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Diversification in the northern neotropics: mitochondrial and nuclear DNA phylogeography of the iguana *Ctenosaura pectinata* and related species

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Abstract

While Quaternary climatic changes are considered by some to have been a major factor promoting speciation within the neotropics, others suggest that much of the neotropical species diversity originated before the Pleistocene. Using mitochondrial and nuclear sequence data, we evaluate the relative importance of Pleistocene and pre-Pleistocene events within the evolutionary history of the Mexican iguana *Ctenosaura pectinata*, and related species. Results support the existence of cryptic lineages with strong mitochondrial divergence (> 4%) among them. Some of these lineages form zones of secondary contact, with one of them hybridizing with *C. hemilopha*. Evolutionary network analyses reveal the oldest populations of *C. pectinata* to be those of the northern and southern Mexican coastal regions. Inland and mid-latitudinal coastal populations are younger in age as a consequence of a history of local extinction within these regions followed by re-colonization. Estimated divergence times suggest that *C. pectinata* originated during the Pliocene, whereas geographically distinct mitochondrial DNA lineages first started to diverge during the Pliocene, with subsequent divergence continuing through the Pleistocene. Our results highlight the influence of both Pliocene and Pleistocene events in shaping the geographical distribution of genetic variation within neotropical lowland organisms. Areas of high genetic diversity in southern Mexico were detected, this finding plus the high levels of genetic diversity within *C. pectinata*, have implications for the conservation of this threatened species.

Keywords: coalescent, demography, hybridization, Mexico, population structure, speciation

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Introduction

Research to infer past processes that have contributed to the geographical distribution of genetic variation at the intraspecific level has mainly focused on terrestrial taxa distributed within the Northern Hemisphere (Hewitt 2000). However, the geographical distribution and evolutionary origins of genetic variation within neotropical species has recently started to attract more attention (e.g. Sullivan *et al*. 1997; Edwards & Bradley 2002; García-Moreno *et al*. 2004; Zaldivar-Riverón *et al*. 2004; Hasbún *et al*. 2005; Mateos 2005; Devitt 2006; Mulcahy *et al*. 2006; Quijada-Mascareñas *et al*. 2007). Broadly, at the level of entire biotas within the lowlands of tropical America, distributions have been influenced by changes in temperature, precipitation and sea level, the interaction of these with each other, and the complex topography of the region (Toledo 1983; Pennington *et al*. 2000; Nores 2004; Pennington *et al*. 2004; Noonan & Gaucher 2005; Rull 2006). While it is generally recognized that these factors have also contributed to both speciation and genetic structuring within species, there is an ongoing debate regarding the timing of their influence in the tropics (Rull 2006). While Quaternary climatic changes are considered as a major factor leading to speciation (Noonan & Gaucher 2005; Rull 2006), tropical species are typically regarded as lineages originating before the Pleistocene (Hewitt 2000; references therein). Evidence to date suggests that speciation has occurred at different rates and times in the neotropics. Within birds molecular phylogenetic data suggest that speciation rates in the lowlands were higher during the Miocene and have decreased since, whereas in the highlands

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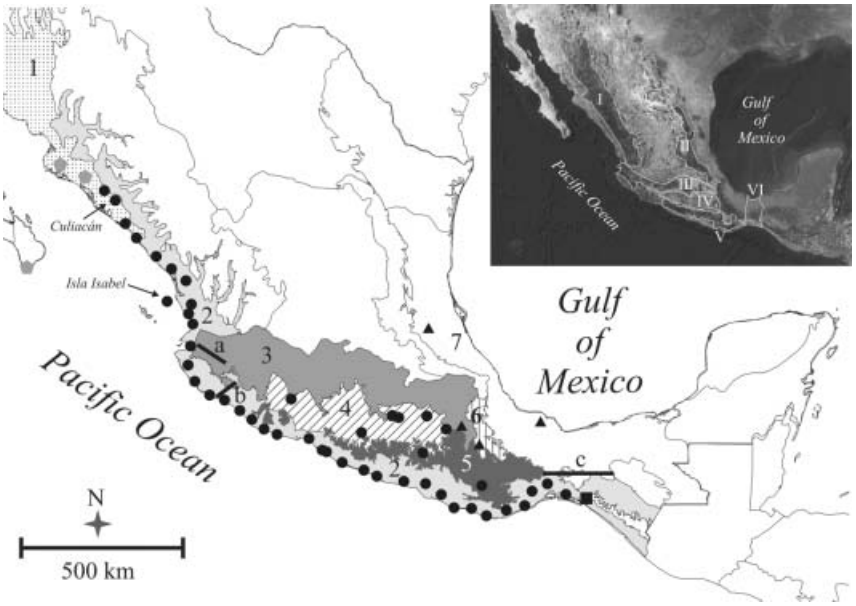

