METHODS AND RESOURCES ARTICLE

A genus-wide analysis of genetic variation to guide population management, hybrid identification, and monitoring of invasions and illegal trade in *Iguana* (Reptilia: Iguanidae)

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Abstract

Biodiversity and wild populations are globally threatened by a wide range of actors. The genus *Iguana*, widely distributed throughout the Americas, is under threat by invasive species, hybridization, the global pet trade, and habitat destruction. This holds especially true for the insular lineages, with the Critically Endangered *I. delicatissima* having experienced a > 75% range decrease, primarily through hybridization with non-native iguanas. We collated published microsatellite data and genotyped samples from new localities to construct a distribution-wide *Iguana* dataset built from 190 individuals for 17 microsatellite loci. This enabled us to identify patterns of genetic differentiation within and among populations, and identify key loci and private alleles for use in conservation management. Our analyses reveal clear separation between *I. delicatissima* and the *I. iguana* complex, highlighting the power of eight key microsatellite loci for the study of hybridization dynamics. Genetic differentiation within *I. delicatissima* identifies four clusters that aid decision making for conservation management action. Within the *I. iguana* complex, we increase mainland localities by 11-fold and recover 3.5 × more alleles across all loci than previously known. Overall, we identify 112 (48% private) and 76 (25% private) alleles for mainland and island lineages, respectively. We highlight loci sets to identify (1) non-native or hybrid iguanas in insular populations and their genetic origin, and (2) genetic origin of insular iguanas in the global pet trade. Overall, we provide a reference for *Iguana* microsatellite loci in order to allow standardization and comparison among studies, aiding broader assessment of research and conservation hypotheses.

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Introduction

Genetic tools are broadly utilized to gain understanding of wild populations and their conservation needs; e.g., examining metapopulation dynamics (Rowe et al. 2003), tracking illegal collection and trade (Coetzer et al. 2017), and characterizing genetic variation and inbreeding (Luís et al. 2007; Engelhardt et al. 2017). Although development of novel techniques continues, 2nd generation methodologies (microsatellites) are still broadly applied (Vashistha et al. 2020) as these are overall cheap to obtain data from, are preferred for small sample sizes, and can be processed and analyzed rapidly. Especially within conservation science, where funding is limited and results tend to be time sensitive, microsatellites are an important go-to genetic tool.

Among Iguaninae, *Iguana* is the widest ranging genus, with mainland populations occurring from central Mexico

through to central Brazil, and widespread presence on Caribbean islands (Belezian, Colombian, Honduran, Mexican, Nicaraguan, and Tobago and Trinidad islands, as well as in the Southern and Lesser Antilles; Iguana Taxonomy Working Group 2016; Hedges et al. 2019). Taxonomically, this genus consists of two well defined lineages, *I. delicatissima* and the *I. iguana* complex. Within the latter, hypotheses of species boundaries are unresolved, though are currently under investigation, see Table 1 for nomenclature and geographic ranges (Stephen et al. 2013; Breuil et al. 2019, 2020, submitted, van den Burg et al. in prep.).

As is true for many Iguanids (IUCN 2020), insular lineages within *Iguana* are highly endangered. The main threat to these species is the continuous spread and recurrent incursion of non-native iguanas, both through natural events and, since at least the 19th Century (Breuil 2003, 2013), anthropogenic assistance, which is expected to continue in the absence of stricter biosecurity measures (Falcón et al. 2012; Bock et al. 2018; van den Burg et al. 2018a, 2020a, 2020b, 2021b; Breuil et al. 2019, 2020; Knapp et al. 2020). These invasive, non-native iguanas have profoundly impacted native insular iguana populations throughout the Lesser Antilles, as the former have larger clutch and body sizes and show higher aggressiveness than native iguanas (van den Burg et al. 2018a; van Wagensveld and van den Burg 2018; Breuil et al. 2019). These incursions from different nonnative sources have resulted in region-wide hybridization, best documented for the Critically Endangered *I. delicatissima* (Vuillaume et al. 2015; van den Burg et al. 2018a, 2018b; Pounder et al. 2020), and to a lesser extent for the distinct lineages within the *I. iguana* complex (Breuil et al. 2019). Given this lengthy, continuous period of hybridization and subsequent within-region movement of non-native iguanas (Censky et al. 1998), morphologically distinguishing between native and (hybridized) non-native iguanas is often not straightforward (Breuil 2013; Vuillaume et al. 2015). This status quo clearly demonstrates the urgent need for robust genetic tools to distinguish native from non-native (or hybrid) animals. Additionally, the increased activity in illegal trade of iguanas from Lesser Antillean islands

(Noseworthy 2017; van den Burg and Weissgold 2020) further highlights the need for island-level identification.

