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Genetic drift or natural selection? Hybridization and asymmetric mitochondrial introgression in two Caribbean lizards (*Anolis pulchellus* and *Anolis krugi*)

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Abstract

Hybridization and gene introgression can occur frequently between closely related taxa, but appear to be rare phenomena among members of the species-rich West Indian radiation of *Anolis* lizards. We investigated the pattern and possible mechanism of introgression between two sister species from Puerto Rico, *Anolis pulchellus* and *Anolis krugi,* using mitochondrial (ND2) and nuclear (DNAH3, NKTR) DNA sequences. Our findings demonstrated extensive introgression of *A. krugi* mtDNA (*k*-mtDNA) into the genome of *A. pulchellus* in western Puerto Rico, to the extent that *k*-mtDNA has mostly or completely replaced the native mtDNA of *A. pulchellus* on this part of the island. We proposed two not mutually exclusive scenarios to account for the interspecific matings between *A. pulchellus* and *A. krugi*. We inferred that hybridization events occurred independently in several populations, and determined that *k*-mtDNA haplotypes harboured in individuals of *A. pulchellus* can be assigned to four of the five major mtDNA clades of *A. krugi*. Further, the spatial distribution of *k*-mtDNA clades in the two species is largely congruent. Based on this evidence, we concluded that natural selection was the probable driving mechanism for the extensive *k*-mtDNA introgression into *A. pulchellus*. Our two nuclear data sets yielded different results. DNAH3 showed reciprocal monophyly of *A. pulchellus* and *A. krugi*, indicating no effect of hybridization on this marker. In contrast, the two species shared nine NKTR alleles, probably due to incomplete lineage sorting. Our study system will provide an excellent opportunity to experimentally assess the behavioural and ecological mechanisms that can lead to hybridization in closely related taxa.

Introduction

Hybrid zones are of great interest to behavioural ecologists and evolutionary biologists because these areas provide natural systems for studies of characters and processes involved in divergence, reproductive isolation and speciation (Abbott *et al.*, 2013). Hybridization may be a more prevalent phenomenon than is generally believed. Indeed, a literature review (Mallet, 2005) estimated that at least 10% of animal species (usually species that diverged from each other relatively recently) hybridize with heterospecifics. The generation and maintenance of a hybrid zone requires mismatings and at least partially successful reproduction between individuals that differ in one or more heritable traits. Natural hybridization may occur sporadically between closely related, broadly syntopic species or be confined to particular contact zones (Jiggins & Mallet, 2000). Species that only hybridize in parts of their overlapping ranges provide an excellent opportunity to investigate possible mechanisms responsible for this pattern (Noor, 1999; Seehausen, 2004; Mallet, 2005; Grant & Grant, 2006; Good *et al.*, 2008).

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One possible consequence of hybridization is replacement (i.e. introgression) of the maternally inherited mitochondrial DNA (mtDNA) of one species by that of another species (Ruedi *et al.*, 1997; Ballard & Whitlock, 2004; Good *et al.*, 2008; Ambrose *et al.*, 2012). In zones of sympatry or parapatry, the foreign mtDNA is transferred by fertile or partially fertile female hybrids that backcross with males of the paternal species (Plötner *et al.*, 2008). In some cases, the foreign mtDNA can spread over significant geographic distances and even completely replace the native mtDNA of a species (Ballard & Whitlock, 2004; Melo-Ferreira *et al.*, 2005; Bachtrog *et al.*, 2006). Two main mechanisms have been suggested to account for mtDNA introgression. The predominant view is that introgression is typically driven by neutral processes (genetic drift; Ballard & Kreitman, 1995). However, some authors have proposed that natural selection on nonsynonymous mutations may favour the foreign mtDNA and thus account for extensive mtDNA introgression in some species (Wilson & Bernatchez, 1998; Melo-Ferreira *et al.*, 2005; McGuire *et al.*, 2007; Dowling *et al.*, 2008). An interesting pattern that has emerged from studies evaluating mtDNA introgression is that this replacement often has no effect on organismal phenotypes or nuclear genomes (Ferris *et al.*, 1983; Tegelström, 1987; Hird & Sullivan, 2009; Liu *et al.*, 2010).

West Indian *Anolis* lizards (i.e. anoles) are a model system for studies of evolutionary and behavioural ecology (e.g. Roughgarden, 1995; Losos & Schluter, 2000; Harmon *et al.*, 2003; Losos *et al.*, 2003; Takimoto *et al.*, 2008; Cox & Calsbeek, 2010; Leal & Powell, 2012). Anole communities can be highly diverse, with up to 15 species coexisting in some localities (Losos, 2004), and it is common for most syntopic species to encounter in a single day (or at least in their lifetime) individuals from three to as many as eight congeneric taxa (Losos, 2009). Despite these frequent interactions, interspecific hybridization in *Anolis* seems to be a rare event; only eight species pairs have been suggested to hybridize, and available evidence indicates that perhaps only two of these cases result in genetic introgression (hybrids being unknown or sterile in the other six species pairs; Losos, 2009). The paucity of hybridization in anoles has been attributed to strong premating isolation mediated by effective species recognition signals (reviewed in Losos, 2009), particularly the dewlap (i.e. an extensible colourful throat fan) and/or the temporal pattern of head-bobbing displays (Rand & Williams, 1970; Jenssen, 1977; Williams & Rand, 1977). Both signals are commonly used by males as part of their courtship displays, and syntopic species always differ in some aspect of the coloration of the dewlap (Fleishman, 1992; Ord & Martins, 2006; Nicholson *et al.*, 2007).

As part of our research on patterns of genetic differentiation in *Anolis* lizards from Puerto Rico and nearby islands in the eastern Caribbean Sea (Rodríguez-Robles *et al.*, 2007, 2008, 2010; Jezkova *et al.*, 2009), we discovered that some individuals of *Anolis pulchellus* Duméril and Bibron, 1837 possess mtDNA of *A. krugi* Peters, 1876. *Anolis krugi* and *A. pulchellus* are sister species (Gorman *et al.*, 1983; Poe, 2004; Nicholson *et al.*, 2005) that have a similar body morphology, but that can be readily distinguished due to their distinctive colour patterns (Rivero, 1998). Additionally, the dewlap of *A. krugi* has a solid coloration that varies from a saturated peach to a relatively pale yellow, whereas the dewlap of *A. pulchellus* has a relatively pale purple centre surrounded by a reddish edge. However, individuals of *A. pulchellus* with the foreign mtDNA of *A. krugi* are morphologically indistinguishable from nonintrogressed *A. pulchellus*. Both anoles are locally abundant and widespread in Puerto Rico (Schwartz & Henderson, 1991), where *A. krugi* is typically associated with mesophilic, moderate to relatively shaded areas, and *A. pulchellus* generally occurs in more open, illuminated habitats (Rivero, 1998; Thomas, 1999). Yet, the species are often syntopic throughout Puerto Rico. (*Anolis krugi* is restricted to Puerto Rico, whereas *A. pulchellus* also occurs in Vieques, Culebra and the Virgin Islands, off the eastern coast of Puerto Rico.) The objective of our study was two-fold. First, we characterized the pattern of mtDNA introgression in *A. pulchellus* by addressing the following questions. Does the mitochondrial capture seem to have occurred only once, or several times? If mitochondrial capture occurred repeatedly, what is the geographic extent of the mtDNA introgression? That is, did it occur in most (or all) areas where the species are syntopic across Puerto Rico, or only in particular regions of the island? Our second goal was to infer whether genetic drift or natural selection may account for the observed pattern of mtDNA introgression.