Fig. 1 Map showing collection sites of *Ctenosaura pectinata* (black circles), *C. acanthura* (black triangles), *C. hemilopha* (grey pentagons) and *C. similis* (black square); relevant biotic provinces: 1) Sonorense 2) Pacific Coast 3) Mexican Volcanic Belt (MVB) 4) Balsas Depression 5) Sierra Madre del Sur 6) Oaxaca Province and 7) Gulf of Mexico; and previously described biogeographical and phylogeographical breaks (black bars): 'a' and 'b' associated to the MVB, and 'c' in the Isthmus of Tehuantepec (see text for details); and satellite image of the study area (inset) where main geographical features are indicated: Sierra Madre Occidental (I), Sierra Madre Oriental (II), Mexican Volcanic Belt (III), Balsas Depression (IV), Sierra Madre del Sur (V) and Isthmus of Tehuantepec (VI) (Image: CONABIO 2003).

divergence events significantly increased over the last million years (Weir 2006). Within Central America molecular phylogenetic data suggests that forest plant species encompass both pre-Pleistocene and Pleistocene speciation events, while in South America species appear to be mostly pre-Pleistocene in origin (Pennington *et al*. 2004). Additionally, in contrast to North American populations, populations of western Amazonian mammals show no genetic signs of demographic expansions associated to late-Pleistocene climate changes (Lessa *et al*. 2003). With regard to genetic structuring within species and its causes, one might hypothesize that if Pleistocene events are leaving genetic signatures above the species level within the neotropics, widespread neotropical species may encompass substantial genetic differentiation and structure, with the possibility of cryptic lineages.

Although few in number, recent studies of neotropical and subtropical terrestrial species are revealing the existence of genetically and geographically distinct lineages (Riddle *et al*. 2000; Zaldivar-Riverón *et al*. 2004; Devitt 2006; Mulcahy *et al*. 2006). This provides some support for the hypothesis of cryptic lineages, specifically within taxa distributed across the lowlands of the tropics. The generality of this phenomenon can only be assessed by the study of additional lowland organisms and in different areas of the tropics. Additionally, the accuracy of delimiting regionally distinct genetic associations, combined with inferring the processes contributing to their formation, can only come from detailed sampling across the range of a species with appropriate genetic markers. While environmental perturbations of the Pleistocene would seem to be a likely driver of intraspecific divergence, earlier climatic and geological events may also have played a role. Thus, to differentiate between Pleistocence and pre-Pleistocene events, genetic markers should also ideally offer the possibility of temporal calibration.