Within *Iguana*, microsatellite research has focused on a wide range of topics; invasion biology, population dynamics, as well as conservation (Valette et al. 2013; Vuillaume et al. 2015; Martin Judson et al. 2018; van den Burg et al. 2018b; De Jesús Villanueva et al. 2021). However, different genetic laboratories have not standardized their data sets and employ different loci sets (Table 2), limiting the vital ability to compare results, efficiently build upon prior work, and improve our understanding of these vulnerable populations. This knowledge gap of variation from across the genus' range limits our ability to meaningfully assess population structure within lineages, and hinders the essential identification of *Iguana* hybrids and conservation of endangered lineages. From the perspective of hybridization issues, the research community currently lacks an understanding of allelic variation among known *Iguana* species and clades because data from native samples are almost entirely limited to island populations. The one exception is a 2015 study by Vuillaume et al. that included a single sample from each of two mainland localities with known origin. Hence, a genus-wide dataset is urgently needed in order to provide a foundation upon which all stakeholders can build, and use to guide conservation management decisions.

Materials and methods

The microsatellite loci set that has been most implemented among *Iguana* studies is that of Valette et al. (2013). We compiled data generated from all or a subset of these 25 loci (Valette et al. 2013; Vuillaume et al. 2015; Breuil et al. 2019, 2020, submitted). Furthermore, we collected data from new mainland (23) and insular (5) localities for a total of 53 native iguanas, to represent a wide-spread *Iguana* dataset (Fig. 1, Table 1). We also included six morphologically identified hybrids between *I. delicatissima* and non-native iguanas; two samples from St. Barths, and Basse Terre and Grande Terre (Guadeloupe) (Vuillaume et al. 2015).

Table 1 Range of extent for currently named *Iguana* species and lineages, including sample sizes as in IguanaBase v1 (van den Burg et al. 2021a)

Species/lineage	Range	Sample size
Iguana delicatissima	Lesser Antilles (Anguilla – Martinique)	67
I. rhinolopha (Clade III sensu Stephen et al. 2013)	Central America, N of Costa Rican calderas (van den Burg et al. in prep)	16
I. aff. iguana Clade IIA (sensu Stephen et al. 2013)	mainland South America, between Costa Rican calderas and Andes (van den Burg et al. in prep)	12
I. aff. iguana Clade I (sensu Stephen et al. 2013)	ABC islands and Maracaibo delta (van den Burg et al. in prep)	9
I. iguana (Clade IIB sensu Stephen et al. 2013)	mainland South America, E of Andes	14
I. insularis	Saint Lucia, Saint Vincent and Grenadines	52
I. melanoderma	Saba, Montserrat, and northern Venezuela	20

Table 2 Research using any subset of the 25 microsatellite loci identified by Valette et al. (2013)

Publication	Focus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Valette et al. (2013)	H																								×	×
Vuillaume et al. (2015)	H		×	×	×	×	×		×	×		×	×		×		×		×		×			×	×	
Moss et al. (2017)	H											×	×		×			×		×	×	×			×	
van den Burg et al. (2018b)	H											×	×		×			×		×	×	×	×		×	
Breuil et al. (2019)	I		×	×		×	×		×	×				×	×	×	×	×	×	×	×			×	×	
Breuil et al. (2020)	I		×	×		×	×		×	×				×	×	×	×	×	×	×	×			×	×	
De Jesús Villanueva et al. (2021)	H				×										×			×		×	×		×			
Breuil et al. (submitted)	H	×	×	×		×	×		×	×				×	×	×	×	×	×	×	×			×	×	
van den Burg et al. (current study)	H+I	×	×	×		×	×		×	×				×	×	×	×	×	×	×	×			×	×	×

Specific loci used and the primary focus, hybridization (H) or intraspecific variation (I), are noted for each study

Laboratory procedures were performed at Labofarm-GenIndexe (France) where PCR settings and primers outlined by Valette et al. (2013) were used to amplify 17 loci (named as IgdL1-25 or L1-25 in previous studies; Valette et al. 2013; Vuillaume et al. 2015). Subsequently, PCR products were ran with Thermo ROX500 and analyzed using an ABI 3130 XL; alleles were next scored using Genemapper, while samples IGU039–43 were used to standardize allele sizes with aforementioned studies (see van den Burg et al. 2021a).

Data analyses

After sample processing, we retained specimens with ≥ 80% scored loci for data analyses (for sample sizes used in analyses see Table 3 and figure captions). Considering our sample-set characteristics, only one population, from Ilet Chancel (29 samples), was suitable to test the presence of null alleles. Following Dabrowski et al (2014), we implemented both Genepop and micro-checker software to assess the presence of null alleles (Raymond and Rousset 1995; van Oosterhout et al. 2004). The remaining sample-set included groups not amenable to this question as they were comprised of; (1) wide geographic sampling within lineages (producing a Wahlund Effect), or (2) low sample size of breeding populations (non-representative of existing variation), or (3) impacted by recent bottlenecks (strong effects of genetic drift).

Data were split into two sets, as previous studies have used different loci (Table 2). Our 12-loci dataset includes all native *Iguana* locations and the six *Iguana-delicatissima* hybrids, and our 17-loci dataset includes all native locations from the *I. iguana* complex. For these datasets we then calculated relative allelic richness using the *GenPopReport* package within the *R* environment (R Core Team 2019).

