



Molecular data exclude current hybridization between iguanas *Conolophus marthae* and *C. subcristatus* on Wolf Volcano (Galápagos Islands)

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Abstract

Natural hybridization may influence population fitness and responsiveness to natural selection, in particular in oceanic island systems. In previous studies, interspecific hybridization was detected between the Galápagos iguana species *Amblyrhynchus cristatus* and *Conolophus subcristatus*. Further, possible hybridization was also suggested to occur between *C. subcristatus* and *C. marthae* at Wolf Volcano on Isabela Island. In this work, we investigated the level of hybridization between *C. subcristatus* and *C. marthae* using a large set of microsatellite markers. Results indicated strong differentiation between species and, while we cannot rule out hybridization in the past, there is no evidence of ongoing hybridization between *C. marthae* and *C. subcristatus*. These findings have great importance for the design of management actions and conservation plans, in particular for the purposes of a head start program. However, because potential for hybridization may change under different environmental and demographic conditions, genetic characterization of newly marked individuals of *C. marthae* and *C. subcristatus* in Wolf Volcano should not be interrupted.

Keywords Hybridization · Introgression · Reproductive isolation mechanisms · Pink Iguana

Introduction

Oceanic island populations are generally characterized by geographic isolation, limited distribution and small population size. These factors are largely responsible for the formation of genetically unique endemic entities (Pruett et al. 2010; Wilson et al. 2009). Such entities often require intensive management, so that understanding their taxonomic status, ecology, demography and genetic delimitation is important to ensure successful conservation plans, preserve species and avoid extinction (Frankham 2005; Frankham et al. 2009; Armstrong and Seddon 2008).

Natural hybridization may influence population fitness and responsiveness to natural selection, particularly in oceanic islands systems (Arnold 2006; Fitzpatrick and Shaffer 2007; Kisel and Barraclough 2010), where it acts as a powerful natural evolutionary factor and promotes new evolutionary trajectories (Schwenk et al. 2008; Hedrick 2013). Paradigmatic examples are found in Galápagos Islands, where patterns of hybridization of Darwin's finches (*Geospiza* spp.) correlate with ecological conditions (Grant and Grant 2016), with hybridization acting rapidly when selection favors the persistence of hybrids, leading to the extinction of a parental species (*Camarhynchus* spp., Kleindorfer et al. 2014). However, evolution may also promote prezygotic and/or postzygotic reproductive isolation mechanisms (RIMs) that prevent hybridization. The formation of reproductive barriers can be either adaptive or a by-product of genetic differentiation between lineages (Henrich and Kalbe 2016). Additionally, hybridization may also cause genetic introgression and contamination of pure populations, endangering natural populations and species. In particular, hybridization events induced by man-mediated environmental changes and introductions of invasive species can eventually lead to biodiversity erosion and loss (Allendorf et al. 2001).