Materials and methods

Taxon sampling and laboratory methods

We collected tissue samples from 309 individuals of *Anolis pulchellus* from 54 localities in Puerto Rico and in Vieques and Culebra, two small islands off the eastern coast of Puerto Rico (Fig. 1; Table S1). For the purposes of visualization and genetic analyses, we combined localities within a 5-km radius, which resulted in 47 'general' localities (Fig. 1). Sample size varied from one to 13 lizards per locality (one to 17 per general locality), with 32 of 54 (59%) populations being represented by five or more individuals.

We isolated total genomic DNA from frozen tissue samples (heart, liver or tail fragments) using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA). For the 309 samples of *A. pulchellus*, we used the primers LVT_Metf.6_AnCr (AAGCTATTGGGCCCA TACC) and LVT_5617_AnCr (AAAGTGYTTGAGTTGCA TTCA; Rodríguez-Robles *et al.*, 2007, 2008) to amplify

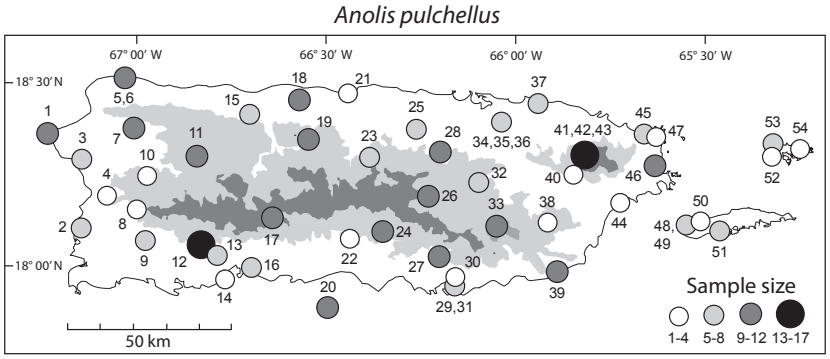

Fig. 1 Map of Puerto Rico, with the 200-m (light grey) and 600-m (dark grey) elevation contours indicated. Circles represent the approximate sampling localities of the specimens of *Anolis pulchellus* included in this study (see Table S1, for specific locality information). Circle size is proportional to sample size.

ca. 1,150 base pairs (bp) of the mtDNA nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 2 and adjacent tRNAs ($tRNA^{Trp}$, $tRNA^{Ala}$), hereafter referred to as the 'ND2' gene region. For a subset of samples representing all major native and foreign mtDNA clades of *A. pulchellus* (see Results), we also generated sequences of two nuclear genes: dynein axonemal heavy chain 3 (DNAH3) for 70 samples and natural killer-triggering receptor (NKTR; Townsend *et al.*, 2008; Benavides *et al.*, 2009) for 60 samples. We used the primers DNAH3_F1 (GGTAAAATGATAGAAGAYTACTG) and DNAH_R6 (CTKGAGTTRGAHACAAT KATGCCAT; Benavides *et al.*, 2009) to amplify *ca.* 700 bp of DNAH3, and we designed species-specific primers NKTR_intL1_AnPu (CAAGATTGTCTTCCAGCAAGG) and NKTR_intH2_AnPu (GGGTCACATCTACTTCATTC), which lie just inside the universal NKTR_F1 and NKTR_R3 primers (Benavides *et al.*, 2009), to amplify *ca.* 1100 bp of NKTR.

We incorporated into our data set 211 ND2 sequences of *A. krugi* representing 54 localities across the entire geographic range of the species in Puerto Rico (Rodríguez-Robles *et al.*, 2010). For this study, we added the adjacent tRNA sequences to all *A. krugi* ND2 sequences, to match our *A. pulchellus* mtDNA data set. We sequenced DNAH3 for a subset of 29 samples, and NKTR for a subset of 24 samples (Table S1). For these two nuclear markers, the samples included individuals from the five major mtDNA clades of *A. krugi* (Rodríguez-Robles *et al.*, 2010).

We carried out PCR in 12.5 μL volumes consisting of 1 μL of template DNA, 0.5 μL of each primer (10 μM), 6.25 μL of Takara *Ex Taq*™ Polymerase Premix (Takara Mirus Bio Inc., Madison, WI, USA) and 4.25 μL of ddH_20. DNA was denatured initially at 95 °C for 2.5 min, and then, 40 cycles of amplification were performed under the following conditions: denaturation at 95 °C for 1 min, annealing at 57 °C (for ND2), 52 °C (for DNAH3) or 55 °C (for NKTR) for 1 min, and extension at 72 °C for 1 min, followed by a final 5-min elongation at 72 °C. Two microlitres of all PCR products were electrophoresed on a 0.8% agarose gel stained with ethidium bromide to verify product band size. We cleaned the double-stranded PCR products with ExoSap –IT® (USB Corporation, Cleveland, OH, USA). We sequenced the ND2 fragment using the primers LVT_Metf.6_AnCr and LVT_L5002_AnPu (AACCAAACACARACTCGAAAAAT; Rodríguez-Robles *et al.*, 2007, 2008) and sequenced the DNAH3 and NKTR fragments using the same primers used for amplification. We used the Big Dye Terminator Ready Reaction kit 1.1 and 3.1 (Applied Biosystems, Foster City, CA, USA) for cycle sequencing and ran the sequences on an ABI 3130 automated sequencer. The final data sets for *A. pulchellus* and *A. krugi* comprised 1138 bp of ND2 (GenBank accession numbers KC677040–KC677562), 690 bp of DNAH3 (GenBank accession numbers KC689362–KC689491, KC689493–KC689570) and 1002 bp of NKTR (GenBank accession numbers KC689571–KC689736; Table S1).

Phylogenetic and median-joining network analyses of mtDNA sequences

We used the program COLLAPSE (version 1.2; available at http://darwin.uvigo.es) to collapse the 309 mtDNA (ND2 + tRNA) sequences to 184 unique haplotypes, and the star-contraction method in the program NETWORK (version 4.200) to further reduce this data set for phylogenetic analyses (Forster *et al.*, 2001). The latter method identifies starlike clusters of haplotypes representing newly emerging mutations ('satellite haplotypes') around a founder (ancestral) node and reduces a cluster to its founder node (Forster *et al.*, 2001). Using a contraction value of five mutational steps, the star-contraction method reduced the data set from 184 to 125 sequences, of which 121 are original haplotypes and 4 are median vectors that represent unsampled or extinct ancestral haplotypes necessary to connect the associated satellite haplotypes in the most parsimonious way (Bandelt *et al.*, 1999). The reduced data set of 125 sequences was used for the maximum likelihood (ML) and Bayesian inference (BI) analyses. Based on previous studies (Gorman *et al.*, 1968, 1983;

Brandley & de Queiroz, 2004; Poe, 2004; Nicholson *et al.*, 2005), we used *Anolis gundlachi* Peters, 1876, *A. poncensis* Stejneger, 1904, and *A. cristatellus* Duméril and Bibron, 1837 as outgroup taxa.

We partitioned the mtDNA data set by gene (ND2 and tRNA), and the ND2 further by codon (1st and 2nd codon positions combined; 3rd codon position). We identified the best-fitting model of nucleotide substitution for each partition using MRMODELTEST (version 2.2; Nylander, 2004). Hierarchical likelihood ratio tests and Akaike information criteria identified GTR + I + G for the 1st and 2nd codon positions, GTR + G for the 3rd codon position, and HKY + I + G for tRNAs as the most appropriate models of nucleotide substitution for the ingroup taxa.