Allele variation across all samples was assessed and visualized in color-coded tables to identify genetic variation within and between *Iguana* lineages (see Results). Within these tables we also include allelic data from the non-native and hybrid iguanas from Vuillaume et al. (2015) and Breuil et al. (2019).

Genetic differentiation and clustering were assessed using STRUCTURE as implemented within GenoDive 3 (Pritchard et al. 2000; Meirmans 2020). For the 12-loci dataset, we applied this methodology to assess its utility to successfully assign native pure and hybrid specimens, as well as to assess the genetic structure among *I. delicatissima* populations. For the 17-loci dataset, we assessed genetic structuring within the *I. iguana* complex, for which we standardized sample sizes among lineages by reducing the sample size of *I. melanoderma* and *I. insularis* to those of the other lineages by randomly sampling while maintaining sample size-island proportions. STRUCTURE runs used identical parameters
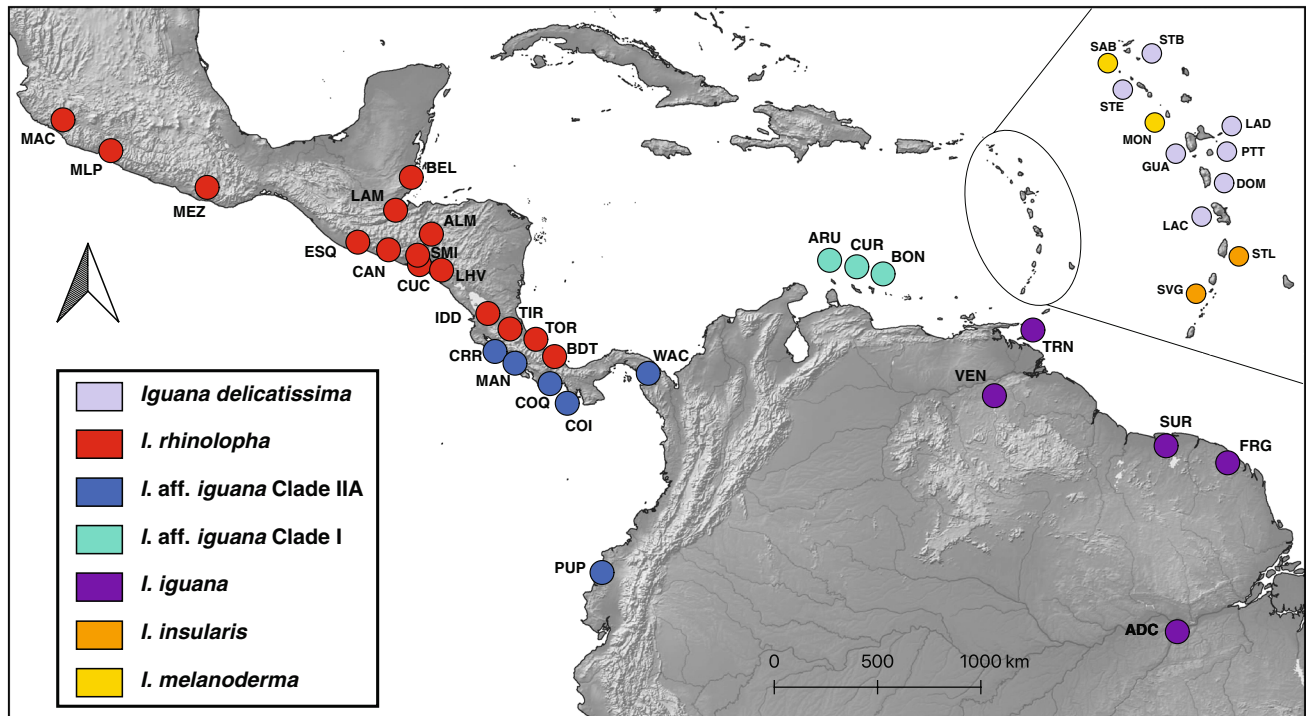
Fig. 1 Map showing sample localities and current taxonomic status with *Iguana*: Inset shows the Lesser Antilles. Further geographic details for each lineage can be found in Table 1. 'aff.' indicates the

uncertain taxonomic status, which is undergoing assessment (van den Burg et al. in prep)

Table 3 Mean allelic richness from two microsatellite loci sets for each taxonomic unit or population, including number of samples sequenced for > 80% of loci

	12 loci	17 loci
Iguana delicatissima		
St. Barths (3)	1.03	–
St. Eustatius (3)	1.07	1.12
Dominica (3)	1.14	1.27
Guadeloupe (12)	1.13	–
La Désirade (2)	1.05	–
Petite Terre (15)	1.13	–
Ilet Chancel (29)	1.16	–
Iguana rhinolopha (15)	1.22	1.61
Iguana aff. *iguana* Clade IIA (11)	1.43	1.98
Iguana aff. *iguana* Clade I (9)	1.40	1.85
Iguana iguana (14,12*)	1.40	1.85
Iguana insularis		
I. i. insularis (38)	1.11	1.30
I. i. sanctaluciae (14)	1.05	1.06
Iguana melanoderma (18)	1.24	1.50

*Data for *I. iguana* represents 14 and 12 samples for the 12- and 17-loci datasets, respectively

including an admixture model, correlated allele frequencies, and without linkage. Runs composed of 10 repeats for K ranges 1–10 using 100 K burnin and 500 K MCMC repeats.