The black spiny-tailed iguana (*Ctenosaura pectinata*) is distributed across 2000 km of lowlands, mainly covered by seasonally dry tropical forest (SDTF), in western and central Mexico and the northernmost tropics in America (Fig. 1). Herpetologists do not recognize subspecies within *C. pectinata* (Uetz & Hallermann 1995; Köhler 2002; Hollingsworth 2004), with the most recent morphological review regarding this species as a continuously distributed unit (Köhler 1996). However there is substantial colour pattern variation across the range of *C. pectinata* (Eugenia Zarza personal observation), and phylogeographical studies of other species exhibiting a similar distribution to *C. pectinata* (Zaldivar-Riverón *et al*. 2004; Devitt 2006), or broadly distributed across other zones of Mexico (Riddle *et al*. 2000; Morse & Farrell 2005; Mulcahy *et al*. 2006) have found deeply separated phylogroups. More specifically, palynological information suggests that the SDTF community has undergone a complex dynamic associated with Pleistocene climatic fluctuations. Fragmentation and subsequent range expansion of forest fragments have been hypothesized to have occurred in different areas within the continental distribution of the SDTF (Toledo 1983; Graham & Dilcher 1995; Pennington *et al*. 2004), and *C. pectinata* is thus expected to have been affected by these. However, there is evidence for tropical lowland biotas containing much older lineages that diverged prior to the Pleistocene (Pennington *et al*. 2004; Weir 2006). This suggests that earlier geological and climatic events within the region may have also contributed to deeper phylogeographical breaks within *C. pectinata.*

The distribution of *C. pectinata* is particularly interesting because it covers several recognized biogeographical

provinces: Sonorense, Pacific Coast, Balsas Depression and Sierra Madre del Sur (CONABIO 1997; Morrone & Márquez 2001) (Fig. 1). These provinces are seen as natural entities and are defined by concordant distribution patterns of species in association with geographical features. *Ctenosaura pectinata* may exhibit population structure influenced by the geographical boundaries of these provinces, or the differing environments within them, or a combination of these. Studies of taxa distributed within the Mexican western lowlands, have detected deep splits between mitochondrial DNA (mtDNA) phylogroups north and south of the Mexican Volcanic Belt (MVB) (Fig. 1, 'a', Mateos 2005; 'b', Devitt 2006). Given that this geological feature spans the Pacific Coast province (Fig. 1), it brings into question the continuity and homogeneity that biogeographical classifications attribute to this province (Espinosa-Organista *et al*. 2000; Morrone & Márquez 2001).

To date, most vertebrate phylogeographical studies focusing on Mexico have employed only mtDNA markers to test or generate hypotheses (Sullivan *et al*. 1997; Riddle *et al*. 2000; Edwards & Bradley 2002; Hasbún *et al*. 2005; Mateos 2005; Devitt 2006; Mulcahy *et al*. 2006). Combining mtDNA and nuclear DNA (nDNA) sequence data offers the opportunity for a more integral view of the evolution of a species, both in space and time (Moore 1995; Hoelzer 1997; Lyons-Weiler & Milinkovitch 1997; Creer *et al*. 2003; Leache & McGuire 2006). Here we use both mtDNA and nDNA sequence data for a detailed phylogeographical analysis of *C. pectinata*, employing fossil calibration times to estimate divergence times. Specific questions we address are: (i) did the *C. pectinata* evolutionary lineage originate during or before the Pleistocene? (ii) does *C. pectinata* contain cryptic and geographically distinct genetic lineages, as would be expected if early Pleistocene climatic changes have been a major driver of speciation in the neotropical lowlands? (iii) does *C. pectinata* show recent demographic signatures of fragmentation and/or range expansion as would be expected if Pleistocene climatic changes have been affecting the SDTF? (iv) does *C. pectinata* exhibit geographical structuring of genetic diversity congruent with other codistributed organisms, specifically the disjunction associated to the MVB?

Materials and methods

Sampling

During 2004 and 2005, 10 individuals were collected from sites separated by approximately 300 km intervals within the range of *Ctenosaura pectinata*, and 2–5 individuals were sampled at two intermediate localities between these. Geographic coordinates of sampling sites were recorded with a global positioning system. Animals were caught using tomahawk traps, noosing or by hand. A blood sample of 0.150 mL was extracted from the ventral coccygeal vein and blood was preserved in 1 mL of buffer (EDTA/SDS) or in 1.850 mL of 100% ethanol. When blood was difficult to obtain, a small piece of tissue from the tail was taken and preserved in 96% or 100% ethanol. Tissue was also taken from dead iguanas found on roads. Iguanas were released in the same place they were captured. We also obtained samples of species known to be closely related to *C. pectinata* (Hasbún 2001): *C. acanthura*, *C. hemilopha* and *C. similis*. One individual of *Iguana iguana* was sampled to be used as the out-group.