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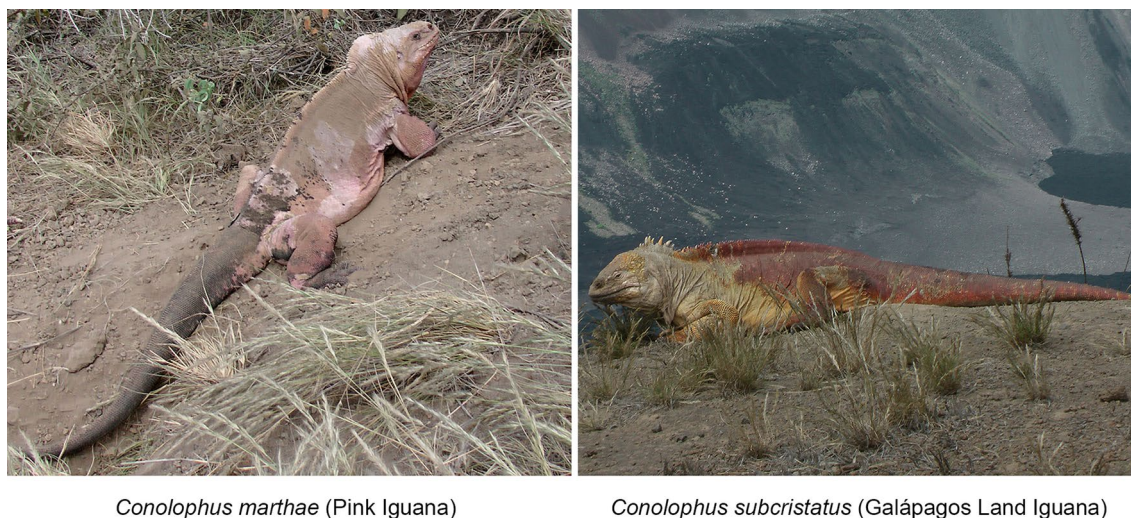
Squamate lizards seem particularly susceptible to hybridization not only between closely related species, but also between genetically divergent taxa (Jančúchová-Lásková et al. 2015). Hybridization has already been documented for iguanas, both between congeneric species such as invasive *Iguana iguana* and the critically endangered *I. delicatissima* in the Lesser Antilles (Breuil et al. 2010; van den Burg et al. 2018), and between different genera. The sister taxa *Amblyrhynchus cristatus*—the Galápagos marine iguana—and *C. subcristatus*, estimated to have diverged about 4.5 Mya (Macleod et al. 2015) occasionally hybridize at Plaza Sur Island and generate viable F1 hybrids (Rassmann et al. 1997; Di Giambattista 2016). Recently, evidence for crossbreeding between invasive *Iguana iguana* and the native rock iguana (Genus *Cyclura*) was provided on Little Cayman Island (Moss et al. 2018). The fact that diversification of island taxa mostly occurred in allopatry could be invoked to explain the weak reinforcement of reproductive barriers (Malone et al. 2000).

From a conservation perspective, interspecific hybridization deserves attention as it can ultimately result in loss of rare and important taxa. In fact, hybridization with *I. iguana* actually represents the greatest threat to *I. delicatissima* (Knapp et al. 2014). Hybridization can change genetic variation maladaptively, alter demography, increase the degree of mortality and infertility of parental species, especially when one of the taxa is more abundant than the other (Burke and Arnold 2001; Dittrich-Reed and Fitzpatrick 2013; Dubois 2006; Petit 2004; Rhymer and Simberloff 1996; Allendorf et al. 2001; Schwartz et al. 2004). This is the case of the Galápagos pink land iguana *Conolophus marthae* (Fig. 1), a recently discovered species endemic to the Galápagos archipelago (Gentile and Snell 2009). Only one small population of the species exists, and its distribution is limited to the

northwestern slopes of Wolf Volcano on Isabela Island. It occurs syntopically (sensu Rivas 1964) with a larger population of *C. subcristatus* (Fig. 1), the most abundant species of land iguanas in Galápagos, distributed in many of the central and western islands of the archipelago (Fabiani et al. 2011). The two species differ genetically and in several morphological traits. They also perform different head bob (nodding) behaviour. The only known population of pink iguana is affected by several threats that include small population size, extremely limited distribution, possible competition with *C. subcristatus*, and introduced predators. For these reasons, it was listed as “Critically endangered” in the IUCN Red List (Gentile 2012).

Given that the highly divergent sister taxa *Amblyrhynchus cristatus* and *C. subcristatus*, estimated to have diverged about 4.5 Mya (Macleod et al. 2015) occasionally hybridize, it is reasonable to hypothesize that hybridization could also occur between *C. marthae* and *C. subcristatus*, which are estimated to have had a common ancestor only 1.5 Mya (Macleod et al. 2015).

In fact, a previous study provided some evidence of hybridization and introgression between the two species. Gentile et al. (2009) investigated 57 individuals from Wolf Volcano (42 *C. subcristatus* and 15 *C. marthae*) using nine microsatellite markers and potentially identified a possible second generation hybrid. They concluded that introgressive hybridization appeared to be rare and insufficiently strong to erode genetic differentiation between the two species. However, the limited number of markers and the small sample size did not allow them to estimate the probability of assigning the putative hybrids to a correct genotypic class. The proper evaluation of the frequency of hybridization and the level of genetic introgression between *C. marthae* and *C. subcristatus* is important, not only to understand the role



Conolophus marthae (Pink Iguana)

Conolophus subcristatus (Galápagos Land Iguana)

Fig. 1 Wolf volcano (Isabela Island). Large adult males of *Conolophus marthae* (left) and *C. subcristatus* (right). Photos G. Gentile

that hybridization might play in the evolution of the two species. It also has crucial implications for future conservation plans, in particular for the purpose of a possible head start program. For these reasons, the need of research aimed at clarifying the issue is explicitly indicated as a priority action in the IUCN risk assessment for *C. marthae*.