Subsequently, we assessed and visualized delta-K estimates for different K values using Structure Harvester (Evanno et al. 2005; Earl and vonHoldt 2012), and employed *distruct* to create final structure barplots (Rosenberg 2004). Lastly, within *R* through the loadingplot() command in the *adegenet* package, we assessed which loci and alleles most contribute to differentiation of *I. delicatissima* and non-*delicatissima* iguanas through a discriminant principal component analysis (DAPC; Jombart 2008).

Results

For the 12-loci dataset, mean allelic richness among *I. delicatissima* populations ranges between 1.03–1.16, and between 1.05–1.43 within the *I. iguana* complex. For the 17-loci dataset, mean allelic richness shows higher values with a similar pattern among the *I. iguana* lineages, 1.06–1.98 (Table 3). Within the complex, *I. melanoderma* and *I. insularis* show lower mean allelic richness compared to taxonomic units that mostly have a mainland range, though *Iguana* aff. *iguana* Clade I is here represented only by island populations.

Null-allele detection tests show inconsistent results with micro-checker suggesting presence for five loci (L3, L5, L12, L18, L24), whereas Genepop only for L20. We retain all loci in further discussion given that no single
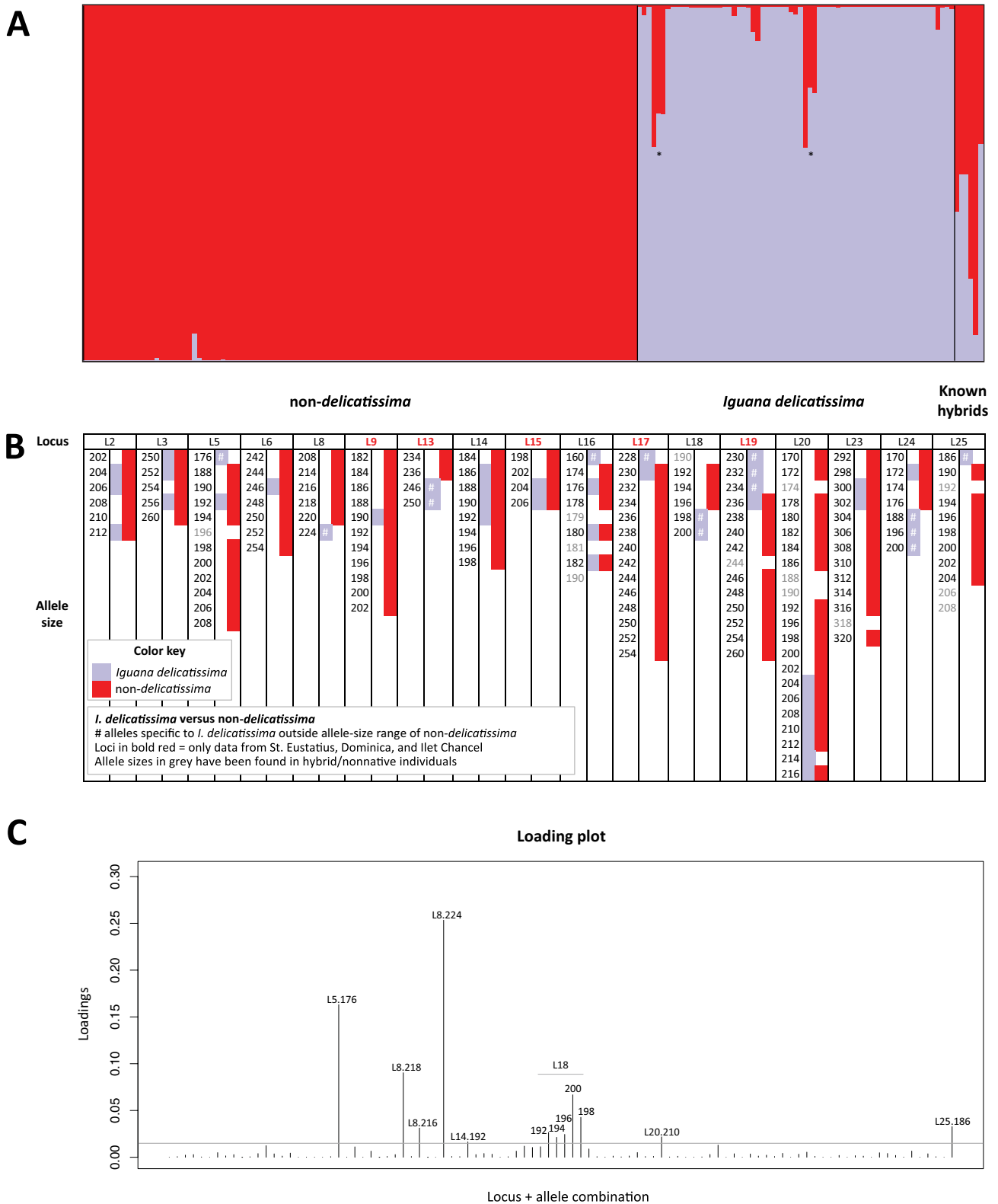
Fig. 2 Genetic differentiation results to study hybridization in *Iguana delicatissima*, using 12 loci dataset. **a** Structure plot for pure *I. delicatissima* (67), pure non-*delicatissima* (117), and hybrid (6) samples, see Table 3; for K = 2. Asterisks indicate individuals from St. Eustatius and Dominica. **b** Overview of allele variation between *I. delicatissima* and non-*delicatissima* individuals, excluding hybrids. **C** DAPC loading contribution of loci and alleles for clustering between *I. delicatissima* – non-*delicatissima*, excluding hybrids
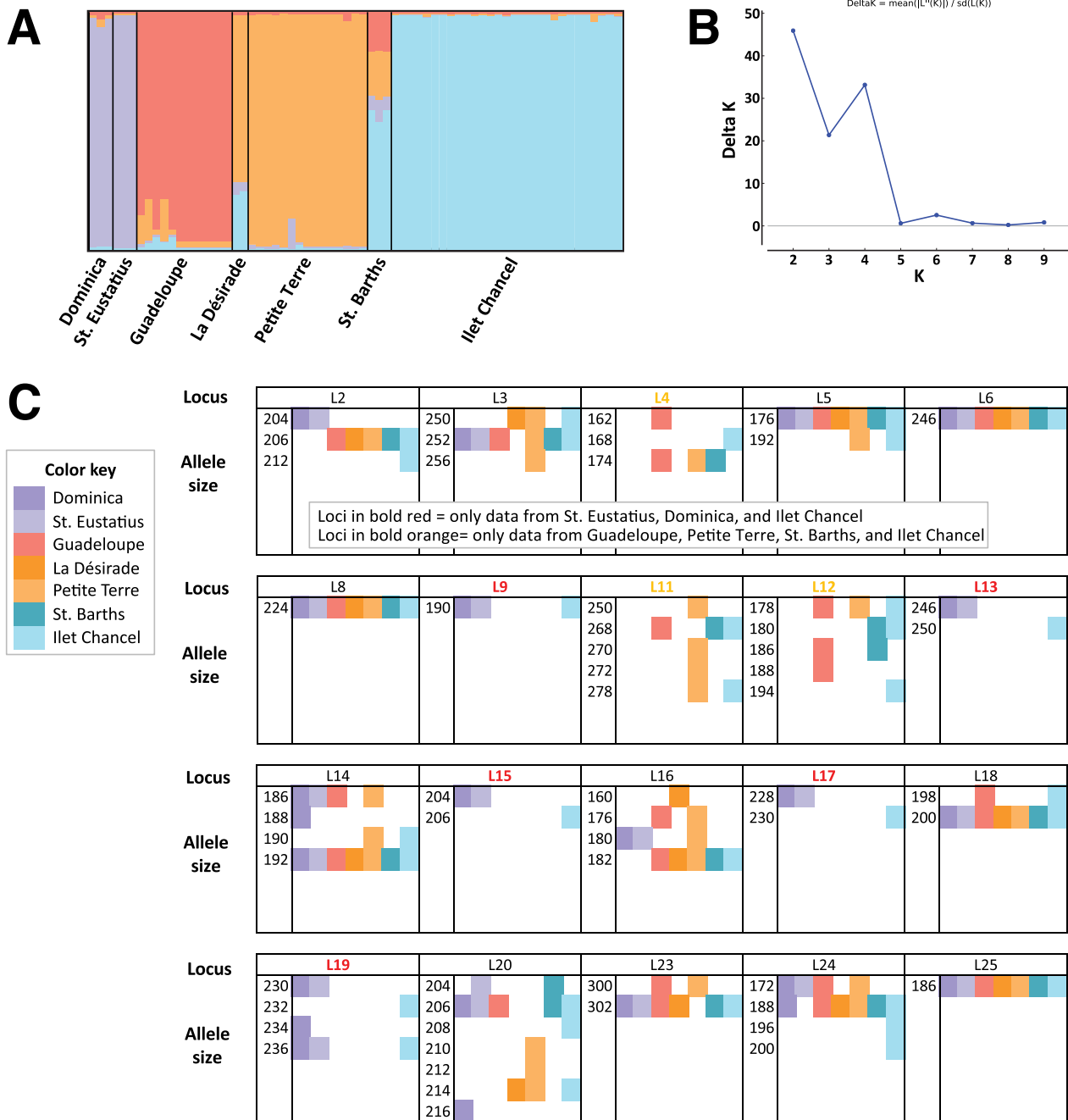
Fig. 3 Genetic variation among *Iguana delicatissima* island populations. **a** Structure and **b** delta K plot for population differentiation among pure *I. delicatissima* individuals (67; Table 3), using 12 loci dataset; K = 4. **c** Allele variation among seven island populations for 20 loci; islands assigned to identical Structure cluster (**a**) have equal-tone colors

locus was flagged by both methods. We recommend these analyses be performed on definable populations that have been adequately sampled and meet the a priori assumption of Hardy–Weinberg equilibrium.