We conducted ML analyses using the program TREEFINDER (Jobb *et al.*, 2004). We used the 'Bootstrap Analysis' option in TREEFINDER (200 replicates, consensus level, 50) to build a tree and determine nodal support for the mtDNA (ND2 + tRNA) data sets of *A. pulchellus* and *A. krugi*. We also assessed tree topology and clade support for both data sets using the program MRBAYES (version 3.1.1; Ronquist & Huelsenbeck, 2003). We initiated the BI analyses from a random starting tree with uniform (uninformative) priors (Brandley *et al.*, 2006). We produced posterior probability distributions by allowing four Monte Carlo Markov chains with a heating value of 0.05 (to increase swapping among trees) to proceed for five million generations each, with samples taken every 100 generations, a procedure that yielded 50 000 trees. After visual evaluation (Leaché & Reeder, 2002), we discarded the first 1 250 000 generations (12 500 trees) as 'burn-in' samples (trees obtained before parameter stabilization occurred) and combined the remaining samples to estimate tree topology, posterior probability values and branch lengths. We ran the Bayesian analyses twice to ensure that the chains were not trapped on local optima.

We constructed a median-joining network for sequences of *A. pulchellus* and *A. krugi* using the program NETWORK (http://www.fluxus–technology.com; Bandelt *et al.*, 1999). The median-joining method uses a maximum parsimony approach to search for all the shortest phylogenetic trees for a given data set (Bandelt *et al.*, 1999). To construct the network, we weighted transversions twice as high as transitions. After generating the initial network, we used the MP option in NETWORK to remove excessive links and median vectors and construct the final median-joining network (Polzin & Daneshmand, 2003).

We estimated the minimum number of introgression events of *A. krugi* mtDNA into the genome of *A. pulchellus* from the ML and BI trees for the combined data sets of all mtDNA sequences of *A. krugi*, and the *A. krugi* mtDNA sequences of *A. pulchellus*. In the ML tree, we first collapsed all branches with bootstrap values <70 (to calculate a less conservative estimate), and then collapsed all branches with bootstrap values <90 (to calculate a more conservative estimate). In the BI tree, we collapsed all branches with a posterior probability <95. The number of introgression events was estimated as the minimum number of character state changes (i.e. shifts from mtDNA of *A. pulchellus* to mtDNA of *A. krugi*) necessary to explain the observed pattern of distribution of *A. krugi* mtDNA in each tree, under the assumption that the introgression only occurred in one direction, from *A. krugi* into *A. pulchellus*. For example, when two or more introgressed individuals of *A. pulchellus* shared the same most recent common ancestor (i.e. formed a monophyletic clade), we assumed that the clade originated from a single introgression event.

Phylogenetic and median-joining network analyses of nuclear sequences

We separated the DNAH3 and NKTR nuclear sequences into alleles using the program PHASE (Stephens & Donnelly, 2003), as implemented in DnaSP (Librado & Rozas, 2009). For the DNAH3 data set, we collapsed the alleles into unique sequences using COLLAPSE and conducted ML and BI analyses as described above using the HKY model of nucleotide substitution for the unpartitioned data set. We relied on the program MEGA (Tamura *et al.*, 2011) to calculate the mean distances between clades inferred by the DNAH3 sequences. We constructed a median-joining network for NKTR alleles using NETWORK. We excluded from the analyses the three indels present in the NKTR data set. DnaSP was used to calculate number of polymorphic sites and nucleotide diversity for the DNAH3 and NKTR data sets.

Results

Geographic patterns of mtDNA introgression into *Anolis pulchellus*

The phylogenetic analyses revealed extensive introgression of *A. krugi* mtDNA into the genome of *A. pulchellus* (Figs 2 and 3). We estimated between 10 and 15 independent introgression events from the ML tree (Fig. S1) and 13 introgression events from the BI tree (data not shown). The introgression is almost exclusively restricted to western Puerto Rico, where 83 individuals of *A. pulchellus* sampled from 14 localities (Fig. 3; localities 1–10, 12–14, 16) only possessed mtDNA of *A. krugi* (hereafter referred to as '*k*-mtDNA'). In other words, not a single phenotypically identified individual of *A. pulchellus* from these localities possessed native mtDNA of *A. pulchellus* (hereafter referred to as '*p*-mtDNA'). However, in eastern Puerto Rico the observed pattern is dramatically different. In this region of the island,