Here, we used a set of 22 genetic microsatellite markers to investigate levels of hybridization between *C. marthae* and a large sample of the syntopic *C. subcristatus* population from Wolf Volcano on Isabela. We also used data from Tzika et al. (2008) to extend the investigation to *C. subcristatus* populations from other locations in Isabela Island. The results are discussed in the light of possible interspecific prezygotic and/or postzygotic reproductive isolation mechanisms (RIMs).

Materials and methods

Sample collection and genotyping

For this study, 108 *C. marthae* and 163 *C. subcristatus* from Wolf Volcano were sampled between different sampling seasons from 2005 to 2009, for a total of 271 individuals. Sampling sites are shown in Fig. 2.

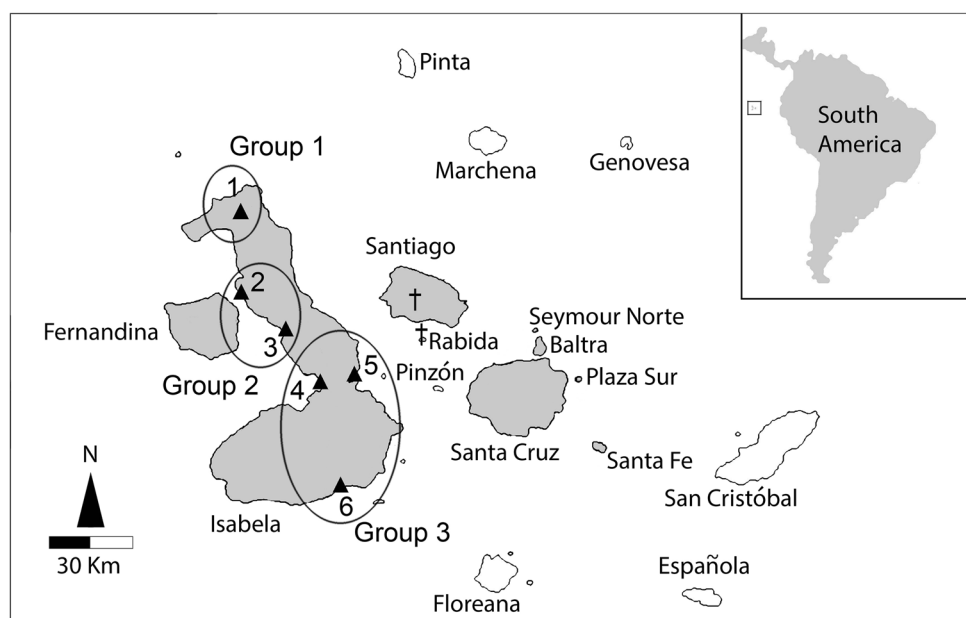
Approximately 1 mL of blood was collected from the caudal vein and preserved in 5 mL lysis buffer (100 nM Tris, 100 nM EDTA, 2% SDS). Total genomic DNA was extracted from blood using the DNeasy Tissue Kit (QUIAGEN) according to the manufacturer's protocol. DNA quality was checked by agarose gel electrophoresis.

All individuals were genotyped for 22 microsatellite markers (Steinfartz and Caccone 2006; Rosa et al. 2009), using PCR protocols described in references. PCR products were analyzed using ABI3100 and ABI 3730 sequencers (Applied biosystems).

