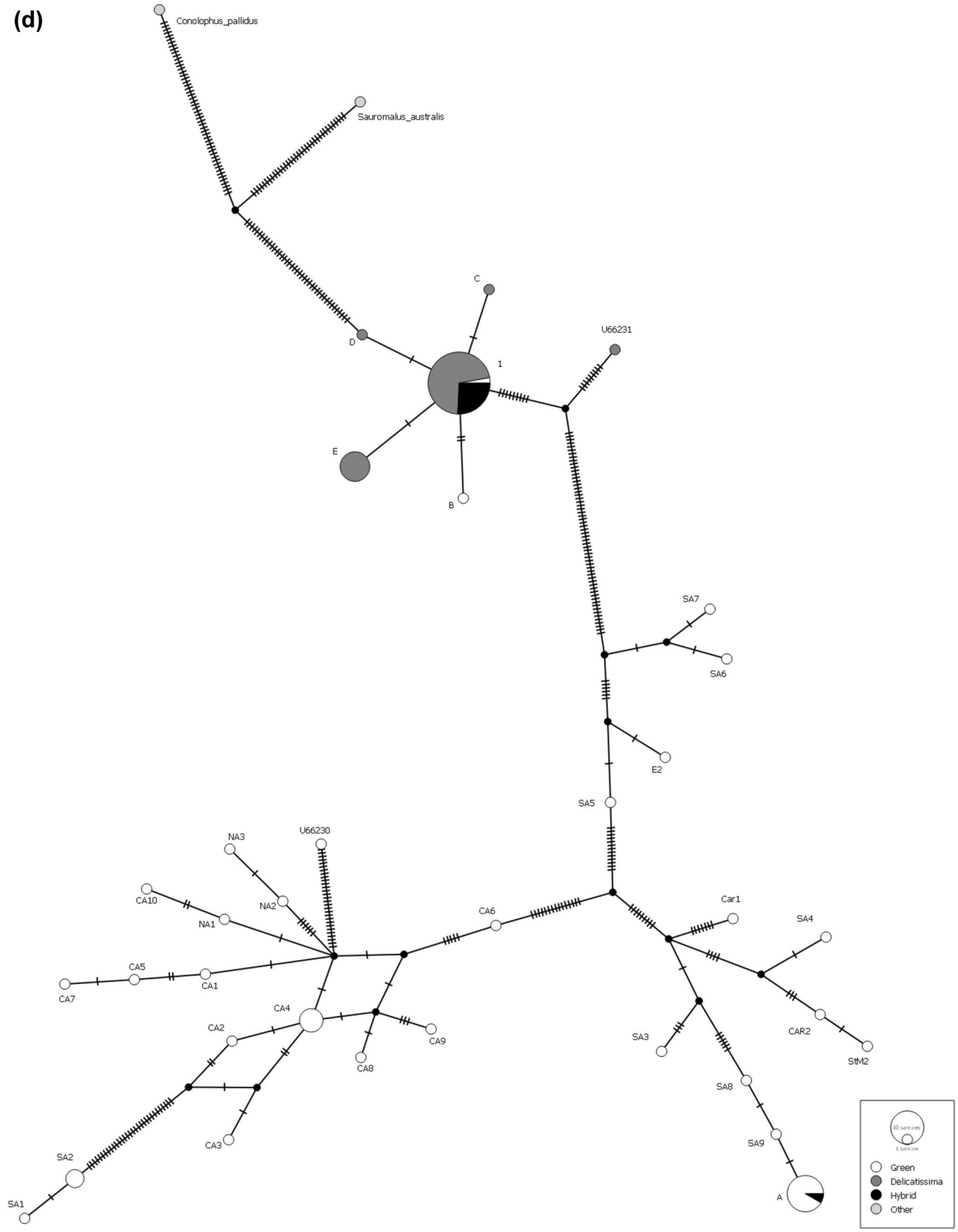

Fig. 5 (continued)

Table 2 Presence of species-specific nucleotide variants for each individual sampled on the main island of Anguilla and St Barthélemy