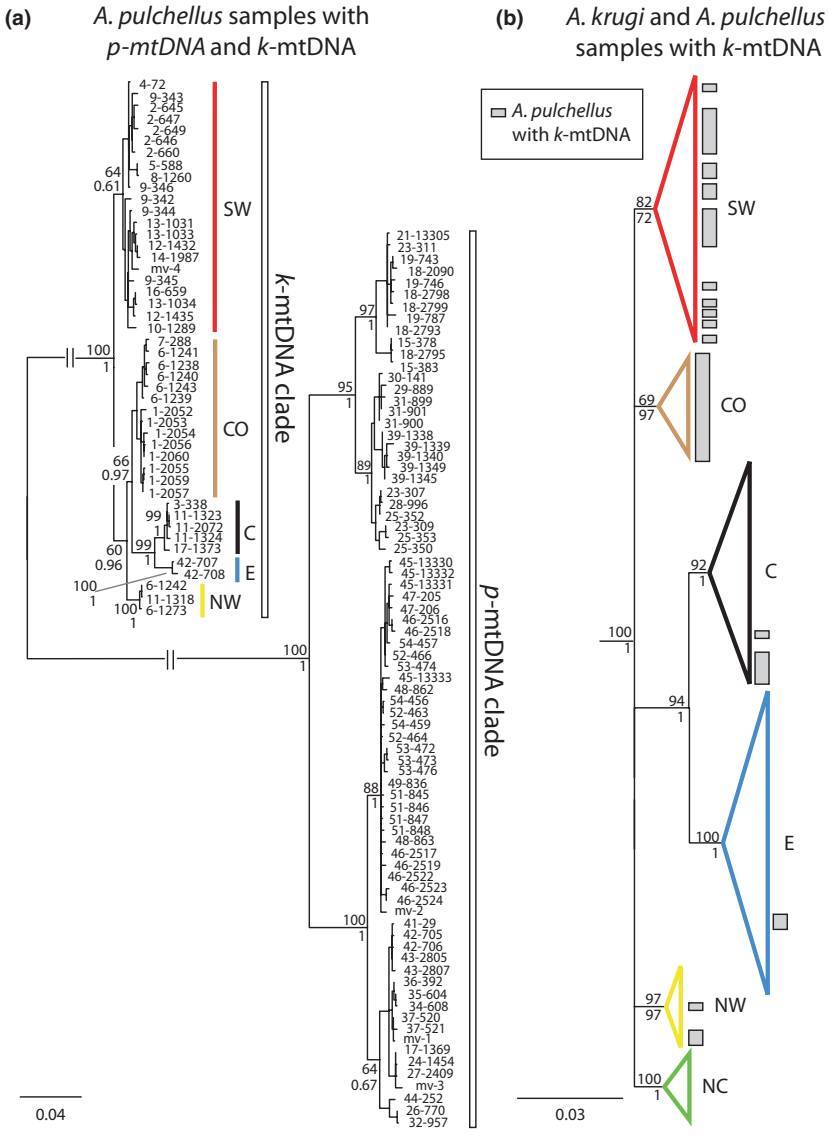

Fig. 2 (a) Maximum likelihood tree for 117 unique mtDNA haplotypes and four median vectors (mv) of *Anolis pulchellus* possessing mtDNA of *A. krugi* (*k*-mtDNA clade), and *A. pulchellus* with native mtDNA (*p*-mtDNA clade). Nodal support was assessed with nonparametric bootstrap values for ML analyses (numbers above node), and with Bayesian posterior probabilities (numbers below node). The colour-coded clades correspond to four (eastern, central, north-western, south-western) of the five mtDNA clades of *A. krugi* identified in Rodríguez-Robles *et al.* (2010), and to the new Coastal mtDNA clade identified in this study. (b) Maximum likelihood tree for the combined mtDNA haplotype data set of *A. krugi* and of *A. pulchellus* possessing *A. krugi* mtDNA. The five (central, eastern, north-central, north-western, south-western) mtDNA clades of *A. krugi* identified in Rodríguez-Robles *et al.* (2010) and the new Coastal mtDNA clade identified in this study are indicated. The grey rectangles indicate haplotypes of *A. pulchellus*, with each rectangle representing one estimated introgression event. (These estimates were made after collapsing all branches with bootstrap values <70.) Abbreviations used are: C, central clade; CO, coastal clade; E, eastern clade; NC, north-central clade; NW, north-western clade; SW, south-western clade.

A. pulchellus populations exhibit the native mtDNA, with the exception of locality 42 (Figs 1 and 3), where we identified two individuals (JAR 707 – MVZ 235575; JAR 708 – MVZ 235576) that possessed *k*-mtDNA. Three populations of *A. pulchellus* (two in western Puerto Rico and the aforementioned one in eastern Puerto Rico; Fig. 3; localities 11, 17, 42) exhibit a mixture of *k*-mtDNA and native *p*-mtDNA.

The *k*-mtDNA in *A. pulchellus* shows pronounced geographic structuring. The phylogenetic analyses revealed that the individual *k*-mtDNA haplotypes can be assigned to four of the five major mtDNA clades of *A. krugi* (central, eastern, north-western, south-western) previously identified (Rodríguez-Robles *et al.*, 2010; Figs 2 and 3). The haplotypes found in localities 1, 5 and 6 in north-western Puerto Rico constitute a heretofore undetected *A. krugi* mtDNA clade (herein referred to as 'coastal clade'; Figs 2 and 3). The fact that our recent phylogeographic assessment of *A. krugi* (Rodríguez-Robles *et al.*, 2010) did not recover this coastal clade suggests that these haplotypes went extinct in *A. krugi*, or simply that we did not sample them. The spatial distribution of the major mtDNA clades of *A. pulchellus* and *A. krugi* shows a remarkable degree of geographic congruence. In *A. pulchellus*, the four clades of *k*-mtDNA are found in the same general areas where these clades occur in *A. krugi* (Fig. 3). These findings indicate that *k*-mtDNA introgression happened multiple times throughout the range of *A. pulchellus* in western Puerto Rico, and suggest that individuals possessing *k*-mtDNA tended to remain within the general areas where the introgression events occurred. The likelihood that introgressed individuals of

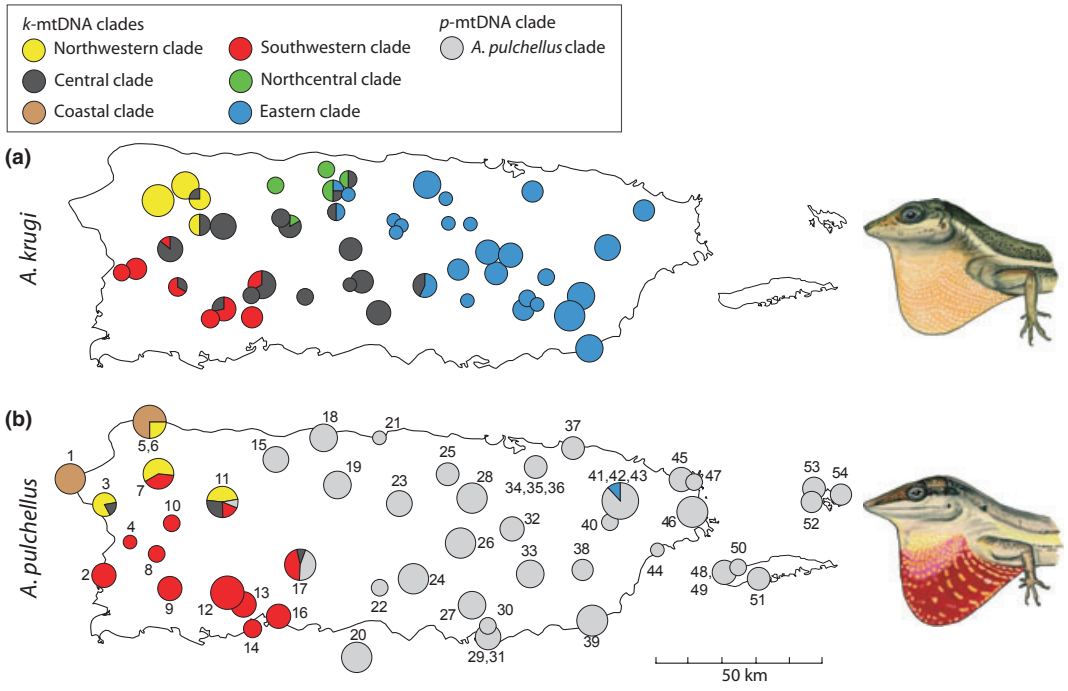

Fig. 3 Approximate geographic locations of the mtDNA haplotype clades of (a) *Anolis krugi* (modified from Rodríguez-Robles *et al.*, 2010) and (b) *Anolis pulchellus*, identified by maximum likelihood and Bayesian inference phylogenetic methods. The mtDNA haplotype clades match those in Fig. 2. Circle size is proportional to sample size, with the smallest circles (i.e. 4, 21, 44) representing one sample, and the largest one (i.e. 41, 42, 43) representing 17 samples.

A. pulchellus exhibited philopatry is congruent with our findings for *A. cooki* Grant, 1931 and *A. poncensis* in south-western Puerto Rico, where the strong geographic structuring of mtDNA is also indicative of limited dispersal in these two anoles (Jezkova *et al.*, 2009). The mean genetic distance (uncorrected for within-species divergence) between *A. pulchellus* with native mtDNA and *A. krugi* is 15.4%, but it is only 3.3% between *A. pulchellus* with *k*-mtDNA and *A. krugi*. The median-joining haplotype network (Fig. 4) shows in more detail the distribution of *k*-mtDNA in *A. pulchellus* in relationship to the mtDNA of *A. krugi*. Fourteen *k*-mtDNA haplotypes are shared between *A. pulchellus* and *A. krugi*.