Hybridization

Figure 2 provides an overview of results for hybridization purposes between native *I. delicatissima* and native non-*delicatissima*, as well as six hybrids. Structure results are
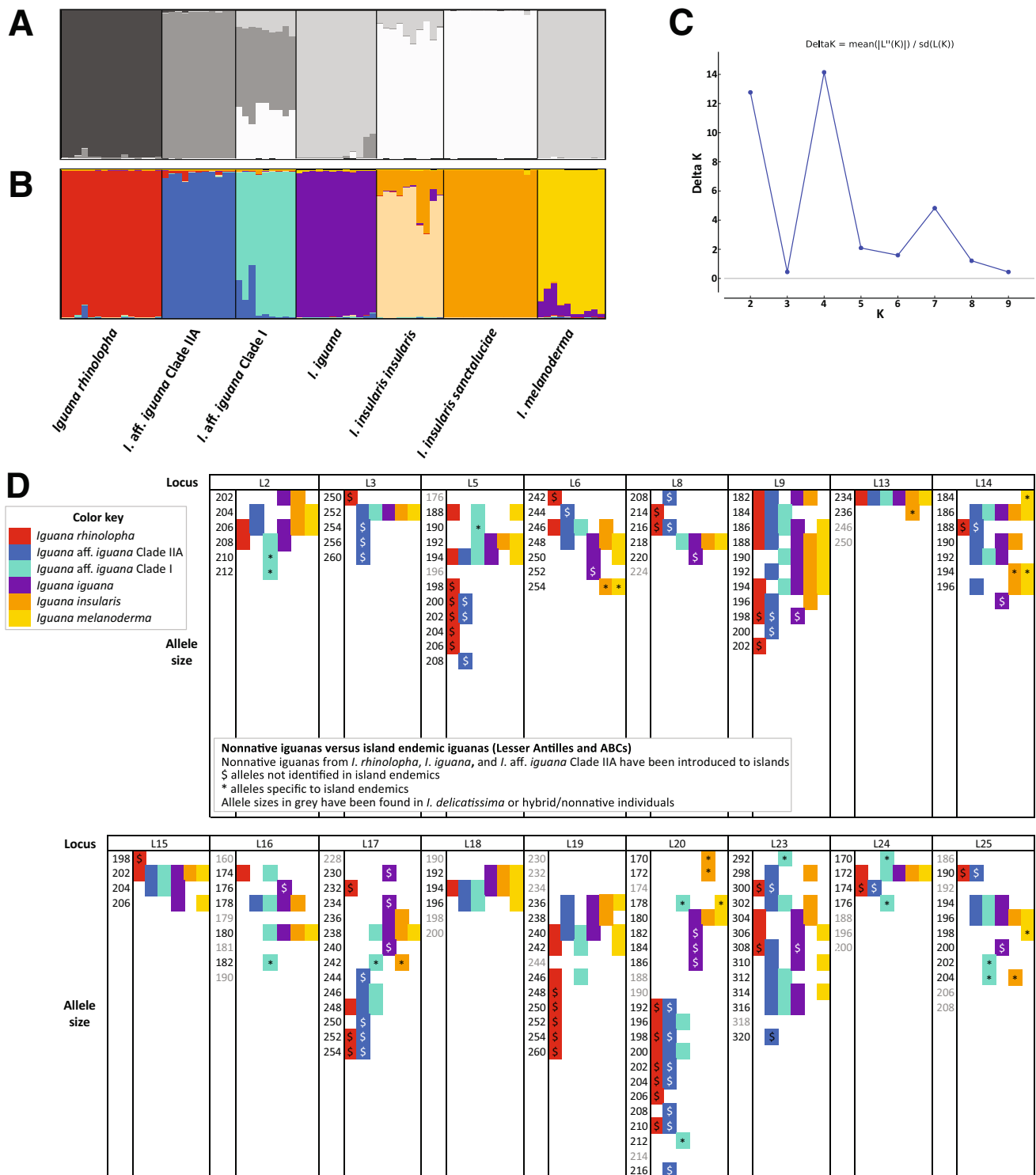
Fig. 4 Genetic variation within *Iguana iguana* complex for 17 microsatellite loci. **a + b** Structure and **c** delta K plots for population differentiation among seven taxonomic units; optimal K = 4, suboptimal K = 7. **d** Allele variation among seven taxonomic units. Sample sizes as in Table 3, except for *I. insularis insularis* (10), and *I. melanoderma* (10)

visualized for K = 2, which clusters *I. delicatissima* and non-*delicatissima* separately, except for the six known hybrids