Genetic analysis

Considering the possible negative effects on estimation of population differentiation and individual assignment caused by scoring errors due to microsatellite stuttering, large allele dropout and null alleles (Chapius and Estoup 2007; Carlsson 2008), the software MICRO-CHECKER (Van Oosterhout et al. 2004) was used to test the dataset for such inconsistencies. The software CONVERT v.1.31 (Glaubitz 2004) was used to compute allele frequencies and identify private alleles (i.e., alleles occurring at only one population) and diagnostic alleles (i.e., alleles with frequency > 10% in one species and < 10% in the other). The software GENEPOP v.4.2 (Raymond and Rousset 1995) was used to compute the inbreeding coefficient F_{IS} , as in Weir and Cockerham (1984). The software was also used to test for Linkage Disequilibrium (LD) among loci. The software GENALEX v.6.502 (Peakall and Smouse 2012) was used to estimate the fixation index between populations (F_{ST} ; Weir and Cockerham 1984), via AMOVA, as in Peakall et al. (1995). Given that F_{ST} shows dependency on within-population diversity, we also estimated F'_{ST} (Hedrick 2005), that is the F_{ST} standardized by the maximum value it can obtain, given the observed within-population diversity. To compare differentiation between *C. marthae* and *C. subcristatus* populations from other locations in Isabela Island, we pooled data

Fig. 2 Sampling locations and sample sizes (n) for *Conolophus subcristatus* in Isabela Island: (1) Wolf Volcano (n=163); (2) Caleta Tagus (n=6); (3) Bahia Urbina (n=45); (4) Bahia Elizabeth (n=13); (5) Bahia Cartago (n=108); (6) Villamil (n=2). Islands where *C. subcristatus* occurs or has occurred in historic times are in grey. Crosses indicate extinction



of *C. subcristatus* from Isabela Island used by Tzika et al. (2008) with our original data. The *C. subcristatus* individuals in the resulting dataset were divided into three groups according to latitude and increasing distance from Wolf Volcano (Fig. 2). Because Tzika and collaborators used 9 of the 22 markers here considered, we used only the same microsatellite markers to calculate F_{ST} , F'_{ST} , and D genetic distance (Nei 1972; GENALEX) between *C. marthae* and each group. We also estimated gene flow between *C. marthae* and each group as $Nm = (1 - F_{ST})/4F_{ST}$ (Wright 1969) and $Nm_{MH} = (1 - F'_{ST})/4F_{ST}$ (Meirmans and Hedrick 2011).

The rationale of this approach was derived from Grant et al. (2005) who hypothesized that: “in the absence of interbreeding, sympatric populations of two species should be no more similar to each other genetically than each one is to allopatric populations of the other. In contrast, the introgression hypothesis predicts that a species is more similar genetically to a sympatric relative than to allopatric populations of that relative, as a result of exchanging alleles”. We tested such a prediction in *Conolophus* from Isabela Island.

Hybridization analyses

To analyze the degree of hybridization between *C. subcristatus* and *C. marthae* different IBC (Individual-Based Clustering) methods were adopted. First, the dataset was analyzed using an allele frequency-based method to infer the admixture between populations (STRUCTURE v.2.3.1); then, a genotypic frequency-based method was used to infer the genotypic hybrid or parental class to whom individuals belonged (NEWHYBRIDS).

STRUCTURE v.2.3.1 (Pritchard et al. 2000; Falush et al. 2003) uses an algorithm that estimates the probability, for each individual, to be assigned to K assumed clusters. To determine the optimal K for the dataset, a method developed by Evanno et al. (2005) and implemented in the software STRUCTURE-HARVESTER (Earl and vonHoldt 2012) was used. Clustering was performed without a priori information, according to Pritchard et al. (2000), with admixture model and uncorrelated allelic frequencies. The analysis was performed through 1,000,000 MCMC repetitions and burn-in of 100,000 repetitions.

The clustering software NEWHYBRIDS (v.1.1 beta) (Anderson and Thompson 2002) was used without priori population information. For each individual, the software estimates the probability to belong to each considered genotypic class: the two parental species (Pure_0 and Pure_1), F1 hybrid (Pure_0 × Pure_1), F2 hybrid (F1 × F1), Bx_0 (F1 × Pure_0) and Bx_1 (F1 × Pure_1). The analysis was performed through 1 million MCMC repetitions and 100,000 burn-in repetitions.

Considering that linkage disequilibrium among loci and/or deviation from Hardy–Weinberg equilibrium (HWE)

may affect the clustering accuracy of both STRUCTURE and NEWHYBRIDS, a multivariate technique, the Factorial Analysis of Correspondences (FCA), was conducted as implemented in GENETIX v.4.05 (Belkhir et al. 2004). Contrary to STRUCTURE and NEWHYBRIDS, FCA does not assume HWE or linkage equilibrium.