Nuclear markers

The DNAH3 nuclear data set (*A. pulchellus* with *p*-mtDNA, $n = 36$; *A. pulchellus* with *k*-mtDNA, $n = 34$; *A. krugi*, $n = 29$) comprises 10 unique alleles with 13 polymorphic sites, 11 of which are phylogenetically informative. Nine of these sites represent synonymous mutations, and four constitute nonsynonymous mutations. The ML and BI analyses of DNAH3 revealed reciprocal monophyly of phenotypically identified individuals of *A. pulchellus* (regardless of whether they carried *p*-mtDNA or *k*-mtDNA) and *A. krugi* (Fig. 5a), indicating no impact of hybridization on this gene. The exception is one specimen of *A. pulchellus* (JAR 708 – MVZ 235576) from locality 42 in eastern Puerto Rico (Fig. 5a; sample indicated by an asterisk). This individual possessed one allele from the *A. pulchellus* clade and one allele from the *A. krugi* clade, likely indicating a recent hybridization event, independent of the mtDNA introgression observed in western Puerto Rico. The mean genetic distance between the *A. pulchellus* and *A. krugi* clades, uncorrected for within-group divergence, is 1.1%, whereas the mean genetic distance between the two clades corrected for within-group divergence is 0.8%. Nucleotide diversity is higher in *A. krugi* (0.0014 ± 0.0002) than in *A. pulchellus* (0.0006 ± 0.0002).

The NKTR nuclear data set (*A. pulchellus* with *p*-mtDNA, $n = 27$; *A. pulchellus* with *k*-mtDNA, $n = 33$; *A. krugi*, $n = 24$) comprises 91 unique alleles with 76 polymorphic sites, 61 of which are phylogenetically informative. Fourteen of these sites represent synonymous mutations, and 20 constitute nonsynonymous mutations. Overall, nucleotide diversity in NKTR is higher than in DNAH3, but in contrast with the latter, nucleotide diversity in NKTR is higher in *A. pulchellus* (0.0094 ± 0.0004) than in *A. krugi* (0.0040 ± 0.0006). The two species do not form monophyletic clades with regard to NKTR, and nine alleles are shared between

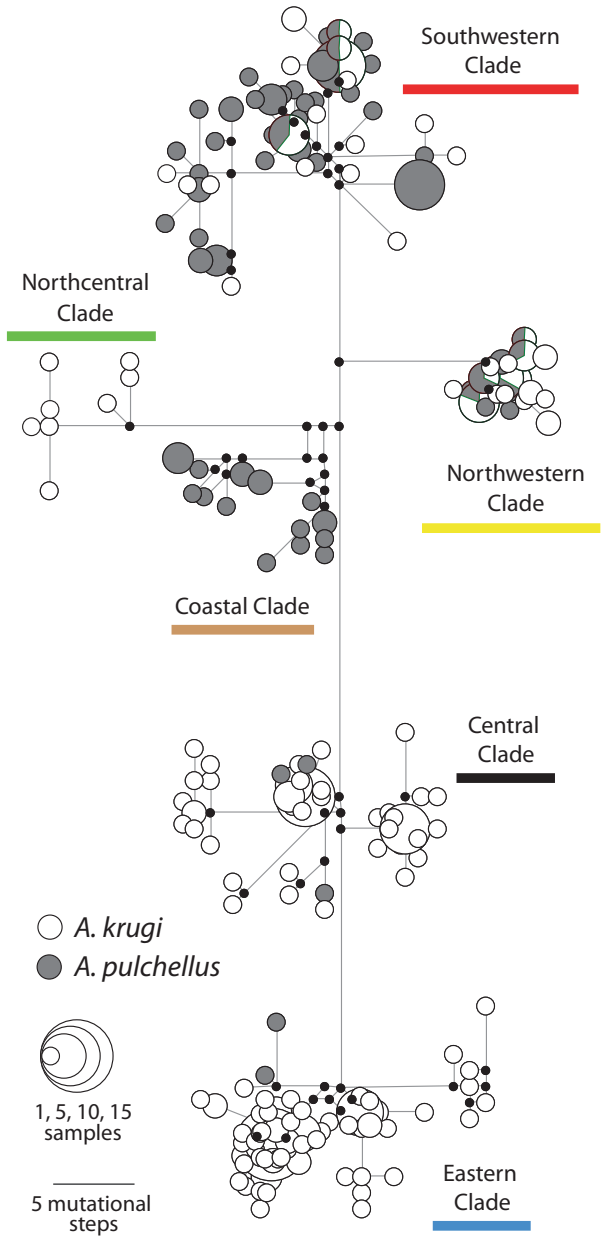

Fig. 4 Median-joining network representing the relationships between mtDNA haplotypes of *Anolis krugi* (n = 211) and of *Anolis pulchellus* possessing mtDNA of *A. krugi* (n = 101). The native mtDNA haplotypes of *A. pulchellus* are not depicted in this figure. Circle size is proportional to haplotype frequencies.

them (Fig. 5b). These shared alleles are widely distributed, not clustered within a geographic area. For instance, the most common shared allele is found throughout Puerto Rico, in the five mtDNA clades of *A. krugi* and in four of the six mtDNA clades of *A. pulchellus* (including the native *p*-mtDNA clade; data not shown), a pattern indicative of incomplete lineage sorting, not interspecific hybridization (cf. van Oppen *et al.*, 2001; McCracken & Sorenson, 2005). If the sharing of NKTR alleles between *A. krugi* and *A. pulchellus* were the result of interspecific hybridization, individual alleles of both species would be clustered within the same geographic area. In other words, the spatial distribution of individual alleles in the two species would be congruent, similar to the spatial congruence observed in the mtDNA.

Discussion

We described the first known case of extensive mtDNA introgression in *Anolis* lizards, a well-known example of an adaptive radiation (Losos, 2009). Our finding was unexpected, because one of the characteristic features of the *Anolis* radiation is the apparent rarity of interspecific hybridization, contrary to the pattern exhibited by other evolutionary radiations (Seehausen, 2004). We documented that the introgression in *A. pulchellus* occurred nearly exclusively in western Puerto Rico (Fig. 3) and that it took place at numerous localities. In fact, our broad sampling did not detect *A. pulchellus* with native mtDNA among 83 individuals from 14 localities in western Puerto Rico. The multiple independent episodes of hybridization suggest that a historical event contributed to the decrease in the effectiveness of premating isolation mechanisms, the barriers that are widely recognized as the main reason why hybridization seems to be an infrequent event in *Anolis*. Our findings also demonstrate that the introgression was unidirectional, with *k*-mtDNA occurring in individuals of *A. pulchellus* (but not vice versa). The extensive introgression of *k*-mtDNA into *A. pulchellus* suggests the possibility of a selective advantage for hybrid individuals.

Geographic patterns of mtDNA introgression into *Anolis pulchellus*

We detected extensive introgression of *Anolis krugi* mtDNA into the genome of *A. pulchellus* in western Puerto Rico (Fig. 3). The original introgression resulted from interspecific matings between *A. krugi* females and *A. pulchellus* males. Subsequent backcrossing of the hybrid females with 'pure' *A. pulchellus* males produced the observed pattern of mitochondrial capture. The low genetic divergence among and presence of shared haplotypes in the mitochondrial genomes of *A. krugi* and *A. pulchellus* (i.e. closely related or identical haplotypes occur in both species) imply that the introgression happened after these species diverged from their most recent common ancestor, a split estimated to have occurred *ca.* 3–6.4 million of years ago (Brandley & de Queiroz, 2004).