(Fig. 2a). Surprisingly, assignment of Dominica and St. Eustatius samples to *I. delicatissima* was < 75% (Fig. 2a),

likely due to their low sample sizes and alleles with proportionally higher representation in non-*delicatissima* samples; all samples in these two populations were fixed for unique *I. delicatissima* alleles. Visual inspection of patterns of allele variation and size range overlap between *I. delicatissima* and non-*delicatissima* reveals four loci with a complete lack of allele variation overlap, three of which include data from all *I. delicatissima* locations: L8, L18, and L25 (Fig. 2b). An additional two loci (L5 and L24) also indicate a high degree of usefulness for hybridization studies, but show a single allele overlap. Lastly, DAPC analysis identified a set of six loci with the highest contribution to differentiating *I. delicatissima* and non-*delicatissima*: L5, L8, L14, L18, L20 and L25 (Fig. 2c).

Iguana delicatissima

Within *I. delicatissima*, delta-K identified four clusters with highest support (Fig. 3b): (1) Ilet Chancel and St. Barths, (2) Dominica and St. Eustatius, (3) Petite Terre and La Désirade, and, (4) Guadeloupe. The first two groups contain islands not in close geographic proximity. For delta-K of 2 (but see Janes et al. 2017), assignment followed that of Ilet Chancel and St. Barths vs. all other islands. Allelic variation is highest for larger islands or those with high population sizes; Dominica, Guadeloupe, Petite Terre and Ilet Chancel (Table 3, Fig. 3c).

Iguana iguana complex

Genetic differentiation and allele variation within the *I. iguana* complex are presented in Fig. 4. Optimal delta-K support indicated four clusters, with sub-structural support for seven clusters (Fig. 4a-c). Allele variation patterns between mainland (generally non-native) and insular lineages highlight loci of interest for identifying the genetic origin of non-native iguanas within those insular lineages; L3, L5, L6, L8, L9, L14 L16, L17, L19, L20, L23–25 (Fig. 4d). Loci that appear to be useful for characterizing insular groups, differs per lineage; *I. melanoderma*, L6, L14, L20, L25; *I. insularis*, L6, L13, L14, L17, L20; *I.* aff. *iguana* Clade I, L2, L5, L16, L17, L20, L23–25.

Discussion

Here we present a nuclear microsatellite database for *Iguana* that includes all current taxonomic groups with broad geographic representation across its range, demonstrating clear population genetic structure within *I. delicatissima* and the *I. iguana* complex. Furthermore, our results identify key sets of loci and private alleles useful in the study of conservation-hybridization issues and for narrowing down

the geographic origin of commercially available pet iguanas and illegally transported individuals. This database fills an important gap given that nuclear loci sequence in prior work show low variability within the *I. iguana* complex (Stephen et al. 2013), prohibiting the fine scale variation at biparentally inherited loci required to address such issues. Overall, our work demonstrates the successful amplification of these markers and their utility within the *Iguana* genus. Importantly, the benefits of this database will increase dramatically with further input from the *Iguana* research community, which we promote through free availability on *figshare* (van den Burg et al. 2021a).

Assignment of loci and private alleles to address research questions is dependent on sampling scheme, both its geographical width and density, as well as sample size per population. Given the geographic range of *Iguana,* the coverage of the presented database is wide, though mainland sampling density is still low, with few samples per location. Notably, the database greatly increases coverage and lineage inclusion compared to Valette et al. (2013), which assigned 19 loci as useful for *I. delicatissima* hybridization studies based on one *I. delicatissima* (Ilet Chancel, Martinique), and one introgressed (*I. delicatissima* – *I. iguana*) population (Grande-Terre, Guadeloupe). Here, including data from almost all *I. delicatissima* populations (only lacking Anguilla), we identify 5–8 loci of specific use for identification of hybrids between *I. delicatissima* and the *I. iguana* complex (Fig. 2b, c). Although these loci are fewer in number, their identification is based on a significant data increase from throughout the genus' range; allowing conservationists to target fewer, highly informative loci and lower research costs. Furthermore, the need to standardize allele sizes across different studies and laboratories is exemplified in our finding that the three samples from St. Eustatius rescored in this study had different allele size for all five loci compared to results in van den Burg et al. (2018b), shifting higher or lower by 5–22 bps, depending on the locus. Similarly, sample reanalyzes in the same laboratory can indicate allele size deviations, as data of several loci presented by Vuillaume et al. (2015) have since been updated (2–16 bps; Breuil et al. 2019). Lastly, with ongoing conservation priorities aimed at insular lineages, the characterization of their allele diversity will carry on, though we address the importance of characterizing new mainland localities. This continuous data collection highlights the need for a "live" dynamic database that provides a crucial, up-to-date, *Iguana* conservation tool.