	C-mos 2 Loci	NT3 4 Loci	PAC 11 Loci	ND4 35 Loci	Species assignment	Number of *I. delicatissima* morphological features	Number *of I. iguana* morphological features	Both *I. delicatissima* and *I. iguana* features
I. iguana (consensus)	**2/0**	**4/0**	**11/0**	**35/0**	*I. iguana*	12	0	–
I. delicatissima (consensus)	0/2	0/4	0/11	0/35	*I. delicatissima*	0	12	–
D1	0/2	0/4	**11/0**	0/35	Hybrid	11	1	–
D2–23, DB1–10	0/2	0/4	0/11	0/35	*I. delicatissima*	12	0	–
GR1–5, 7, 8, 12–14, 20, 21	**2/0**	**4/0**	**11/0**	**35/0**	*I. iguana*	0	12	–
GR6	**2/0**	**4/0**	**11/0**	**35/0**	*I. iguana*	1	11	–
GR9	2/2	3/4	**11/0**	0/35	Hybrid	5	7	–
GR10	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	12	–
GR11	2/2	3/4	**11/0**	0/35	Hybrid	4	6	2
GR15	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	11	1
GR16	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	11	1
GR17	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	11	1
GR18	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	11	1
GR19	2/2	**4/0**	**11/0**	0/35	Hybrid	3	9	–
GR22	**2/0**	**4/0**	**11/0**	0/35	Hybrid	0	11	1

Each cell contains a ratio of *I. iguana*:*I. delicatissima* variants, with individuals with primarily *I. iguana* variations of a gene denoted by bold type and those with *I. delicatissima* variations as normal type. Combined nucleotide variant values greater than the number of species specific loci (highlighted in row 1) in that gene demonstrate the presence of polymorphisms. Columns 7–9 indicate how many morphological features of *I. delicatissima* and *I. iguana* were identified

interspecific sites and was thus identical to previously reported *I. iguana* haplotype IihapSA7 (JN811116) and clustered along with other *I. iguana* sequences rather than *I. delicatissima* (Fig. 5c; Table 2; Supplementary Fig. 1c). The absence of *I. delicatissima* sequence suggests this individual was not a first generation (F1) hybrid. All *I. iguana*, along with hybrids that possessed primarily *I. iguana* features, clustered among other *I. iguana* PAC sequences (Fig. 5c; Supplementary Fig. 1c) (Stephen et al. 2012). However, the presence of several haplotypes within the *I. iguana* population on Anguilla was notable relative to other genes.

ND4

The ND4 gene was identical for all 22 morphologically elected *I. delicatissima* and hybrid D1, showing that they carried *I. delicatissima* mtDNA (Fig. 5d; Table 2; Supplementary Fig. 2). The samples from Anguilla were identical to previously reported *I. delicatissima* haplotype 1 and A (AF217783 and KJ561221; Malone et al. 2000 and Martin et al. 2015 respectively), isolated from individuals across the West Indies on Anguilla, Dominica, Guadeloupe, Îles des Saintes, La Désirade, Petite Terre, and St Eustatius. The majority (12 out of 13) of *I. iguana* individuals possessed mtDNA consistent with their morphological classification, whereas a single individual had *I. delicatissima* mtDNA (GR10). Of the nine individuals that were considered hybrids but showed primarily *I. iguana* features, eight had *I. delicatissima* DNA, and one (GR6) had *I. iguana* maternal DNA (Table 2). This suggests that the direction of cross is predominantly male *I. iguana* mating with *I. delicatissima* females (Table 2).

Genetic identity of St Barthélemy iguanas

All 10 *I. delicatissima* individuals from St Barthélemy were found to have identical haplotypes to those circulating in the Anguillan *I. delicatissima* population for the three nuclear loci. Two ND4 haplotypes were identified with three individuals being identical to the Anguillan *I. delicatissima* haplotype, and seven identical to haplotype E previously reported from St Barthélemy (Martin et al. 2015). No indication of hybridisation was reported in the iguana's sampled from St Barthélemy.

Discussion

This present study evaluated the genetic integrity of the *I. delicatissima* population on Anguilla. We demonstrated that the remnant population on the Anguilla mainland has

already hybridised with the introduced *I. iguana*, with evidence of hybridisation in 12% of individuals captured in the last remaining pocket of habitat containing *I. delicatissima*. In addition, there is evidence of hybrid matings occurring more than one generation ago. Our work further highlights that hybridisation with *I. iguana* is a major threat facing *I. delicatissima* populations and is of even wider significance in light of recent reports of *I. iguana* hybridizing with other native and endangered *Iguana* and *Cyclura* populations in the region (Moss et al 2018).