Results

Genetic analyses

The loci MIG-E13 and MIG-E16 were monomorphic for the same allele in the two species. For this reason, they were excluded from the subsequent analyses resulting in 20 loci for analysis. MICRO-CHECKER analysis highlighted the presence of null alleles at four loci in *C. marthae* (CS8, MIG-E3, MIG-E4, MIG-E15) and six loci in *C. subcristatus* (CS2, CS8, MIG-E3, MIG-E4, MIG-E8, MIG-E14). Private alleles were found in both populations. *C. marthae* showed seven private alleles (1 for loci CS3, MIG-E6 and MIG-E10; 2 for loci MIG-E3 and MIG-E4), while *C. subcristatus* had 28 private alleles (1 for loci CS1, CS2, CS3, CS4, CS9, MIG-E4, MIG-E6, MIG-E12 and MIG-E14; 2 for CS5 and MIG-E2; 3 for CS10 and MIG-E3; 4 for MIG-E8; 5 for MIG-E10). Two markers (CS7 and MIG-E15) were diagnostic for the distinction of the two species. Statistically significant LD was found at loci CS7-CS9 and CS4-MIG-E8 for *C. marthae* and at loci CS2-CS8, MIG-E3-MIG-E4, MIG-E8-MIG-E12 and MIG-E4-MIG-E14 for *C. subcristatus*. The composite inbreeding coefficient (F_{IS}) estimated using 20 microsatellite loci was 0.009 (not statistically significant) for *C. marthae* and 0.043 ($p < 0.0025$ after Bonferroni correction) for *C. subcristatus*. F_{IS} values of individual loci are reported in Table 1.

The F_{ST} and F'_{ST} values between the two syntopic populations, computed using 20 markers, were 0.280 and 0.763 ($p < 0.01$), respectively. The F_{ST} , F'_{ST} , D , and gene flow values between *C. marthae* population and the three groups of *C. subcristatus* populations from the whole Isabela Island, obtained using 9 loci (as in Tzika et al. 2008), are reported in Table 2.

Hybridization analyses

The Evanno method indicated $K = 2$ as the best grouping and the STRUCTURE analysis clearly divided individuals according to morphotype. Each animal was assigned to either one or the other cluster, with Q values > 0.998 (Fig. 3A).

NEWHYBRIDS did not assign any sample to the hybrid genotypic classes considered. Each individual was assigned to either one or the other parental species class

Table 1 Expected (H_E) and observed (H_O) heterozygosity and F_{IS} values for the 20 loci in the two species

Locus	<i>C. marthae</i>			<i>C. subcristatus</i>		
	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}
CS1	0.648	0.667	0.029	0.804	0.799	-0.006
CS2	0.421	0.486	0.136*	0.595	0.777	0.235*
CS3	0.389	0.339	-0.149	0.367	0.402	0.089
CS4	0.204	0.190	-0.072	0.667	0.668	0.002
CS5	0.556	0.526	-0.056	0.767	0.768	0.002
CS6	0.713	0.690	-0.034	0.747	0.667	-0.121
CS7	0.729	0.751	0.029	0.840	0.860	0.024
CS8	0.204	0.273	0.254*	0.546	0.772	0.293*
CS9	0.333	0.326	-0.023	0.749	0.804	0.069
CS10	0.194	0.179	-0.089	0.779	0.825	0.056
MIG-E3	0.500	0.703	0.290*	0.564	0.771	0.268*
MIG-E11	0.439	0.434	-0.012	0.778	0.773	-0.006
MIG-E4	0.187	0.276	0.325*	0.117	0.205	0.428*
MIG-E6	0.833	0.499	-0.675*	0.877	0.593	-0.481*
MIG-E10	0.546	0.523	-0.044	0.863	0.883	0.023
MIG-E8	0.491	0.467	-0.050	0.615	0.858	0.284*
MIG-E15	0.713	0.847	0.159*	0.933	0.608	-0.536*
MIG-E2	0.414	0.488	0.152	0.879	0.919	0.043*
MIG-E12	0.729	0.652	-0.119*	0.847	0.811	-0.044
MIG-E14	0.804	0.816	0.015	0.710	0.911	0.221*
All loci	0.503	0.507	0.009	0.702	0.734	0.043*

