An overlooked pink species of land iguana in the Galápagos

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Despite the attention given to them, the Galápagos have not yet finished offering evolutionary novelties. When Darwin visited the Galápagos, he observed both marine (*Amblyrhynchus*) and land (*Conolophus*) iguanas but did not encounter a rare pink black-striped land iguana (herein referred to as ''rosada,'' meaning ''pink'' in Spanish), which, surprisingly, remained unseen until 1986. Here, we show that substantial genetic isolation exists between the rosada and syntopic yellow forms and that the rosada is basal to extant taxonomically recognized Galápagos land iguanas. The rosada, whose present distribution is a conundrum, is a relict lineage whose origin dates back to a period when at least some of the present-day islands had not yet formed. So far, this species is the only evidence of ancient diversification along the Galápagos land iguana lineage and documents one of the oldest events of divergence ever recorded in the Galápagos. Conservation efforts are needed to prevent this form, identified by us as a good species, from extinction.

genetic isolation | molecular phylogeny | evolution | islands | lizards

Fig. 1. Galápagos Islands. The islands where land iguanas occur or have occurred in historic times are in gray. The locations of sampling sites are reported in *Materials and Methods*.

Since Darwin's pioneering work in the archipelago, the Galápagos Islands have been a major scientific resource for evolutionary biologists. This archipelago, currently consisting of about a dozen volcanic islands and more than 100 associated islets, is located on the equator, about 1,000 kilometers west of the South-American coast (Fig. 1). Given their volcanic origin, the Galápagos Islands host unique flora and fauna that have evolved over millions of years in geographic isolation, generating a variety of endemic species with unique and varied ecological, morphological, and behavioral adaptations (1, 2).

Land iguanas are among the most spectacular representative species of the Galápagos Islands. They once lived in many areas of the Galápagos archipelago (Fig. 1). Currently, many factors contribute to their threatened status (3), one of which may be incomplete taxonomy (4). Two species of Galápagos land iguanas are currently recognized: *Conolophus pallidus* and *Conolophus subcristatus,* with the former occurring only on Santa Fe, whereas *C. subcristatus* occurs on Fernandina, Isabela, Santa Cruz, Plaza Sur, Seymour Norte (a translocated population), and Baltra.

Charles Darwin visited the Galápagos in 1835. During the 5 weeks of his stay in the archipelago, he did not explore Volcan Wolf (the northernmost volcano in Isabela). Thus, although he noticed and commented on both marine and land iguanas (5), he did not encounter a distinct form of land iguana that occurs only on that volcano. Perhaps even more surprising, this form (herein referred to as "rosada,"[†] meaning "pink" in Spanish) remained unrecorded despite many other scientists having visited Volcan Wolf over the past century. Since it was accidentally seen by some Galápagos National Park rangers in 1986, this form has received no attention. The rosada form is characterized by a distinct phenotype (Fig. 2). It can be clearly distinguished from the syntopic yellow form (*C. subcristatus*) by evident idiosyncrasies in morphology and color.
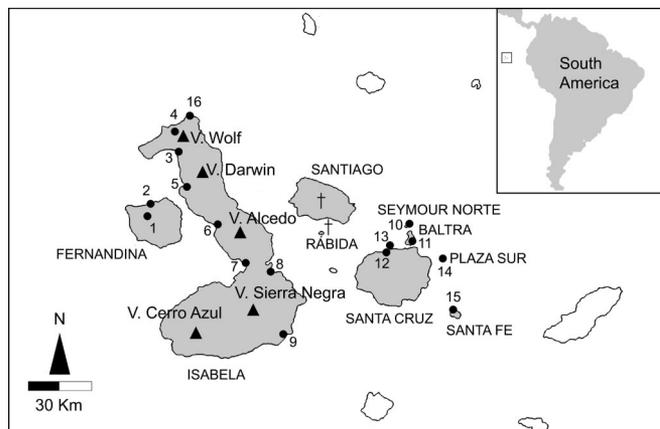
Earlier genetic studies suggest that the split of the marine and land iguana lineages could have occurred as late as 10.5 million years ago, when the archipelago did not have the current configuration and none of the present islands had yet emerged. Such studies also suggested that the present pattern of diversification of land iguanas originated recently, during the Pleistocene Epoch (6). However, no previous studies included the rosada form. Here, we address its genetic distinctiveness and taxonomic status by means of mtDNA sequencing and microsatellite genotyping.