Morphological and genetic comparison

Two mismatches between morphological and genetic identity were identified. One individual (GR6) was morphologically identified as a hybrid due to one *I. delicatissima* feature (presence of rounded scales and a row of oval scales between the labials and the sub-labials) but clustered with *I. iguana* in all four genes analysed and could not be identified genetically as a hybrid. In contrast, GR10 was morphologically identified as *I. iguana* but genetically was found to have *I. delicatissima* mtDNA. However, this individual was a subadult so may not have yet developed all of the distinguishing morphological features. These two exceptions, which constitute 7.7% of the sampled animals, indicate that the ancestral hybridisation event(s) occurred several generations ago, which could explain discrepancies in morphological identification, and highlights why morphological identification should not be relied upon on its own, particularly if several generations have passed since the invasion of *I. iguana* and in the identification of juveniles.

Ancestry of hybrids

Of the 10 hybrids genetically identified in this study, 9 exhibited *I. delicatissima* ancestry through the maternal lineage. This evidence demonstrates the direction of cross between these two species, with male *I. iguana* mating with female *I. delicatissima*. Previous studies have reported the more vigorous, larger *I. iguana* males achieving more matings than male *I. delicatissima* (van den Burg et al. 2018). A similar behaviour has been reported in the Galápagos Islands between land iguanas (*Conolophus* sp.) and marine iguanas (*Amblyrhynchus cristatus*), where unmated female land iguanas are forced to mate with male marine iguanas which are larger and more aggressive (Rassmann et al. 1997). An alternate explanation for this is that male *I. delicatissima* are more selective when it comes to mating opportunities. However, van den Burg et al. (2018) reported hybrids on the nearby island of St Eustatius with *I. iguana* mtDNA, showing that male *I. delicatissima* may also mate with female *I. iguana*. Nevertheless, we cannot exclude the notion that the small population size of *I. delicatissima* on mainland Anguilla increases the likelihood of a female *I. delicatissima* being encountered by a male *I. iguana* rather than a male *I. delicatissima*. While the sex ratio of *I. delicatissima* caught in this study was skewed at 0.57 males to every female (8 males:14 females plus 1 juvenile), we only captured 1 male *I. iguana* in this area, and 3 hybrids—2 of which were subadults. Thus, there does not ostensibly seem to be a greater potential for female *I. delicatissima* to encounter a male *I. iguana* than a male *I. delicatissima*, at least in this restricted range.

Whilst several hybrids were identified on Anguilla through genetic analysis, none demonstrated evidence of hybridisation in all of the three nuclear loci. Two hybrids were identified at two of these loci, one by two genes, and seven in just one gene (Table 2). This discordance among the genes analysed suggests that none of the 10 hybrids genetically identified are first generation hybrids and directly points to more distant hybridisation in their ancestry, and in fact the hybridisation event could have even taken place prior to its ancestors arriving on Anguilla (Censky et al. 1998). It is also worth noting that ancestral polymorphisms and incomplete lineage sorting can disrupt phylogenetic inference and may contribute to differences observed (Maddison 1997; Steiner et al. 2012). In the future, next generation technologies such as RADseq could be utilised to amplify thousands of loci from across the genome to further explore and provide insights into the relatedness and ancestry of individuals.

Genetic diversity of Anguillan iguana populations

It is apparent from the genetic markers used in this study that there is a significant paucity of genetic diversity within the Anguillan *I. delicatissima* population with only one haplotype being identified in each of the four loci. This is perhaps not surprising as other studies have demonstrated a lack of unique haplotypes between geographically isolated populations (Day and Thorpe 1996; Martin et al. 2015; Stephen et al. 2012), and has also been reported in the populations on St Eustatius, Dominica and the French West Indies (Judson et al. 2018; van den Burg et al. 2016; Vuillaume et al. 2015). Other genetic markers such as microsatellites could be informative in understanding the structure and genetic diversity of a population, as well as estimating effective

populations sizes (Valette et al. 2013; van den Burg et al. 2016), but this was not an objective of the current study.

In contrast to the low genetic diversity observed in Anguilla's *I. delicatissima* population, the *I. iguana* population exhibits more variation and indicates a larger founder population (Crawford and Whitney 2010). This is not surprising as *I. iguana* are reported to have arrived into Anguilla through several routes, such as being transported on hurricane debris (Censky et al. 1998) and by boat traffic from the neighbouring island of Saint Martin/Sint Maarten which lies only 12 km from Anguilla (Mukhida personal observation).