Laboratory procedures

DNA was extracted with DNeasy tissue and Blood (QIAGEN) extraction kits or by following a modified salt precipitation protocol (Sunnucks & Hales 1996; Aljanabi & Martínez 1997). An initial pilot study of single copy nDNA genes was undertaken to assess levels of variation within *C. pectinata.* Sequences of the genes GapD, β-fibrinogen (Friesen *et al*. 1997; Dolman & Phillips 2004) and α-enolase (Friesen *et al*. 1997) were obtained from four individuals collected in four geographically distant localities. Among these genes, intron VIII of the α-enolase gene displayed suitable variation for phylogeographical analysis.

This intron was polymerase chain reaction (PCR) amplified using combinations of primers EnolL731, EnolH912 (Friesen *et al*. 1997), H and L (Table 1). A 25 μL PCR reaction was performed which included a final concentration of 10× NH_4 buffer, 3 mM MgCl, 0.3 mM of each dNTP, 0.25 μM each primer, plus 0.5 units of BIOTAQ DNA Polymerase and approximately 50 ng of DNA. Temperature and time cycles for PCR amplifications are shown in Table 2. Sequence reactions were performed using primers H and L.

A fragment of the Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 (ND4) gene was used for an mtDNA phylogeographical analysis. The target fragment was amplified with primers ND4, ND4Rev (Arévalo *et al*. 1994), ND4F1 (modified from Arévalo *et al*. 1994) and ND4R623 (Hasbún *et al*. 2005) (Table 1). The 25 μL PCR mix included reagents in the same concentrations as those specified above for α-enolase amplification. Temperatures and times for PCR amplification are shown in Table 2. The above-mentioned primers and, when necessary, two internal primers Fwd402ND4seq and Rev228ND4seq were used for sequencing. Single strand sequencing reaction mix for both nDNA and mtDNA fragments contained 6.35 μL of H_2O, 1.5 μL of sequencing buffer, 0.15 μL of forward or reverse primer at 3.5 μM, 1 μL of BIGDYE TERMINATOR version 3.1 and 1 μL of PCR product totalling a 10 μL final volume. Sequence cycles were as indicated in Table 2. PCR and sequence reactions for both the nDNA and mtDNA regions were performed in a DNA Engine Tetrad 2, Peltier thermal cycler and sequences obtained using an ABI 3700 capillary sequencer according to manufacturer instructions.

Table 1 Primers sequences (5′–3′) employed in this study

Locus	Primer name	Primer sequence (5′–3′)
α-enolase	EnolH912 (Friesen *et al*. 1997)	CCAGGCACCCCAGTCTACCTGGTCAAA
	EnolL731 (Friesen *et al*. 1997)	TGGACTTCAAATCCCCCGATGATCCCAGC
	H	GTCTACCTGGTCAAAAGGATCC
	L	ATCCCAGCCGCTACATCTCTGC
ND4	ND4 (Arévalo *et al*. 1994)	CACCTATGACTACCAAAAGCTCATGTAGAAGC
	ND4Rev (Arévalo *et al*. 1994)	TATTAGGAGATGTTCTCG
	ND4F1(modified from Arévalo *et al*. 1994)	CACCTATGACTACCAAAAGC
	ND4R623 (Hasbún *et al*. 2005)	ATGTGAAGAGCTATGATTAGATGTTCTC
	Fwd402ND4seq	TAACCAACATAGCACTCCC
	Rev228ND4seq	GGTGTTTGGATTAGGC

Table 2 Temperature (Temp.) and time conditions for PCR amplification and sequencing of the two loci employed in this study. 'Number of cycles' refers to denaturing-annealing-extension cycles