The geographic distribution of the *k*-mtDNA clades in *A. pulchellus* corresponds to the distribution of these clades in *A. krugi* (Fig. 3). Further, the *k*-mtDNA

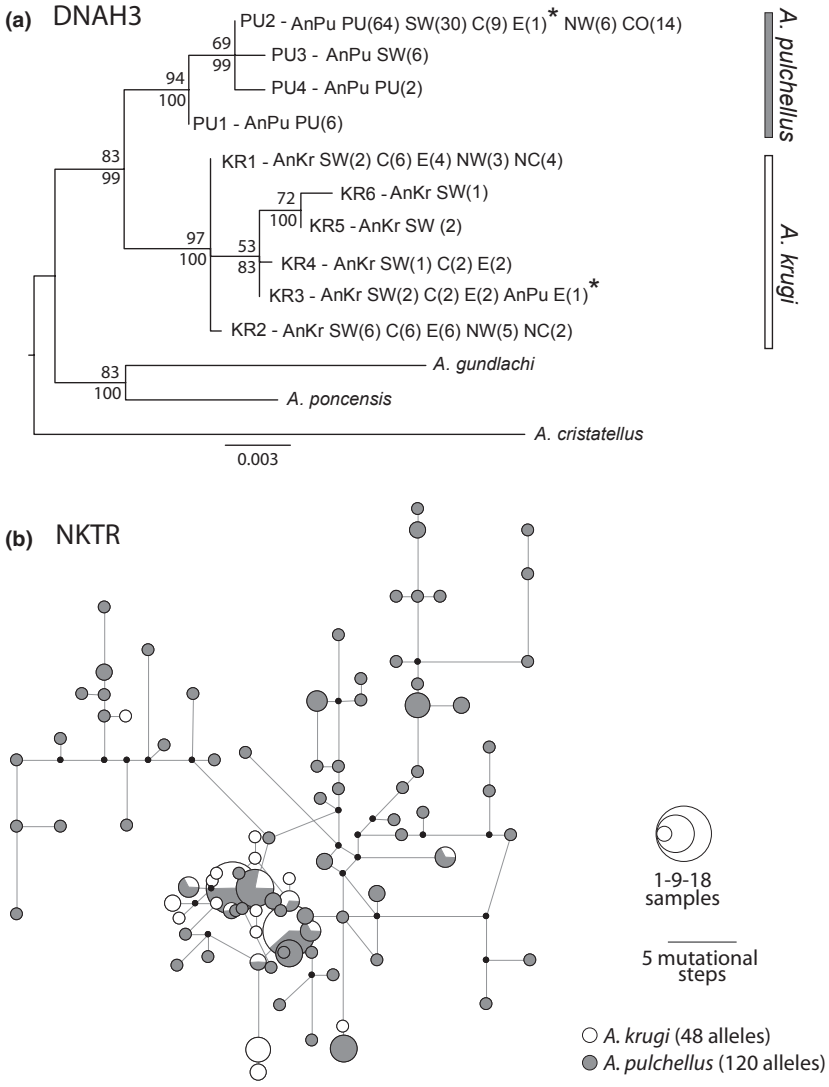

Fig. 5 (a) Maximum likelihood tree for the nuclear DNAH3 data set (*Anolis pulchellus* with native mtDNA, $n = 36$; *A. pulchellus* with *A. krugi* mtDNA, $n = 34$; *A. krugi*, $n = 29$; and three outgroups, *A. cristatellus*, *A. gundlachi*, *A. poncensis*), phased and collapsed into unique alleles (i.e. each sample is represented by two alleles). Nodal support was assessed with nonparametric bootstrap values for ML analyses (numbers above node) and with Bayesian posterior probabilities (numbers below node). The unique denomination of each allele is followed by the species abbreviation (AnPu = *A. pulchellus*, AnKr = *A. krugi*), the abbreviation of the mtDNA clade in which the allele was detected, and the number of individuals (in parentheses) exhibiting that particular allele. Abbreviations used are: C, central clade; CO, coastal clade; E, eastern clade; KR, *A. krugi* clade; NC, north-central clade; NW, north-western clade; PU, *A. pulchellus* clade; SW, south-western clade. (b) Median-joining network representing the relationships between NKTR sequences of *A. pulchellus* (grey, $n = 60$) and *A. krugi* (white, $n = 24$), phased into unique alleles. The smallest, black circles indicate median vectors (Bandelt *et al.*, 1999). Circle size is proportional to haplotype frequencies, and branch length is proportional to the number of mutations separating the alleles.

haplotypes of *A. pulchellus* are identical or closely related to native haplotypes in nearby populations of *A. krugi* (Fig. S2), suggesting that the affected populations have remained in the same general area since the introgression events. We estimated that 10–15 introgression events occurred throughout western Puerto Rico (Fig. 2b). However, the actual number of introgression events is likely greater, because of the possible existence of extinct or unsampled haplotypes of *A. krugi* and the low support of many shallow nodes in our *k*-mtDNA phylogeny (Fig. S1). For instance, due to poor bootstrap support of nodes on the ML tree, we assumed (using the more conservative threshold) that the entire coastal clade plus eight star-contracted haplotypes from the south-western clade represented a single introgression event (Fig. S1), even though samples within this group are up to 3% divergent, six times higher than in any other group of *k*-mtDNA haplotypes representing one introgression event (0–0.5%). If diversification and mutation rates were similar in all *k*-mtDNA clades of *A. pulchellus*, and if the coastal clade plus the eight star-contracted haplotypes from the south-western clade indeed originated from a single introgression event, this introgression would have had to occur much earlier than all other introgression events in *A. pulchellus*. A more likely scenario is that certain poorly supported nodes represent actual cladogenetic events, implying that introgression happened multiple times within these two mtDNA clades.

We did not identify any individuals of *A. pulchellus* with native mtDNA among 83 individuals from 14 localities in western Puerto Rico, indicating that pure *A. pulchellus* on this part of the island are rare and were undetected by our extensive geographic sampling.

However, although the total number of samples is substantial, the number of individuals for some localities is small (Fig. 1; Table S1). Consequently, rare haplotypes, including those of pure *A. pulchellus*, may be underrepresented in our sampling. On the other hand, our findings may accurately reflect the actual circumstances, and *k*-mtDNA may have completely replaced *p*-mtDNA in western Puerto Rico. In any case, available evidence indicates that *A. krugi*'s mtDNA has replaced *p*-mtDNA on this part of the island to a considerable degree, implying that *k*-mtDNA introgression has affected the evolutionary dynamics of western Puerto Rican populations of *A. pulchellus* and, by extension, of *A. krugi* as well.

Replacement of *A. pulchellus*' native mtDNA by *k*-mtDNA occurs even in some areas from which *A. krugi* is currently absent. In particular, being a mesophilic species, *A. krugi* does not occur along the south-western coast of Puerto Rico, one of the most arid regions of the island (Helmer *et al.*, 2002; Daly *et al.*, 2003). Nevertheless, *A. pulchellus* populations from the south-western coast exclusively harbour *k*-mtDNA haplotypes that correspond to those of *A. krugi* from more northern locations (Fig. 3). Ecological niche models constructed using the climatic conditions of the Last Glacial Maximum showed that the south-western coast of Puerto Rico has not been suitable for *A. krugi* since at least the last glacial period (Rodríguez-Robles *et al.*, 2010). Collectively, these findings suggest that *A. pulchellus* dispersed south into this region of the island after the introgression events. Indeed, a landscape genetic analysis revealed very small genetic distances among *A. pulchellus* populations with *k*-mtDNA in southern Puerto Rico (Fig. S3), a pattern consistent with an episode of recent range expansion into this area (Hewitt, 1996).