Results

Phylogenetic Relationships and Genetic Divergence. Combined ($P_{\text{incongruence length difference (ILD) test}} = 0.17$) and separate phylogenetic analyses of the mitochondrial control region (CR) and cytochrome *b* (cyt*b*) data sets as well as parsimony, maximum likelihood (ML), and Bayesian methods of inference produced very similar results. Eight nodes (Fig. 3 *A–H*) within the

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Fig. 2. Adult male iguanas of the yellow (*A*, Sierra Negra; *D*, Volcan Wolf) and rosada (*B* and *C*, Volcan Wolf) forms (photograph by G.G.)

Fig. 3. ML phylogenetic tree. The tree is rooted at the midpoint. The branches subtending the node A and the genus *Amblyrhynchus* have been equally shortened. The asterisk denotes a terminal node. Numbers at the nodes indicate statistical support as follows (from *top*): MP, ML, Bayesian analysis. For MP and ML, only bootstrap values higher than 70 are shown. For Bayesian analysis numbers indicate posterior probability values. The number of individuals examined is reported inside each terminal triangle.

Conolophus clade were strongly supported. Strikingly, the primary split was between the rosada form and all other populations. Not surprisingly, the populations of (*i*) Santa Fe, (*ii*) the central islands (Baltra, Santa Cruz, and Plaza Sur), and (*iii*) the western islands (Fernandina and Isabela) each formed a monophyletic group. All topologies alternative to positioning rosada at the base of the *Conolophus* tree were rejected, with $P_{\text{Shimodaira-Hasegawa (S-H) test}}$ ranging from 0.004 to ≪0.001. On the other hand, the S-H test accepted the alternative hypotheses: (*i*) Santa Fe (central islands, western islands): $P = 0.444$ and (*ii*) (Santa Fe, central islands) western islands: $P = 0.434$.

On average, ML average genetic distances among populations within the central islands group and within the western islands group (excluding the rosada form) were 0.0026 ± 0.0011 (SD) and 0.0044 ± 0.0023, respectively. Higher values were observed between the 2 groups (0.0148 ± 0.0008). The ML average genetic distance between *C. pallidus* and *C. subcristatus* was 0.0177 ± 0.0006. The 15 rosada individuals were identical, and their ML average genetic distance from the rest of the land iguanas was 0.0741 ± 0.0018. The ML average genetic distance between *Amblyrhynchus* and *Conolophus* was 0.2576 ± 0.0038.

The recombination detection program (RDP3) analysis did not provide any evidence of mtDNA recombination between marine and land iguanas, including the rosada form (all tests were not significant with *P* set at 0.05 and Bonferroni correction).

Our estimate of the divergence time for the basal split between the rosada and the rest of land iguana lineages (Fig. 3, node A) was ≈5.70 million years (±1.32, SD).

Microsatellite Structure and Variation. The first STRUCTURE analysis indicated K = 2 as the most appropriate number of clusters (Fig. 4*A*). The MICRO-CHECKER analysis performed on the 2 clusters did not indicate scoring error attributable to stuttering or allele dropout. Only locus CS8 showed evidence of null alleles in the second cluster ($P < 0.001$). All rosada individuals were included in the first cluster (rosada group) with $q = >0.99$. All yellow individuals were assigned to the second

Fig. 4. STRUCTURE analyses. (*A*) ΔK values are shown for K ranging between 2 and 6. Maximum value is observed when K = 2. (*B*) Results after the first analysis indicate individuals WA7 and WA11 (yellow morphotypes) as possible hybrids. The second analysis, which was more refined, indicates WA11 as a possible second-generation hybrid (see text).

cluster (yellow group) with an average $q = 0.97$ (0.05, SD). Two yellow individuals assigned to the yellow cluster, WA7 and WA11, exhibited q values equal to 0.76 and 0.80, respectively (Fig. 4B).