	α-enolase		ND4		Sequencing	
Step	Temp.	Time	Temp.	Time	Temp.	Time
Initial incubation	94 °C	5 min	94 °C	5 min	96 °C	60 s
Denaturing	94 °C	30 s	94 °C	30 s	90 °C	10 s
Annealing	64 °C	30 s	52 °C	40 s	50 °C	5 s
Extension	72 °C	30 s	72 °C	60 s	60 °C	4 min
Final extension	72 °C	5 min	72 °C	5 min	—	—
Number of cycles	40		40		25	

Phylogenetic and phylogeographical analyses

All sequences were aligned by eye using BioEdit Sequence Alignment Editor 7.01 (Hall 1999). α-Enolase haplotypes of heterozygous individuals were inferred using PHASE version 2.1 (Stephens *et al*. 2001; Stephens & Scheet 2005). Different methods to detect recombination in this nDNA fragment were applied: RDP method (Martin & Rybicki 2000) Bootscanning (Salminen *et al*. 1995), GENECONV (Padidam *et al*. 1999), Maximum Chi-Square (Smith 1992; Posada & Crandall 2001a), Chimaera (Posada & Crandall 2001a) and Sister Scanning (Gibbs *et al*. 2000), which have been implemented in RDP2 (Martin & Rybicki 2000), as well as the Φ-test (Bruen *et al*. 2006), implemented in SPLITSTREE version 4 (Huson & Bryant 2006).

Evolutionary tree construction

Neighbour-joining trees were generated in PAUP* version 4.0 b10 (Swofford 2002) using the model of nucleotide substitution that best fits the data, determined with MODELTEST version 3.7 (Posada & Crandall 1998). The same model was employed to calculate pairwise genetic distances between mtDNA haplotypes. Maximum-parsimony tree construction was also performed with PAUP*. One hundred replicates of a heuristic search were performed with an initial random stepwise addition of sequences and tree-bisection–reconnection branch swapping. Branch support was estimated from 10 000 replicates of a bootstrap search. Additionally, Bayesian analyses were performed with the program MRBAYES version 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The settings were two simultaneous runs of the Markov chain Monte Carlo (MCMC) for three million generations, sampling every 100 generations, a heating parameter value of 0.30 and a 'burn-in' of 25%. A model of nucleotide evolution was selected with MODELTEST. Summaries of 35% (10 500 samples) of the sampled parameter values and sampled trees were obtained, as well as a majority-rule consensus tree.

Haplotype network construction

Although evolutionary gene trees may be informative at the intraspecific level, relationships resulting from intrinsic processes of population dynamics (e.g. persistence of ancestral haplotypes, multifurcations, recombination and horizontal transfer) are better visualized in reticulated graphs or networks (Posada & Crandall 2001b; Cassens *et al*. 2005). Additionally, phylogenetic networks allow one to represent equally parsimonious hypotheses of sequence relationships (Cassens *et al*. 2003), as well as the examination of spatial and temporal patterns of genetic variation (Templeton 1998). Gene genealogies were inferred using two approaches for haplotype network construction. Median-joining networks (Bandelt *et al*. 1999) were calculated with the program NETWORK version 4.2.0.1 (www.fluxus-engineering.com) keeping the parameter ε = 0. This method starts with minimum spanning trees combined within a single network and then, to reduce tree length, median vectors (consensus sequences) are added. Such vectors can be interpreted as possibly extant unsampled sequences or extinct ancestral sequences (Bandelt *et al*. 1999). In addition, TCS version 1.21 (Clement *et al*. 2000) was

employed to infer haplotype networks using statistical parsimony (Templeton *et al*. 1992) with a confidence limit of 95%.

Nested-clade phylogeographical analysis

The nested-clade phylogeographical analysis (NCPA) discriminates between random and nonrandom associations of haplotypes with geography (Templeton *et al*. 1995). Although widely used in phylogeography, criticisms against this method have recently arisen (Petit 2008). A high frequency of false positives has been detected when analysing simulated populations with NCPA (Knowles & Maddison 2002; Panchal & Beaumont 2007). However, it has not been clarified whether false positives were originated by the simulation procedure or by the application of the NCPA (Templeton 2008). Thus, the results obtained with this analysis should be cautiously taken. Complementary analysis (e.g. mismatch distribution) can be employed to further support conclusions derived from NCPA (Garrick *et al*. 2008)

Median-joining networks were employed as the template for an NCPA. Loops found in the networks were not broken in order to maintain all information provided by the reticulated relationships. Clades were nested according to the rules for ambiguous and unambiguous relationships (Templeton *et al*. 1987; Templeton & Sing 1993). Permutational contingency and geographical distance analyses were executed in GEODIS version 2.4 (Posada *et al*. 2000) to reject the null hypothesis of no association between haplotype variation and geography (Templeton *et al*. 1995).