Possible mechanisms of hybridization and introgression in *Anolis pulchellus*

Given that hybridization has only been documented in eight of the more than 380 species of *Anolis*, and that introgression is only known to occur in two of these species pairs (Losos, 2009), our discovery of extensive natural hybridization between *A. krugi* and *A. puchellus* was surprising. What historical factors or conditions may have facilitated interspecific matings in these anoles is an open question. However, our current understanding of the ecology, visual physiology and communication of West Indian *Anolis*, including *A. krugi* and *A. pulchellus* (Leal & Fleishman, 2002, 2004; Fleishman *et al.*, 2009), allows us to propose two not mutually exclusive scenarios that would have facilitated hybridization between these sister species.

First, climatic factors may have caused a change in the vegetation profile in western Puerto Rico that resulted in an increase in the amount of open, grassy habitats and a reduction in shaded, forested areas. This broad-scale change in habitat structure would have led to a shift in habitat light geometry, which affects signal detectability and discrimination in anoles (Fleishman *et al.*, 2006). In contrast to forests, the light geometry in grassy areas is dominated by the intensity of the background light (also known as the radiance background), not by the intensity of downwelling (incident) light, due to the prevalence of background spectral properties in open habitats. The latter conditions have been shown to favour the spectral properties of the dewlap of *A. pulchellus* (Fleishman *et al.*, 2009). On the contrary, the dewlap of male *A. krugi* experiences a decrease in detectability in open habitats. Accordingly, in open habitats, *A. krugi* females would have been more likely to detect the dewlaps of *A. pulchellus* males than those of conspecific males. This scenario would have favoured mismatings between the two species, due to a failure of their species recognition system. A breakdown of species recognition systems due to changes in habitat light conditions has been demonstrated to promote hybridization across multiple species of African cichlid fishes (Seehausen *et al.*, 1997).

A second possibility is that the proliferation of open grassy areas led to an increase in the relative density of *A. pulchellus* in areas of sympatry with *A. krugi* in western Puerto Rico. Theoretical models suggest that when relative abundances are severely uneven among closely related, syntopic species, the probability of mismatings is low for common species and high for their rarer counterparts (McPeek & Gavrilets, 2006). If the density of *A. pulchellus* increased disproportionately in western Puerto Rico and the dewlaps of *A. krugi* were less conspicuous than those of *A. pulchellus*, *A. krugi* females would have experienced higher encounter rates with *A. pulchellus* males than with conspecific males and would have been more likely to accept male *A. pulchellus* as mates. At a locality in north-eastern Puerto Rico, *A. pulchellus* was estimated to reach a population density of up to 20 000 individuals per hectare (Gorman & Harwood, 1977), one of the highest estimates for any reptile. In comparison, *A. krugi*'s density at six locations in north-central Puerto Rico was estimated to be 100 individuals per hectare (Borkhataria *et al.*, 2012). Density estimates of recent populations thus indicate that *A. pulchellus* can be considerably more abundant than *A. krugi*. Differences in density between sympatric species of *Pseudacris* chorus frogs have been suggested to play a major role in the evolution of species recognition signals, particularly in the less common species, to avoid hybridization (Lemmon, 2009). Perhaps the apparent rarity of current hybridization events between *A. krugi* and *A. pulchellus* is the result of an increase in the efficacy of discrimination between potential mates by *A. krugi* females.

Once hybridization occurs, two main mechanisms have been proposed to account for mtDNA introgression: genetic drift and natural selection. Given the supposed selective neutrality of mtDNA, genetic drift is usually considered the more likely mechanism (Ballard & Kreitman, 1995). But can genetic drift account for the observed *k*-mtDNA structure in *A. pulchellus* populations in western Puerto Rico? We detected multiple introgressions of *k*-mtDNA into the genome of *A. pulchellus*, as well as geographic congruence of *k*-mtDNA clades in *A. krugi* and *A. pulchellus*. These patterns are consistent with either genetic drift or natural selection. Specifically, hybridization and subsequent backcrossing occurred at multiple locations throughout western Puerto Rico, and individuals possessing the foreign *k*-mtDNA either persisted by chance or were favoured over *A. pulchellus* with native *p*-mtDNA. However, the multiple and independent instances of *k*-mtDNA introgression into *A. pulchellus* populations, combined with the high frequency of introgressed specimens in western Puerto Rico, suggest that selection favoured individuals with *k*-mtDNA, leading to partial or complete replacement of the native *p*-mtDNA (Fig. 6a). Indeed, some studies have suggested that even rare hybridization events can ultimately lead to fixation of the foreign mtDNA if the latter has a selective advantage (Wilson & Bernatchez, 1998; Shaw, 2002; Staubach *et al.*, 2012).

Partial or complete replacement of the native mtDNA in multiple populations of *A. pulchellus* is unlikely to have been caused by genetic drift. If drift was the mechanism for *p*-mtDNA replacement, we would expect to find a large frequency of introgressed individuals in only a subset of *A. pulchellus* populations, for most populations would exhibit a mixture of native and foreign mtDNA, or only the native *p*-mtDNA, if the introgressed individuals went extinct for stochastic reasons (Fig. 6b). Alternatively, genetic drift could have led to a high incidence of *k*-mtDNA in multiple western populations of *A. pulchellus* if introgression and replacement of *k*-mtDNA occurred in a single, small population of *A. pulchellus*, followed by a range expansion of *A. pulchellus* with *k*-mtDNA into previously unoccupied areas (Fig. 6c). Under such scenario, however, we would expect to find a signal of post-introgression range expansion (Hewitt, 1996), that is, a pattern of small genetic distances among *A. pulchellus* populations with *k*-mtDNA, contrary to our findings (Fig. S3). Further, we would not expect to see geographic congruence between the foreign *k*-mtDNA of *A. pulchellus* and the native *k*-mtDNA of *A. krugi*, as populations of *A. pulchellus* with *k*-mtDNA would be genetically similar to the population where the introgression originally happened.

In a panmictic population, the probability that a particular allele (or haplotype) may drift to fixation is equal to the haplotype's initial frequency (Wright, 1931). Fourteen populations of *A. pulchellus* in western Puerto Rico exclusively harbour *k*-mtDNA, and nine of these populations are represented by 5–13 individuals. Provided that *k*-mtDNA is fixed in these populations, estimating the probability of complete replacement of *p*-mtDNA in all nine *A. pulchellus* populations under genetic drift requires knowing the initial frequency of the *k*-mtDNA haplotypes. Outside of western Puerto Rico (sampling localities 15, 18–47), 2 of 178 (1.12%) individuals of *A. pulchellus* on the island had *k*-mtDNA. If this frequency is representative of the initial hybridization rate between *A. krugi* females and *A. pulchellus* males, the probability of *k*-mtDNA haplotypes in each

