Supporting Information

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SI Text

PCR Conditions for the Amplification of the cyt*b* Gene.
Final volume of 50 μL:
1× reaction buffer EuroTaq (EuroClone),
1.5 mM $MgCl_2$,
0.2 mM dNTP,
0.2 μM primer 1,
0.2 μM primer 2
1 U EuroTaq (EuroClone)
PCR cycle:
94 °C 2′ initial denaturation
30 cycles:
94 °C 30‘‘ denaturation
56 °C 30‘‘ annealing
72 °C 2′ elongation
Final step: 72 °C 4′

Divergence Time Estimation. We used the combined data set (CR + cyt*b* sequence data) and the software R8S 1.70 (see main text). We followed the following steps, as recommended in the manual: (*i*) We performed a phylogenetic analysis to obtain a maximum likelihood tree and 500 bootstrap pseudoreplicates. Parameters and models used are described in the main text. (*ii*) The ML tree was the starting point for the R8S analysis. We first selected the Penalized Likelihood (PL) criterion, which relaxes the stringency of the clock assumptions using an optimized smoothing procedure in the combined likelihood-nonparametric estimation of absolute times of divergence. (*iii*) To set the correct smoothing parameter, a cross-validation was conducted. (*iv*) Based on the results of the cross-validation (which indicated that a model of a molecular clock at predictive cross-validation performed as well as or better than a PL model), we selected a global clock (1) to estimate times of divergence. (*v*) Confidence intervals of divergence times were calculated by using the 500 bootstrap pseudoreplicates of the original ML tree. (*vi*) We used a single calibration point by enforcing a maximum age of 535,000 years for a monophyletic clade that included only individuals from Fernandina and Isabela (node E in Fig. 3). This is the estimated age of the emergence of the oldest volcano, Sierra Negra, in the western islands (see ref. 8 in main text).

Additional Phylogenetic Analysis of Original and Rassmann's cyt*b* Haplotypes. In this analysis, we combined our data set (141 land iguanas + 10 marine iguanas) with Rassmann's data set (see main text). Because Rassmann's data set used only the first 446 bases of the cyt*b*, we trimmed our data set accordingly. This resulted in 36 cyt*b* haplotypes. Divergence was investigated by performing a ML phylogenetic analysis. We used TREEFINDER 2006. The GTR+Γ model was used, with all parameters estimated separately for first, second, and third positions. Nodal support was tested by bootstrapping (see ref. 29 in main text). Results are shown in Fig. S1.

The average absolute number of differences (including maximum and minimum values) within and between the different species are reported in Table S1.

1. Langley CH, Fitch W (1974) An estimation of the constancy of the rate of molecular evolution. *J Mol Evol* 3:161–177.

Recombination Analysis. We tested for possible mtDNA recombination between *Amblyrhynchus* and *Conolophus* by applying the methods (*i*) RDP, (*ii*) GENECONV, and (*iii*) CHIMAERA, as implemented in RDP3 (see refs. 33-35 in the main text).

The 3 methods selected differ in the algorithm used and may perform differently. The choice of the method was primarily based on the fact that, in general, the power of the method depends on the size of the data sets. Those selected are recommended for data sets <50 sequences (see ref. 35 in the main text).

In general, the 3 methods investigate polymorphic sites in a window of a selected size (note that ‘‘window size’’ refers to the number of variable sites included in each window). Evidence of recombination is sought by screening multiple sequence alignments. This is done by examining all possible triplets. Once all detectable recombination signals have been found, the program tries to reconcile them into a minimal set of unique recombination events.

To promote accuracy, we followed the recommendations in the manual and set the following options.

RDP Method. We used the ‘‘no reference option,’’ which means that all sites are examined irrespective of whether they are phylogenetically informative or not. Although this setting provides better power of detection, it is prone to false-positive results. Because our goal was to exclude recombination, we considered this as a conservative approach.

GENECONV Method. We used the options ‘‘scan sequence triplets’’ and ‘‘do not use monomorphic sites.’’

To rule out the hypothesis that results might be affected by the choice of an inappropriate window size, we conducted separate exploratory analyses, by using different window sizes, ranging between 40 and 100. We selected $P = 0.05$ as the highest acceptable probability that sequences could share high identity in recombinant regions by chance. We used the Bonferroni correction as a multiple comparison correction, as recommended in case of data sets <100 sequences sharing >70% identity.

We used our original data set of 2.2 Kb, which included 39 haplotypes from 141 land iguanas and 5 haplotypes from 10 marine iguanas. Note that although the number of marine iguanas haplotypes was limited, it included the most divergent cyt*b* marine iguana haplotypes from Rassmann's data set. Furthermore, the analyses based on these data were very robust. In fact, the phylogenetic analysis performed by combining our data set and Rassmann's data set provided very strong evidence of the strict monophyly of marine and land iguana haplotypes, and the average number of differences between land and marine iguana haplotypes showed strong differentiation.

For consistency, the analyses were repeated by using the 36 cyt*b* short haplotypes (446 bp, our data set + Rassmann's data set). No evidence of recombination was found.

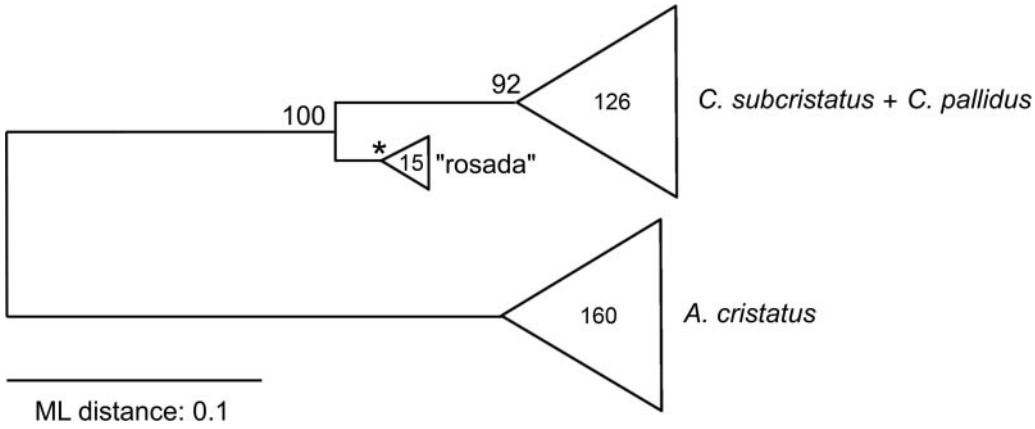

Fig. S1. ML phylogenetic tree based on 446-bp cyt*b* sequence data. The tree is rooted at the midpoint. Nodal support is indicated by bootstrap values above the nodes. The asterisk indicates a terminal node. Land and marine iguana haplotypes form separate and monophyletic groups. The number of individuals examined is reported inside each terminal triangle.

Table S1. Average number of absolute differences between cyt*b* haplotypes (446 bp) within and between land and marine iguana species

Within species			
C. subcristatus	3.32	(7,1)	
C. pallidus	—	—	
rosada form	—	—	
A. cristatus	4.14	(8,1)	
Between species			
	C. subcristatus	*C. pallidus*	rosada form
C. pallidus	6.4		
	(7,5)		
rosada form	26.8	29	
	(30,24)	(—,—)	
A. cristatus	57.4	58.8	59.9
	(62,53)	(61,56)	(62,58)

The numbers in brackets indicate the maximum and minimum values observed, respectively.

Other Supporting Information Files

SI Appendix