Conservation implications

The genetic identification of hybrid iguanas, and the discovery of both hybrids and *I. iguana* in the remaining range of *I. delicatissima* on the Anguilla mainland highlights the urgency of taking immediate conservation action to ensure the continued survival of *I. delicatissima* on Anguilla, and in maintaining this species range across the Lesser Antilles. Since 2016 any individual identified both morphologically and genetically as *I. delicatissima* has been translocated to one of Anguilla's uninhabited and *I. iguana*-free offshore cays: Prickly Pear East (18° 15′ 60.00″ N, − 63° 10′ 60.00″ W) (Fig. 2), which had been previously identified as a suitable site for reintroduction (Day and Subin 1997; Mukhida and Soanes 2016). Population monitoring and biosecurity (to ensure *I. iguana* do not reach the island) will continue to monitor the progress of this translocation and, following a regional *I. delicatissima* conservation planning meeting in March 2018 (Mukhida et al. 2018), discussions have been taking place regarding the translocation of further individuals from sites that support larger populations.

According to Franklin's (1980) somewhat controversial 50/500 rule, an effective population size (defined as a measure of the genetic behaviour of a population relative to that of an 'ideal' population) of under 50 individuals is at high risk of extinction. Other authors have suggested this number should be higher (Reed and Bryant 2000). While we cannot be sure how many individuals would provide a sustainable population size on Prickly Pear East, it is clear that the founders of this newly reintroduced population will need to be supplemented with additional bloodlines to ensure long-term survival (IUCN/SSC 2013). The analysis of samples from St Barthélemy has highlighted the growing population on Îlet Fourchue as one such potential donor site. The genetic analysis of 10 individuals from this population revealed an almost identical genetic composition to those on Anguilla, and no evidence of hybridisation was found in the 10 individuals sampled. If translocation from Îlet Fourchue is deemed appropriate under international translocation guidelines (IUCN/SSC 2013) further genetic analysis will be undertaken of all individuals due to be translocated to avoid inadvertently introducing hybrids. In addition, before any translocations are undertaken under the auspices of 'genetic rescue' (Bell et al. 2019) it is necessary to assess the genomic heterozygosity of the donor population and to examine the history of population (i.e. have historic bottlenecks been recorded) (Robinson et al. 2019). The prevalence and the risk of disease should also be thoroughly assessed (Hellebuyck et al. 2017).

This study has further highlighted the urgency of action to preserve Anguilla's remaining *I. delicatissima* population on offshore islands. Without intervention, the rapid spread of *I. iguana* on Anguilla's mainland and the level of hybridisation already reported here will rapidly lead to the extirpation of Anguilla's mainland *I. delicatissima* population.

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Appendix

See Fig. 6.

Date.	Time.	Location.	Waypoint. Coordinates.

Capture method	

Age: Hatchling / Juvenile / Adult	Sex : Male / Female
	Probe depth /

PIT #
STICK PIT LABEL HERE

Check when complete		
Head photo	DNA sample	Bead ID sequence #

SVL	Vent -tail	Regen tail length	Head length	Head width	Head height	Weight	4th toe length front	4th toe length hind

Checklist of morphological features of the head of *Iguana iguana / Iguana delicatissima*. Refer to annotated head shot guide (circle as applicable, include actual data recorded and notes) i.e (Gula spikes > 10) / Gula spikes < 7-8. 11 Gula spikes

1. Subtympanic plate / No subtympanic plate.

2. Lower sublabial scales forming a mosaic / Sublabial row of scales ± parallel to the labial scales

3. Flat sub-labial / Rounded scales + Row of oval scales between the labials and sub-labials

4. Dewlap edges forming a right angle / Rounded dewlap edge

5. Gular spikes extending into the lower half of the dewlap / Gular spikes on the straight upper edge of the dewlap

6. Gular spikes > 10. Gular spikes <7-8

7. Triangular gular spikes / Conical, long and more or less curved gular spikes

8. Top of head flat / Top of head bumpy + Occipital bumps

9. Eye chestnut brown / Grey eye

10. Nuchal tubercles / Lack of nuchal tubercles

11. Body colour greenish grey / Body colour brownish-grey

Fig. 6 Checklist used for recording the morphological features of each iguana caught during this study

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