The second set of STRUCTURE analyses confirmed the assignment of the individual WA7 to the yellow group (q ranging from 0.87 to 0.95, depending on ν values used), whereas the individual WA11 showed mixed ancestry with a substantial proportion of genes derived from a rosada grandparent (q ranging from 0.87 to 0.46, depending on ν values used; Fig. 4C).

Evidence for linkage disequilibrium between loci CS5 and CS9 ($P < 0.05$, after Bonferroni correction) was found only for the yellow group. This group also showed deviation from Hardy–Weinberg Equilibrium (HWE) at locus CS9 attributable to heterozygosity deficiency ($P < 0.05$). The two clusters showed $F_{ST} = 0.25$ ($P \ll 0.001$). The genetic differentiation was confirmed using the stepwise mutation model ($Rho_{ST} = 0.93$, $P \ll 0.001$), and a comparison between F_{ST} and Rho_{ST} showed a significant role for allele size in determining the level of population differentiation ($P \ll 0.001$).

The levels of variation differed between the 2 groups, with an observed heterozygosity (H_O) value of 0.67 for the yellow group and 0.38 for the rosada group. Similarly, average allelic richness was 6.29 (± 2.46) and 3.78 (± 2.22) in the groups of yellow and rosada clusters, respectively. Private alleles (i.e., alleles whose occurrence is restricted to only 1 group of individuals) constituted 74% of all alleles. Thirteen and 47 private alleles were observed in the rosada and yellow groups, respectively.

Discussion

The most surprising result was the deep divergence of the rosada lineage at the basis of the *Conolophus* clade. This species alters the current thinking about the timing of diversification of land iguanas, which was previously supposed to have occurred in the Pleistocene Epoch (6). Although with a large SD, our estimate sets the origin of this relict lineage back to a period when at least some of the present-day islands had not yet formed. In fact, the oldest extant islands in the archipelago, San Cristóbal and Española, are at least 2.35 and 3.3 million years old, respectively, if not older (7). Thus, given its present distribution, the rosada form clearly represents a conundrum because it occurs only on Volcan Wolf, which is considered younger than Volcan Sierra Negra (0.53 million years, the oldest volcano of Isabela) (8) and almost as old as Volcan Cerro Azul (0.35 million years) (9).

The ML average genetic distance between *C. subcristatus* and *C. pallidus* is much lower than between the rosada form and each of the 2 named species, supporting the distinctiveness of the taxon. Our preliminary data on the morphology of the rosada and yellow forms also indicate differentiation: in addition to their color pattern and independent of their gender, all rosada individuals investigated are distinguished from the other 2 species by flat dorsal head scales and the prominent adipose nuchal crest with small conic scales. The rosada also shows strong differences in the pattern of the "head-bob" (nodding), a behavior important in territoriality (10) and courtship (11).

The microsatellite data also indicated strong differentiation between the rosada and yellow forms, with mutation and genetic drift (in particular for the rosada form) being important determinants. A similar magnitude of microsatellite differentiation was observed among *C. subcristatus* populations from other islands (12). Although the 2 forms still share 26% of alleles, none of the rosada individuals investigated incorporated genes from the syntopic yellow iguanas, at least in the past 2 generations, and only 1 yellow individual shows possible mixed ancestry with a rosada grandparent. Thus, introgressive hybridization appears to be rare and not sufficiently strong to have prevented genetic differentiation. In any case, incomplete reproductive isolation between the rosada and syntopic yellow land forms is not surprising, considering that hybridization can still occur between marine and land iguanas (13), 2 genera morphologically, ecologically, behaviorally, and genetically very distant.

The mtDNA haplotype of the rosada is highly differentiated from those of marine iguanas and the rest of land iguanas. The results of the RDP3 analyses allow us to reject the hypothesis that such differentiation might have occurred by mtDNA recombination after hybridization between land and marine forms. The hypothesis of the origin of the rosada by recent hybridization alone between the 2 forms is not supported either. In fact, a rosada-like haplotype is not found in our sample of yellow iguanas, or in marine iguanas. This is indicated by a phylogenetic analysis that we performed by combining original haplotypes from the present study with those found by Rassmann *et al.* (6) in their sample of 150 marine iguanas from 21 locations on 14 islands [see supporting information (SI)].

