

captured the obvious genetic break between main island and Virgin Island samples (if one exists), and, next, to investigate possible genetic structure among sampling localities within the relatively small island of Vieques.

For our full dataset we analysed a total of 3407 loci with less than 5% missing data using the programs STRUCTURE and DAPC. We used the ΔK method of Evanno *et al.* (2005) to determine that $K = 2$ best fitted the data for our analyses both with and without a location prior. We also investigated $K = 3$ given the known bias towards $K = 2$, especially when hierarchical structure is present (as we might expect to find in our data; Janes *et al.*, 2017). Our DAPC analysis also identified two clusters based on the BIC after comparing different K values (Supporting Information, Fig. S3). DAPC analysis showed that individuals from Vieques and Puerto Rico form one cluster while individuals from the Virgin Islands comprised a different, separate cluster. STRUCTURE analysis did not discriminate between these groupings at $K = 2$, but did at $K = 3$ (Fig. S4). Divergence between Virgin Islands and Puerto Rico/Vieques clusters was 4.1% (Tamura–Nei corrected; Table S2). Due to our widely divergent sample sizes between Vieques Island, the Virgin Islands and Puerto Rico, and particularly because our samples from the main island and the Virgin Islands do not come from one geographical locality but several, we did not feel it was sensible to compute F_{ST} for Virgin Islands and Puerto Rico populations.

For the second part of our analysis, we removed samples from the Virgin Islands and East Puerto Rico from the SNP dataset, resulting in some monomorphic loci. We thus retained 2433 polymorphic loci from the 37 Vieques Island samples. We analysed these data first using STRUCTURE, as above, and then DAPC. A ΔK analysis performed with STRUCTURE revealed $K = 2$ for the dataset. Posterior probabilities for group membership, however, were not well aligned with geography (Fig. 4D). By contrast, DAPC identified two slightly overlapping clusters that generally correspond with the eastern and western halves of the island (and thus crudely with the mtDNA haplogroups; Fig. 4A–C). Nonetheless, concordance between mtDNA and SNP clustering was imperfect as five individuals with eastern Vieques mtDNA haplotypes were assigned to the western Vieques SNP cluster with highest probability. Divergence between the DAPC inferred clusters within the Vieques SNP dataset was 0.5% (Tamura–Nei corrected distances). F_{ST} between the west and east Vieques clusters (as defined by DAPC) was 0.02.

TESTS FOR ISOLATION-BY-DISTANCE

Our test for IBD in the Vieques dataset revealed a significant relationship between calculated Provesti's

genetic distances and UTM-transformed geographical distances (Mantel test; $r = 0.36$, $P = 0.001$; Fig. 5). This shows that individuals sampled closer together on the island tend to be more genetically similar, and thus suggests that gene flow may be reduced between geographically distant individuals on Vieques Island.

In our partial Mantel analysis to test for a marginal effect of mitochondrial clade membership on genomic distance, we found no evidence to support a significant correlation between mitochondrial distance and SNP distance when controlling for geography (partial regression coefficient, $\beta_2 = 0.0007$, $t = 0.126$, $P = 0.927$ from Mantel permutations; Supporting Information, Fig. S6). We also failed to find evidence of a significant correlation between morphological dissimilarity for the attributes measured in this study and geographical or genetic distance within Vieques Island (Appendix S2).

DISCUSSION

We undertook this project after a broad-scale study of phylogeography across the Puerto Rico Bank revealed an intriguing pattern of two deeply divergent mtDNA haplogroups from a modest number of representative samples of *A. cristatellus* obtained on the relatively small island of Vieques. One of these two haplogroups was quite closely allied to samples from eastern Puerto Rico including the metropolitan area of San Juan and the former US Naval Base at Roosevelt Roads (Reynolds *et al.*, 2017). This observation, coupled with the knowledge that Vieques has been subjected to very a high frequency and duration of military, commercial, and public boat and air traffic over the past 60–100 years or more, suggested to us the possibility that members of this haplogroup might have been anthropogenically introduced to Vieques within the last century or so. Furthermore, *A. cristatellus* is a reasonably successful invader in other areas, having been introduced and become successfully established in several localities outside its native range (Hahn & Kohler, 2010).