F_{IS} negative and positive values indicate excess and defect of heterozygotes, respectively

*Statistical significance after Bonferroni correction (new $\alpha=0.0025$)

Table 2 F_{ST} , F'_{ST} , D , Nm , and Nm_{MH} values between *C. marthae* and *C. subcristatus* populations in Isabela Island

	<i>C. subcristatus</i>		
	Group 1	Group 2	Group 3
<i>C. marthae</i>			
F_{ST}	0.239*	0.301*	0.298*
F'_{ST}	0.595	0.688	0.716
D	0.569*	0.736*	0.851*
Nm	0.794	0.580	0.590
Nm_{MH}	0.423	0.259	0.239

F'_{ST} (Hedrick 2005) is the F_{ST} standardized by the maximum value it can obtain, given the observed within-population diversity. D is the genetic distance as in Nei (1972). Gene flow was estimated as $Nm=(1-F_{ST})/4F_{ST}$ (Wright 1969) and $Nm_{MH}=(1-F'_{ST})/4F_{ST}$ (Meirmans and Hedrick 2011)

* $p(\text{random} \geq \text{obs}) \ll 0.001$

(Pure_0 and Pure_1) according to morphotype, with probability > 99.9% (Fig. 3b).

The FCA clearly divided the two populations into two distinct groups that are consistent with morphotype (Fig. 4).

Discussion and conclusions

This study provided no evidence in support of current hybridization between *C. marthae* and *C. subcristatus* on Wolf Volcano. All analyses did not find evidence of hybridization events between the two species in the last two generations. The hybrid status of the individual identified as possible second generation hybrid by Gentile et al. (2009), (and morphologically assignable to *C. subcristatus*), was identified as *C. subcristatus* with this larger set of markers. This result is most likely a consequence of the larger number of markers used in the present study, which allowed a more refined analysis than in Gentile et al. (2009).

Several loci showed homozygote excess in both populations. To some extent, null alleles could be invoked to explain such a pattern, especially at those loci that showed positive F_{IS} in both species. However, the reliability of methods for null allele detection has been recently questioned, because they are often poorly consistent with each other and they seem to produce high numbers of false positives when dealing with small populations (Dąbrowski et al. 2014). Additionally, microsatellite null alleles do not alter the overall outcome of assignment testing and can be included in these types of studies (Carlsson 2008). For these reasons, given the purpose of the present work, markers showing null

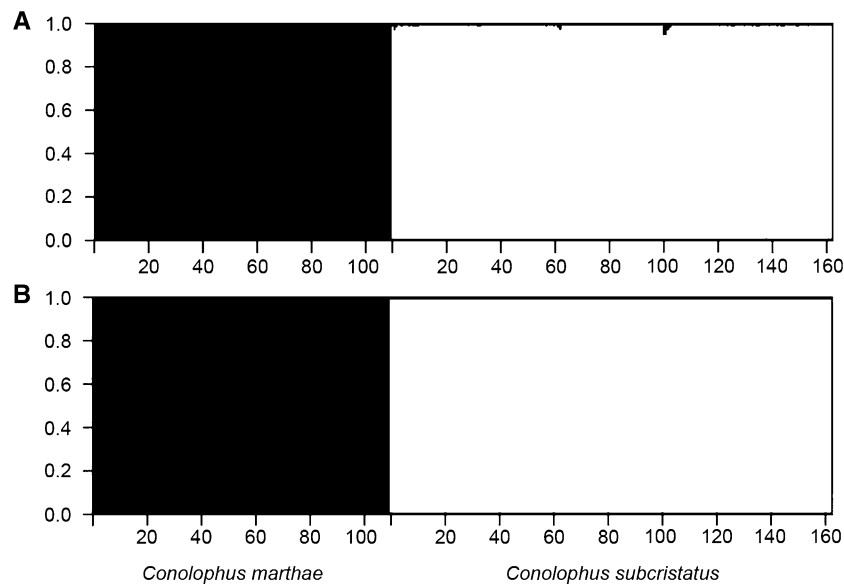


Fig. 3 **A** STRUCTURE graphical output. Each individual is represented by a vertical bar which can be divided in 2 colors, indicating the Q proportions of each cluster for each individual. In black, Q values referred to *C. marthae* individuals; in white, to *C. subcristatus*. **B** NEWHYBRIDS graphical output. Each individual is represented by a vertical bar which can be divided in 6 colors, meaning p values to