The identification and continued usefulness of microsatellites in research on wild populations lies in their rapid mutation rate (Schlötterer 2000), demonstrating their use in studies of recently separated populations (Kuehn et al. 2007), but highlighting their poor informative value for long evolutionary timelines (Carsten et al. 2013). Indeed, this high mutation rate prevents the determination of shared

alleles as synapomorphic or homoplasious, when comparing highly divergent lineages. This is evident in our database for shared alleles (e.g., L2) between *I. iguana* lineages and two *I. delicatissima* populations (St. Eustatius and Dominica) (Fig. 2a). Genetic samples from these *I. delicatissima* populations were obtained prior to first documented hybridization on both islands (van den Burg et al. 2018b, 2020a), though historic hybridization might have occurred. Another explanation for shared L2 alleles is independent mutation as might be expected after a long period of evolutionary divergence.

The Critically Endangered, Lesser Antillean iguana has seen a > 75% decline from its pre-Colombian range due to extirpations subsequent to human settlement, and more recently, hybridization with non-native iguanas of the *I. iguana* complex (van den Burg et al. 2018a). Given the extremely low mtDNA variation throughout the range of *I. delicatissima* (Martin et al. 2015), microsatellites are better suited to guide research and conservation (Vuillaume et al. 2015; Martin Judson et al. 2018; van den Burg et al. 2018b). Small populations will lose diversity quickly and randomly diverge due to founder effects and/or drift over short time periods (Wright 1931), which has been demonstrated in Iguanids as well (Colosimo et al. 2014; Welch et al. 2017). Thus, to maintain long term genetic variation within *I. delicatissima* it is essential that all island populations are genotyped. Our database builds on Vuillaume et al. (2015) by inclusion of two additional island populations, St. Eustatius and Dominica. Genetic clustering among currently sampled populations is not correlated with geographic proximity, given two identified clusters: St. Eustatius–Dominica, and St. Barths–Ilet Chancel (Fig. 3a). Their genetic similarity could originate from within-region dispersal facilitated by hurricanes, Amerindians or post-Colombian colonizers (Censky et al. 1998; Bryan et al. 2007; Bochaton et al. 2016). Although this database represents the most inclusive to date, we call for sampling and genotyping of the Anguilla population and those on isolated islets around St. Barths, as well as additional genotyping of samples from Dominica and La Désirade. These genetic data can furthermore help identify the remaining pure animals on Guadeloupe and Martinique, which are in need of urgent translocation to secure their genetic diversity. Lastly, these data and identified key loci will aid other conservation management decisions as identified by conservation action plans (e.g., translocations (Scott et al. 2020), captive breeding, and reintroductions (Knapp et al. 2014; Angin 2017; ANT/ATE/STENAPA 2018; Breuil 2021)).

We provide an initial assessment of allele variation among recently described lineages within the *I. iguana* complex. Optimal support was found for four genetic clusters, which grouped currently known lineages as (1) *I. rhinolopha*, (2) *I. aff. iguana* Clade IIA, (3) *I. insularis*, (4) *I. iguana* and *I. melanoderma*, and with an admixed

assignment for *I. aff. iguana* Clade I (Fig. 4a, c). A sub-optimal 7-cluster division distinguished *I. iguana* and *I. melanoderma*, as well as uniquely assigned *I. aff. iguana* Clade I (Fig. 4b, c). Allelic diversity indicated higher variation in mainland lineages and *I. aff. iguana* Clade I compared to solely insular lineages, with especially low diversity in *I. i. sanctaluciae* (Table 3). For vulnerable island populations (*I. melanoderma*, *I. insularis*, *I. aff. iguana* Clade I), we find three loci sets (4–8 loci) for origin assessment (see Results text for specifics). For the current dataset, we note the skewed sampling effort towards recently described insular lineages (Breuil et al. 2019 2020, submitted), and highlight the need for improved sampling of the mainland lineages. Although threats within the *I. iguana* complex are less studied compared to those in *I. delicatissima*, it is well-known that mainland populations can be locally threatened due to overharvesting, either for food or to supplement the pet trade (Stephen et al. 2011). More recently, hybridization between non-native iguanas and native island populations has been described, as well as illegal trade of *I. insularis* and *I. melanoderma* (Noseworthy 2017; Breuil et al. 2019; van den Burg and Weissgold 2020). Overall, the identified allelic variation (Fig. 4D) will aid in the discovery of non-native and hybrid iguanas and the geographic origin of these as well as of iguanas in the global commercial hobbyist trade.

In conclusion, this manuscript provides a reference for *Iguana* microsatellite loci in order to allow standardization and comparison among studies, aiding broader assessment of research and conservation hypotheses. Importantly, this work provides an essential tool for conservation of the Critically Endangered *I. delicatissima*, and taxa within the *I. iguana* complex. To ensure future comparability, we strongly urge that future studies utilize: (1) the same allele size ladder, (2) positive controls from localities with fixed alleles, and/or (3) samples scored in prior studies.

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Declarations

Conflict of interest Authors declare no conflict of interest.

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