To investigate this hypothesis we undertook the most thorough geographical sampling of *A. cristatellus* to date on Vieques. We sequenced 1191 bp of mtDNA from 155 samples collected across Vieques, and aligned these samples with 224 previously accessioned sequences from prior studies of *A. cristatellus* and other closely related taxa. In addition, we used GBS to generate SNP data for an additional 3407 polymorphic nuclear loci for 48 individuals: 37 from Vieques, and the remainder from Puerto Rico (six samples) and from the Puerto Rico Bank Virgin Islands (five samples). We analysed these data using gene tree inference by Bayesian and ML methods (mtDNA), and using population genetic methods based on an explicit model

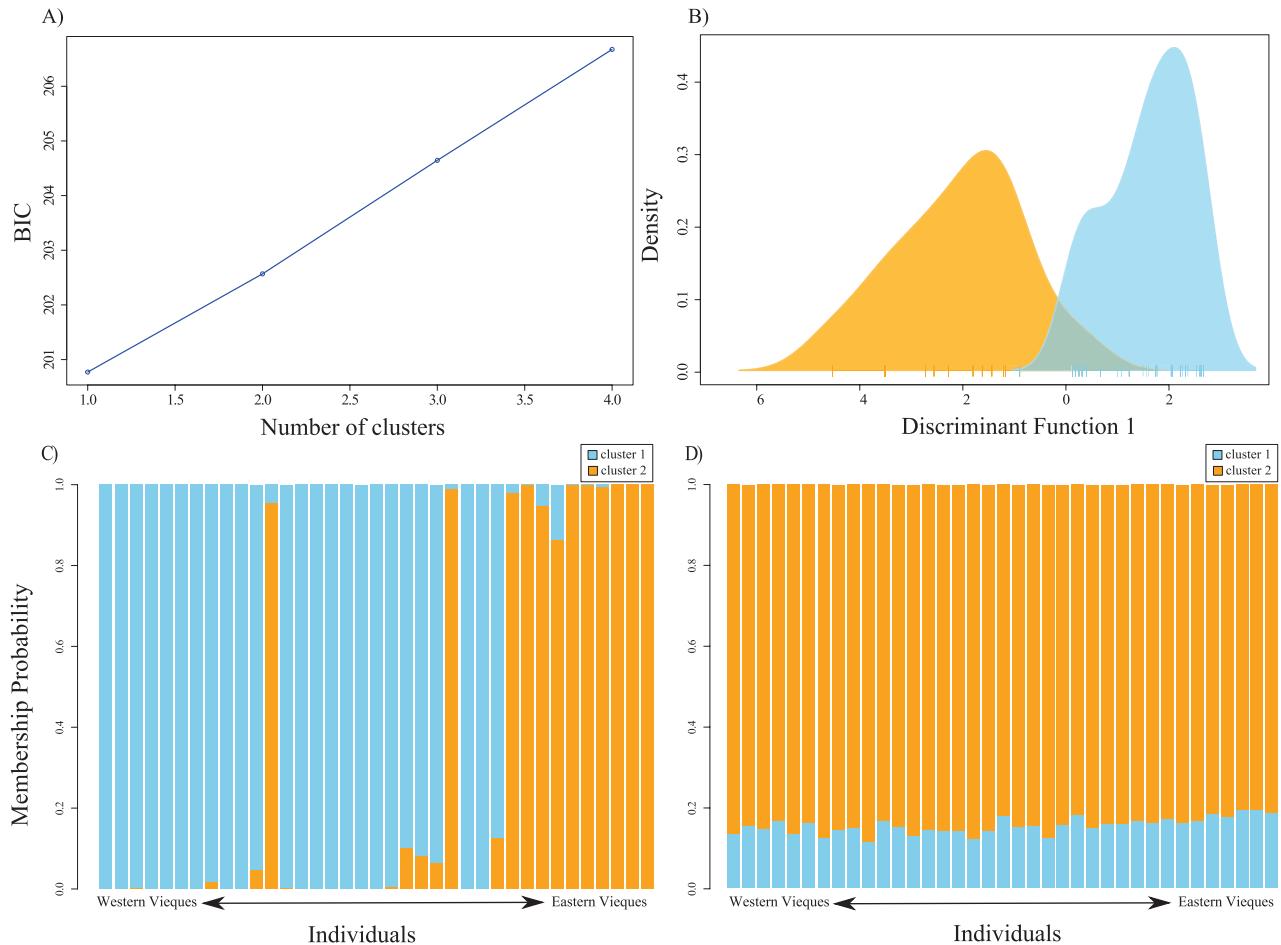


Figure 4. Results from clustering analyses without a location prior for the Vieques SNP dataset. A, BIC vs. number of clusters plot from DAPC analysis. B, DAPC clustering showing two separate clusters from the all-SNP dataset. Membership probability plots from (C) DAPC and (D) STRUCTURE $K = 2$ showing a lack of geographical discrimination between clusters. Each bar represents the genotype of one of the 37 samples from Vieques. The colour fraction of each bar represents the probability of the individual belonging to each of the two clusters. Individuals are ordered left-to-right by values of one canonical axis extracted from a multidimensional scaling analysis; however, this corresponds roughly to their west to east locations on the island.

(STRUCTURE) and on no particular model (DAPC) of among-population divergence.