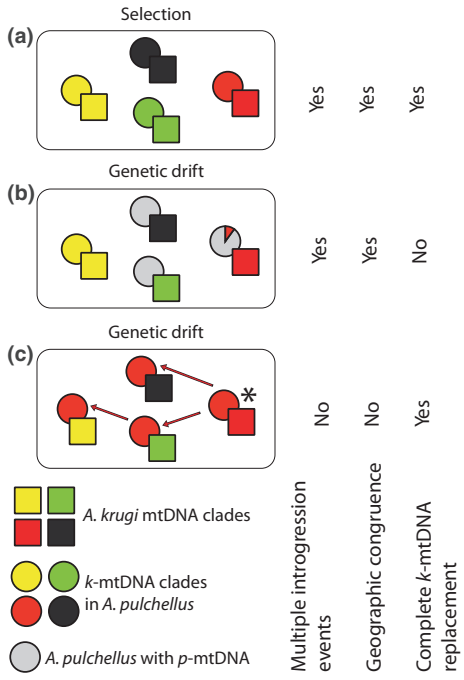

Fig. 6 Scenarios of phylogeographic structure of populations of *Anolis pulchellus* possessing mtDNA of *Anolis krugi* (*k*-mtDNA), with an indication of whether each pattern is consistent with (a) selection or (b, c) genetic drift as the driving mechanism. Clades of *k*-mtDNA are indicated by different colours. (a) Introgression of *k*-mtDNA into multiple populations of *A. pulchellus*, followed by selective sweeps of those haplotypes. This scenario leads to phylogeographic congruence between the clades of *k*-mtDNA harboured in *A. pulchellus* and the native mtDNA clades of *A. krugi*. (b) Introgression of *k*-mtDNA into multiple populations of *A. pulchellus*, leading to persistence and possible fixation of these haplotypes in only a random subset of populations of *A. pulchellus*. (c) Introgression and possible fixation of *k*-mtDNA in a single population of *A. pulchellus* (labelled with a star), followed by range expansion (indicated by arrows) into areas previously unoccupied by *A. pulchellus*. This scenario leads to *k*-mtDNA becoming prevalent or fixed in several populations, but does not lead to phylogeographic congruence between the clades of *k*-mtDNA harboured in *A. pulchellus* and the native mtDNA clades of *A. krugi*.

A. pulchellus population drifting to fixation is 0.0112. The probability that multiple, independent events occur together is equal to the product of their individual probabilities. Therefore, the probability of fixation of *k*-mtDNA in nine populations is 0.0112^9, that is, $2.77e^{-18}$. If we only consider the four populations with sample sizes ≥ 10 individuals, this probability is $1.57e^{-8}$ (0.0112^4). Even if we assume that the initial frequencies of the *k*-mtDNA haplotypes were twenty times higher (22.5%), the probability of fixation of *k*-mtDNA in the nine *A. pulchellus* populations is $1.48e^{-6}$ (0.225^9; 0.0026 for the four populations with sample size ≥ 10 individuals). Taken together, these estimates suggest that the probability is negligible that the observed pattern of *k*-mtDNA occurrence in western *A. pulchellus* populations was caused by genetic drift.

The previous arguments against genetic drift as the mechanism accounting for *k*-mtDNA introgression into *A. pulchellus* imply that natural selection may have caused the observed pattern of mtDNA replacement, and consequently that selection possibly acted on traits affected by the mitochondrial genome. Sequence variation in mtDNA has traditionally been considered selectively neutral (Ballard & Kreitman, 1995), but increasing evidence indicates that selection can act on this genome (e.g. Bazin *et al.*, 2006; Ballard *et al.*, 2007; Meiklejohn *et al.*, 2007; Schon *et al.*, 2012). Besides negative selection against deleterious mutations in mtDNA, studies have demonstrated positive directional selection on the joint mitochondrial-nuclear genotypes (Ellison & Burton, 2006; Dowling *et al.*, 2008; Ballard & Melvin, 2010). Further, evidence indicates that in some cases, introgressed individuals can exhibit higher fitness, which can result in directional selection in favour of individuals with foreign mtDNA (Tegelström, 1987; Niki *et al.*, 1989; Alves *et al.*, 2008; Plötner *et al.*, 2008; Boratynski *et al.*, 2011). Despite the increasing evidence for mitochondrial selection, the effect of mtDNA on fitness is not clearly understood (Ballard & Melvin, 2010). Some studies suggested that sequence polymorphism among mitochondrial-nuclear genotypes corresponds to variation in metabolic performance, which may affect a wide range of life-history traits and therefore have fitness consequences (Zera & Zhao, 2003; Ballard & Melvin, 2010). Some performance differences have been reported between *A. pulchellus* and *A. krugi*, with the latter having faster acceleration and a longer jump than the former (Losos, 1990; Vanhooydonck *et al.*, 2006), but the association between these traits and mtDNA is unclear (Gray *et al.*, 2006).

Phenotype of introgressed *Anolis pulchellus*

Earlier studies suggested that mitochondrial DNA introgression often has little impact on phenotype or the nuclear genome (McGuire *et al.*, 2007; Good *et al.*, 2008; Liu *et al.*, 2010; Zhou *et al.*, 2012; Pagès *et al.*, 2013), a proposition consistent with our findings. In fact, individuals of *A. pulchellus* with *k*-mtDNA were morphologically indistinguishable from specimens harbouring their native *p*-mtDNA. What processes could reduce the effects of *k*-mtDNA introgression on the phenotype of *A. pulchellus*? Initial phenotypical signals of hybridization between *A. krugi* and *A. pulchellus* may have been diminished by continuous backcrossing between female hybrids possessing *k*-mtDNA and pure *A. pulchellus* males. Therefore, the introgressed *k*-mtDNA remained intact, whereas the nuclear signatures of *A. pulchellus* became more prevalent. The backcrossing and the gradual reduction in hybridization signals in morphological traits could have been further facilitated by asymmetrical reproductive ability (i.e. fertile females and sterile males) of F_1 hybrids resulting from crosses of *A. krugi* females with *A. pulchellus* males (*cf.* Liu *et al.*, 2010). This asymmetry would have ensured that hybrid females with *k*-mtDNA could only produce viable offspring with pure *A. pulchellus* males. Such scenario is consistent with Haldane's rule (Coyne, 1985), which states that in interspecific hybrids the heterogametic sex is more likely to be sterile. In fact, karyotypes of *A. pulchellus* and *A. krugi* are characterized by a complex sex chromosome system in which males are the heterogametic sex (X_1 X_2 Y) and females the homogametic sex (X_1 X_1 X_2 X_2; Gorman & Atkins, 1966).

Conclusion

We documented the existence of a large hybrid zone between *Anolis krugi* and *A. pulchellus* in western Puerto Rico. The interspecific matings resulted in extensive unidirectional mtDNA introgression, which led to nearly complete or complete replacement of the native mtDNA of *A. pulchellus* by the foreign *k*-mtDNA at various localities. The prevailing view that hybridization is a rare event in *Anolis* is probably accurate, but our findings suggest that genetic assessments of syntopic populations sampled across their geographic range is necessary to adequately corroborate this perspective. For example, we would not have detected hybridization and the ensuing introgression if our sampling of *A. pulchellus* had been restricted to north-central and south-eastern Puerto Rico. Finally, the possibility that *A. pulchellus* with *k*-mtDNA may have had a selective advantage suggests that hybridization may provide a 'functional novelty' that confers a fitness advantage to introgressed individuals. *Anolis krugi* and *A. pulchellus* may provide an excellent opportunity to elucidate behavioural and ecological mechanisms that may contribute to the breakdown of the species recognition system, and subsequent hybridization and introgression in closely related taxa.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Species, population number, clade, field number, voucher number, GenBank accession numbers, locality, and coordinates of the specimens used in this study.

Figure S1 Minimum number of introgression events of *Anolis krugi*'s mtDNA into the genome of *Anolis pulchellus*, estimated from the ML tree for the combined data sets of all *A. krugi* sequences and the *A. krugi*'s mtDNA sequences in *A. pulchellus*.

Figure S2 Star-contracted *Anolis krugi* mtDNA haplotypes shared between *Anolis pulchellus* and *A. krugi*.

Figure S3 Interpolated genetic distances among populations of *Anolis pulchellus* with *A. krugi* mtDNA.

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