In addition to the taxonomic implications, this form, which we recognize as a good species, is very important because it carries substantial evolutionary legacy, being basal to all other land iguana remnant populations. Thus far, the rosada form is the only evidence of deep diversification along the Galápagos land iguana lineage. No analogous evidence has been found in marine iguanas so far.

These findings call for a conservation program aimed at evaluating the risk of extinction of this newly recognized species, which, based on currently available data, would be assignable to the "critically endangered" category by meeting criteria B and C of the International Union for Conservation of Nature (IUCN) Red List (14).

Materials and Methods

Sampling. The reader is referred to Fig. 1 for the location of sampling sites. For mtDNA analysis, samples were collected as follows (the number of individuals sampled is in square brackets): *C. subcristatus*: (1) Roca Limba [5], (2) Cueva Norte [9], (3) Puerto Bravo [27], (4) Piedras Blanca [31], (5) Caleta Tagus [6], (6) Bahia Urbina [10], (7) Bahia Elizabeth [5], (8) Bahia Cartago [8], (9) Villamil [2], (10) Seymour Norte [3], (11) Baltra [8], (12) Cerro Dragón [4], (13) Venecia [7], and (14) Plaza Sur [12]; *C. pallidus*: (15) Santa Fe [6]; and *Amblyrhynchus cristatus*: (14) Plaza Sur [5] and (16) P.ta Albemarle [5]. For microsatellite DNA, samples were collected at Volcan Wolf at sites (3) Puerto Bravo [29] and (4) Piedras Blanca [28]. Of these, 15 individuals (11 male and 4 female) were of the rosada form.

Blood Drawing and DNA Extraction. Blood (≈ 1 mL) was drawn from the caudal or brachial vein and preserved in 5 mL of lysis buffer (100 mM Tris, 100 mM EDTA, 2% mg/mL SDS). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen).

mtDNA Amplification. We used the primer pair 12S1984-CB437LD (15) to amplify by PCR a 1,126-bp fragment of the CR positions 1370–2495 in GenBank sequence AY948121. PCR conditions were as described in ref. 14. The primers TGLU14121 (5'-CCGAAAAATCCACCTTGTTATTCAAC-3') and TTHRREV (5'-GGGGGTGGTTTAATTCCCAGC-3') were developed and used to amplify by PCR a 1,200-bp fragment that includes the whole cyt*b* gene and small fragments of the tRNAs for glutamic acid and threonine. Sequences were run on an ABI PRISM 3100 (Applied Biosystems) automated sequencer. Conditions for the PCR amplification of the cyt*b* gene are available as supplementary data. Sequences were edited with SEQUENCHER 4.1.2 (Gene Codes). The alignment obtained by using CLUSTAL X (16) was checked by eye. The final data set consists of 1118 bp of CR and 1113 bp of cyt*b*.

Phylogenetic Analyses. Phylogeny inference was performed using the CR and the cyt*b* data sets both separately and combined. We tested for phylogenetic incongruence of CR and cyt*b* by performing the ILD test (17), after removing the invariant characters (18). Analyses were conducted using maximum parsimony (MP) (19), ML (20), and Bayesian inference (21), as implemented in PAUP* 4.0b10 (22), TREEFINDER 2006 (23), and MRBAYES 3.1.2 (24), respectively. MP heuristic parameters were as follows: starting trees obtained by random-addition (10 replicates) and Tree Bisection and Reconnection (TBR) branch swapping. Gaps in the CR were coded as unordered characters at the end of the data matrix (contiguous gaps were treated as 1 single gap). ML trees