In general, our results did not support our a priori hypothesis that the mitochondrial haplotypes found in Vieques that are most closely allied to Puerto Rico East haplotypes result from an anthropogenic introduction of main island genetic material into Vieques. Rather, this pattern seems to have resulted from an historical mitochondrial break on the Puerto Rico Bank palaeo-island, inclusive of the Virgin Islands. This is supported by the high genetic diversity within each mitochondrial haplogroup for both nuclear and mitochondrial DNA, and by the strong pattern of genetic structure among the *ND2* haplotypes (Fig. 3; Supporting Information, Fig. S1). If the presence of individuals with Puerto Rico East clade haplotypes on the island was the product

of recent dispersal, we would expect low diversity and low geographical structure, merely owing to the stochastic sampling of individual migrants and the shortage of time during which to establish a genetically structured geographical distribution in the introduced area (Fitzpatrick *et al.*, 2012). Furthermore, we might expect to see introduced haplotypes co-occurring with native haplotypes, a pattern that is generally not observed (Fig. 3). Instead, we see a consistent tendency for mitochondrial haplotypes allied with main island lineages to be found in the western part of Vieques, while haplotypes closely related to Virgin Island clades are circumscribed to the east (Fig. 3). This pattern is wholly consistent with the geographical configuration of the present and past (palaeo-island as well as with a contemporary ecological gradient on the island,

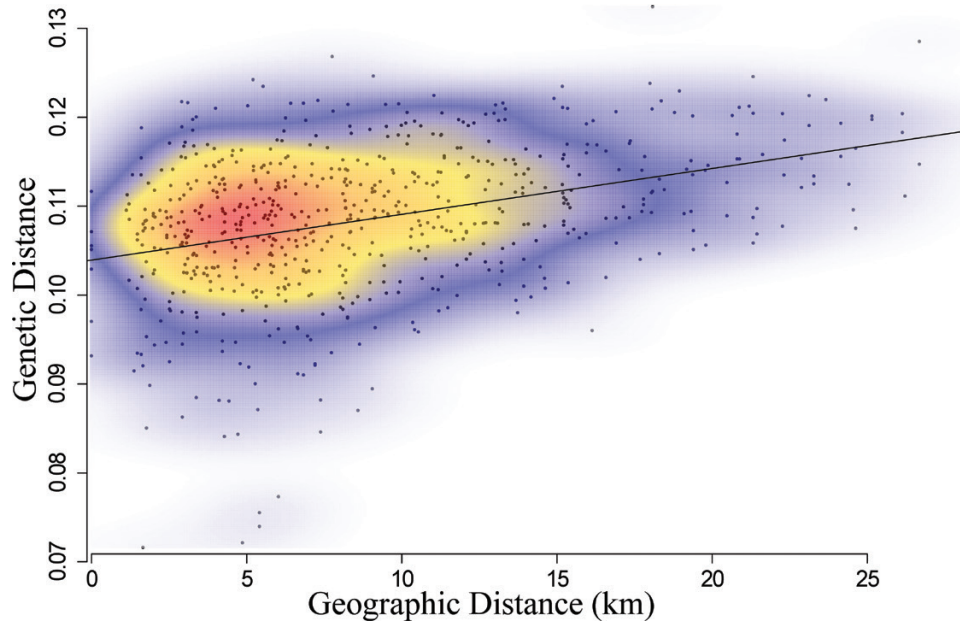


Figure 5. Isolation-by-distance plot for the Vieques SNP dataset from 37 individuals with a total of 2433 loci. Mantel's test for correlation was significant ($r = 0.36$; $P = 0.001$). Genetic distance is calculated with Nei's distance method and geographical distance is calculated from UTM coordinates converted from GPS coordinates. Colours represent the density of data points with red to blue as most to least dense.