belong to each genotypic class. Black, p values to belong to *C. marthae* pure species group; white, *C. subcristatus* pure species group; red, F1 hybrid; green, F2 hybrid; brown, Bx_0; grey, Bx_1. Probability values associates to hybrid and back-cross generations are so small that they are not detectable in this graph

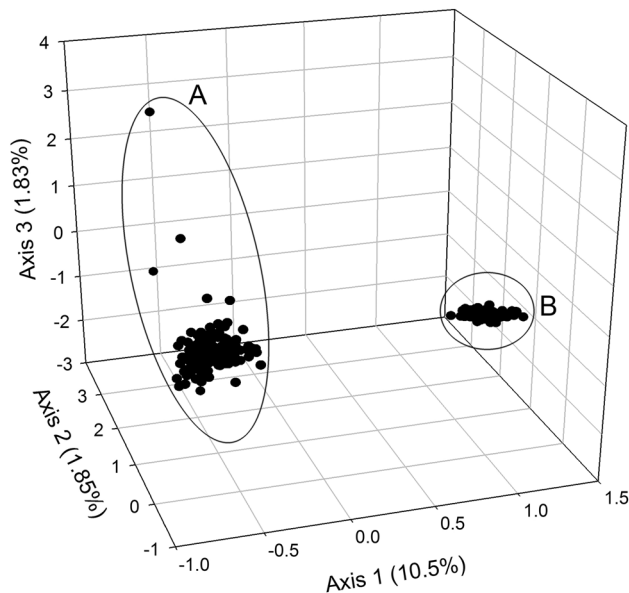


Fig. 4 Factorial analysis of correspondences of iguana populations in Wolf Volcano (Isabela Island). (A) *Conolophus subcristatus*; (B) *C. marthae*

alleles were not excluded from the analyses, and no correction of allele and genotype frequencies was attempted. Similarly, markers showing linkage disequilibrium were not excluded from the analysis as there was no correspondence of loci in LD among populations, suggesting that LD may

in part be related to null alleles and/or demographic events, rather than to chromosomal association.

Homozygote excess could also reflect inbreeding, which would be consistent with the small size of both populations, as estimated in Gentile et al. (2016).

The high number of private alleles in the two species supports the lack of interbreeding between the two groups identified by STRUCTURE. In general, when present, private alleles tended to be found at the extremes rather than in the middle size classes of the allele size distributions, as expected in case of genetic differentiation between populations (Szpiech and Rosenberg 2011). The F_{ST} and F'_{ST} values, are high enough to suggest independent differentiation between the two species. Indeed, when converted to gene flow estimates, F_{ST} and F'_{ST} statistics did not return Nm , and Nm_{MH} equal or very close to zero, as it would be expected in case of complete differentiation. This can be in part reconciled with results from STRUCTURE and NEWHYBRIDS analyses considering that the high mutation rate of microsatellite DNA can cause the occurrence of alleles of the same length in sorted lineages. Actually, non-zero gene flow estimates could also document low level of gene flow occurred in the past. In fact, the estimators of genetic differentiation (F_{ST} , F'_{ST} , and D) between *C. marthae* and *C. subcristatus* were higher in allopatric than in sympatric conditions, whereas the opposite was obviously true for gene flow estimators (Nm , and Nm_{MH}). These results would be consistent with the hypothesis that hybridization occurred in the

past, in accordance with predictions by Grant et al. (2005). As *C. marthae* has been found monomorphic for a single, specific and very divergent mtDNA haplotype (Ciambotta et al. unpublished), hybridization might have occurred only via males of *C. subcristatus*.