were sought via a genetic algorithm, by which method they were less prone to get trapped in local optima (25). Different models were used for the CR and the cyt*b* data. MODELTEST 3.7 (26) was used to select the HKY85+Γ (Ti/Tv rate = 2.464, α = 0.161) for the CR. The GTR+Γ model was instead used for the cyt*b*, with all parameters estimated separately for the first, second, and third positions. The same models were used in the Bayesian analysis. Gaps were recoded as binary data and considered as a separate partition to which a binary (restriction) model of evolution was applied as implemented in MR-BAYES. For each partition, parameters' values were estimated during the search. The first, second, and third positions of the cyt*b* were treated as separate partitions. Such partitioned analyses were aimed at modeling the data more accurately to reduce systematic errors that could mislead phylogenetic analyses (27, 28). For MP and ML, nodal support was tested by bootstrapping (29), with 1,000 pseudoreplicates. *A. cristatus*, the sister taxon of *Conolophus* (30), was used as an outgroup within the *Iguaninae* group. Alternative tree topologies were investigated using the S-H test (31). We estimated times of divergence using a nonparametric approach, as implemented in R8S 1.70 (ref. 32; see *SI* for details).

Tip-to-tip distances along the ML tree were averaged to calculate ML average genetic distances between populations within and between each group resulting from the phylogenetic analysis.

Recombination Analysis. We tested for possible mtDNA recombination between *Amblyrhynchus* and *Conolophus* by applying the following methods: (*i*) RDP (33), (*ii*) GENECONV (34), and (*iii*) CHIMAERA (35), as implemented in RDP3 (ref. 33; see *SI* for details).

Microsatellite Characterization. The extracted DNA was genotyped at 9 microsatellite loci as in Tzika *et al.* (12).

Microsatellite Structure and Variation. MICRO-CHECKER 2.2.3 (36) was used to check for possible typing errors, null alleles, large allele dropout, and errors attributable to stutter peaks. Population structure and individual assignment were performed using a Bayesian method implemented in STRUCTURE 2.2 (37). We first performed a STRUCTURE analysis to assess the number of groups (K). We used a model that assumed admixture and uncorrelated allele frequencies and used no prior population information. The K value that maximized the statistic ΔK (38) was chosen as the optimal K value. Based on the assignment obtained, once the K value was assessed, we reran STRUCTURE using the number of distinct clusters and the phenotype of individuals (yellow vs. rosada) as prior population information. This was aimed to infer the ancestry of all individuals that, from the previous run, could potentially have mixed ancestry. In this run, we used a model that accounted for the occurrence of null alleles (39), as the MICRO-CHECKER analysis suggested. Aware of the resolution limit of the number of loci used (40, 41), we set the parameter GENSBACK = 2 to investigate the hypothesis that each individual belongs to the alternative phenotype or has 1 parent or grandparent with the alternative phenotype. To test for sensitivity of the data to ν (in our case, the probability that an individual may be misclassified or has mixed ancestry within the past 2 generations), we conducted 3 runs by setting the parameter MIGRPRIOR = 0.1, 0.05, and 0.01, as suggested by Pritchard *et al.* (37). For all STRUCTURE analyses, membership coefficients (q) were calculated using 1.0×10^6 repetitions of a Markov chain Monte Carlo simulation, after an initial "burnin" of 5.0×10^5 repetitions. Tests for significant deviations from HWE and genotyping disequilibrium were performed as implemented in GENEPOP 3.3 (42). The level of polymorphism was measured as the mean number of alleles per locus and both H_O and expected heterozygosity, respectively, using the program GENETIX 4.05 (43). Because the observed number of alleles in a sample is dependent on sample size, allelic richness was also estimated with the program FSTAT 2.9.3 (44). Population differentiation was investigated taking into consideration both the infinite and stepwise mutation models. Assuming an infinite mutation model, the Wright's fixation index F_{ST} was assessed by the estimator θ with the program FSTAT 2.9.3. The estimator Rho_{ST} of the R_{ST} statistic, which is based on a stepwise mutation model, was calculated using the program RSTCALC (45). To determine whether stepwise-like mutations contributed to genetic differentiation, we performed a statistical test based on randomization of allele size (46). The test, implemented in the program SPAGeDi (47), can be interpreted as testing whether $F_{ST} = Rho_{ST}$. In case of tests with multiple comparisons, the sequential method of Holm (48), also known as "sequential Bonferroni," was applied as implemented in the MULTIPLICITY PROGRAM 2 (49).

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