whereby eastern Vieques consists of subtropical dry forest with <80 mm of rainfall annually and west Vieques consists of subtropical mesic forest with 80–150 mm of annual rainfall (Ewel & Whitmore, 1973; Brandeis *et al.*, 2009). Additionally, in our broad and dense geographical sampling on the island we found no tendency for either haplogroup to be consistently associated with either ports or densely inhabited areas. Finally, analysis of 3407 nuclear SNPs showed similar, but imperfectly matched, population genetic structure in nuclear markers compared to mtDNA, probably reflecting natural geographical genetic differentiation rather than the introduction of foreign genetic material to the island.

A second question that arises is whether the apparent genetic break we have documented for mtDNA represents historical allopatry (on the Puerto Rico Bank palaeo-island) followed by secondary contact and introgression, or primary intergradation. To answer this question, we used data for 2433 SNPs sampled from 37 individuals dispersed across the island of Vieques including mtDNA sequences from both of the two major *ND2* haplogroups. STRUCTURE analysis of the data showed no evidence for discrete populations on Vieques (Supporting Information, Fig. S5). Analysis using DAPC, which may be more sensitive to genetic clines, showed more evidence for population genetic structure; however, we found no support for a significant effect of mtDNA haplogroup

membership on SNP divergence. This suggests that the lack of correlation between SNP and mtDNA divergence may simply be due to IBD in the former and deep coalescence with geographical structure in the latter, rather than an alternative of correlation owing to concerted evolutionary histories of SNP and mtDNA in lineages evolving under a demographic scenario such as allopatry (see Reynolds *et al.*, 2017). At the time of writing, a new analytical model had just been published to characterize genetic clines in continuously distributed populations (Bradburd *et al.*, 2018). Although we have not applied it to our data herein, this may represent an intriguing avenue of analysis for other similarly motivated studies in the future.

Overall, we found little support for a hypothesis of historical allopatry and thus propose that the pattern we have observed is most probably the result of primary intergradation combined with IBD. Unfortunately, historical allopatry can never be entirely ruled out in this case, because with sufficient gene flow its signal would eventually disappear. In this context, it seems reasonable to acknowledge that IBD and deep coalescence for mtDNA is effectively our null hypothesis, and a non-significant result reflects a failure to reject the null, not definitive evidence that the null is correct. Nevertheless, we show that an unusual pattern of deep mitochondrial divergence across a relatively small island with no

apparent geographical or ecological separation is not particularly well explained by historical allopatry; instead, it may be the product of more nuanced evolutionary and geological factors that can only be understood via analysis of both mitochondrial and nuclear genomes.

Our study underlines the importance of using multiple genetic markers when assessing population structure in a species. In this case results from mtDNA alone may have caused us to erroneously infer either the introduction of foreign genetic material to the island or ancient allopatry followed by recent secondary contact. When combined with data from over 3400 SNPs, the picture changes quite dramatically and we find relatively little support for either hypothesis. Our results suggest instead a process of IBD in which we happen to have sampled across a deep coalescence event in geographically structured mtDNA.

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AUTHOR CONTRIBUTIONS

Q.N.Q., L.J.R. and R.G.R. conceived of the project. Q.N.Q. designed the study and conducted most of the field sampling, data acquisition and analyses, and

drafted the manuscript. R.G.R. designed the GBS protocol. R.G.R. and L.J.R. provided logistical and intellectual input for data analyses and interpretation. All authors contributed to the writing and editing of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Appendix S1. Supplementary methods: laboratory protocol for GBS library preparation.

Appendix S2. Supplementary methods and results for morphological data.

Appendix S3. Supplementary figures.

Appendix S4. Supplementary tables.

SHARED DATA

All data from this study are archived on Dryad (doi:10.5061/dryad.80gb5mkn4, [Quach et al., 2019](#)).