Reproductive isolation mechanisms

If this scenario is correct, it would point towards the existence of effective reproductive isolation mechanisms (RIMs) between *C. marthae* and *C. subcristatus*. Pre-copulatory and post-zygotic mechanisms may be invoked to explain the lack of introgression following hybridization between *Amblyrhynchus cristatus* and *C. subcristatus* at Plaza Sur Island (Rassmann et al. 1997). As regards *C. marthae* and *C. subcristatus* at Wolf Volcano, although it is not possible to completely exclude postzygotic RIMs, past hybridization may have enhanced the evolution of precopulatory RIMs by reinforcement (Servedio 2001; Hoskin et al. 2005).

Precopulatory RIMs might be due to physiological, behavioural, and ecological factors. A non-overlapping breeding season could avoid interspecific hybridization in the two syntopic species. Analyses on steroid hormones (progesterone and 17 β -estradiol) carried out on both species showed that *C. subcristatus* from Wolf Volcano concentrates breeding in the months immediately after the rainy season. Despite sexual activity has been documented for both species in the same period, the number of gravid females is much higher for *C. subcristatus* than for *C. marthae* (Gentile et al. 2016). The above may be explained in terms of lack of recruitment (which has not been observed in *C. marthae*), but it could also hide an opportunistic reproductive strategy of *C. marthae*, which might not have a specific breeding season (Onorati et al. 2016). Clearly, further data are needed to clarify the issue.

Differences in mating behavior and in sexual intraspecific communication behaviour, such as nodding, or differences in chemical signals can also avoid interspecific hybridization, preventing mating and influencing the choice of a partner (Jančuchová-Lásková et al. 2015; Gabirot et al. 2012). Analyses on the nodding of the two species (Gentile et al., in prep) highlighted strong differentiation between the head bob displays performed by *C. marthae* and *C. subcristatus*. Remarkably, the display action pattern (DAP) of *C. subcristatus* population at Wolf volcano is very simplified and different from DAPs of populations of the same species but breeding in other areas of the island. Pronounced behavioural differences in potentially hybridizing syntopic species are not uncommon (Jančuchová-Lásková et al. 2015).

Preliminary analyses of profiles of hydrocarbons extracted from the secretions of femoral pores also suggest differentiation between the two species (Gentile et al.

unpublished). This could be related to possible differentiation in chemical signals important in a reproductive context (Jančuchová-Lásková et al. 2015).

Other hypotheses for possible factors enhancing the avoidance of interspecific hybridization could regard partial habitat segregation that may lead to ecological differentiation between syntopic species (Mebert et al. 2015). Field observations indicated a differential microhabitat use by the two species, with *C. marthae* occupying more shaded areas, possibly to regulate basking, in relation to partial skin depigmentation. In fact, the pink colour is due to blood flowing in the derm of depigmented skin. Moreover, possible differences in the diet of the two species may enhance the ecological differentiation between the two groups, reducing the chances of encounters. In this regard, preliminary results of an analysis of the ratio of Carbon and Nitrogen stable isotopes obtained from nails indicated different fractionation profiles between the two species (Gentile et al., unpublished).

In conclusion, despite the potential for ancient hybridization between *C. marthae* and *C. subcristatus*, our findings are inconsistent with ongoing hybridization. Our data confirm conclusions drawn by Gentile et al. (2009) who pointed out that if hybridization occurs it is indeed rare, having little or no effect on the genetic integrity of two interacting species. Hypotheses can be made on the mechanisms that might prevent hybridization and consequent introgression. Future studies designed to test these distinct hypotheses would be valuable to both the academic and conservation communities.

These findings have great importance for the design of management actions and conservation plans, because at present hybridization between *C. marthae* and *C. subcristatus* does not represent a concern for *C. marthae*, especially for the purposes of a head start program. However, as hybridization is context dependent and potential for hybridization could change under different environmental or demographic conditions, genetic monitoring of newly captured individuals of the two species on Wolf Volcano should not be interrupted.

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Data availability Allele frequencies and genotype data are available in the Supplementary Information.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Ethical approval Animal manipulation and blood sampling were performed according to a protocol that minimized animal stress, in accordance with the European Community guidelines and with the approval of the Galápagos National Park Directorate. Samples were exported and imported under the CITES permits 101/BG and IT/IM/2015/MCE/01711.

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