Mismatch distribution and neutrality tests

For both sequence data sets the Tajima (1989) and Fu & Li (1993) tests were performed in order to detect any possible deviation from neutrality which could be either an effect of natural selection or the result of a past demographic expansion. To explore the latter possibility, distributions of the number of observed pairwise differences between haplotypes were obtained. In addition, mismatch distributions were simulated under the sudden-demographic expansion and the spatial-demographic expansion models. Statistically significant differences between observed and simulated distributions were evaluated with the sum of square deviations (SSD) and the Harpending's raggedness index (hg) to reject the hypothesis of demographic expansion. All tests were performed in ARLEQUIN version 3.0b (Excoffier *et al*. 2005).

Estimation of divergence times

Molecular mutation rates have previously been proposed for reptiles. However, these rate estimates either do not apply to mtDNA regions, or if they do, they have been calibrated based on assumptions regarding the origin or colonization of islands or vicariance events (e.g. Rassmann 1997; Zamudio & Greene 1997), as opposed to fossil data. There are both advantages and limitations to using palaeontological information as external calibration information for molecular clocks (Benton & Ayala 2003; Reisz & Muller 2004). Advantageously, it is possible to estimate absolute divergence times when assigning the age of a fossil to a node in a molecular phylogeny which would be considered as a minimal age for that lineage (Marshall 1990; Magallón & Sanderson 2001; Near *et al*. 2005). However, due to the imperfect nature of fossil records, divergence times obtained from palaeontologically calibrated phylogenies should be regarded as underestimates (Benton & Ayala 2003). Other errors associated with palaeontological information may also exist, such as the incorrect placement of a fossil in a phylogeny, or incorrect fossil dating (Near *et al*. 2005). However, the incorporation of more than one palaeontological calibration point can generate more reliable divergence estimates because time ranges are taken into account instead of single dates (Reisz & Muller 2004).

We have taken a Bayesian approach to estimate an ND4 substitution rate for the subfamily Iguaninae using fossil data as external calibration points. This was not possible for α-enolase sequences due to an absence of sequence data for representatives of this subfamily. The analysis was executed in the program BEAST version 1.4.2 (Drummond & Rambaut 2007) which implements an MCMC framework for the inference of time-measured phylogenies using an uncorrelated relaxed molecular-clock model (Drummond *et al*. 2006). Substitution model parameter values were selected according to the results of MODELTEST version 3.7 (Posada & Crandall 1998). Upper and lower bounds around these parameters were defined as 120% and 80%, respectively, of the original value (Emerson 2007). Mutation rate was not fixed and an uncorrelated lognormal relaxed molecular-clock model was selected. The analysis started with a UPGMA tree and considered the Yule process tree prior. Input files were generated with BEAUTI version 1.4.2 (Rambaut & Drummond 2007a). Two runs of one million generations each were executed, sampling every 100 generations and a burn-in of 10% of samples. Results of the two runs were displayed and combined in TRACER version 1.3 (Rambaut & Drummond 2005) to check for stationarity. From this analysis, a mean age estimate, and a 95% credibility interval around it, for the *C. hemilopha*, *C. pectinata* and *C. acanthura* clade were calculated. An estimate of the mean mutation rate and a 95% credibility interval within Iguaninae were also obtained. These parameters and their credibility intervals were then used as prior information for a subsequent analysis to estimate divergence times within *C. pectinata*. In this second analysis, a normal distribution for the root height and mean rate priors were assumed. Mean and

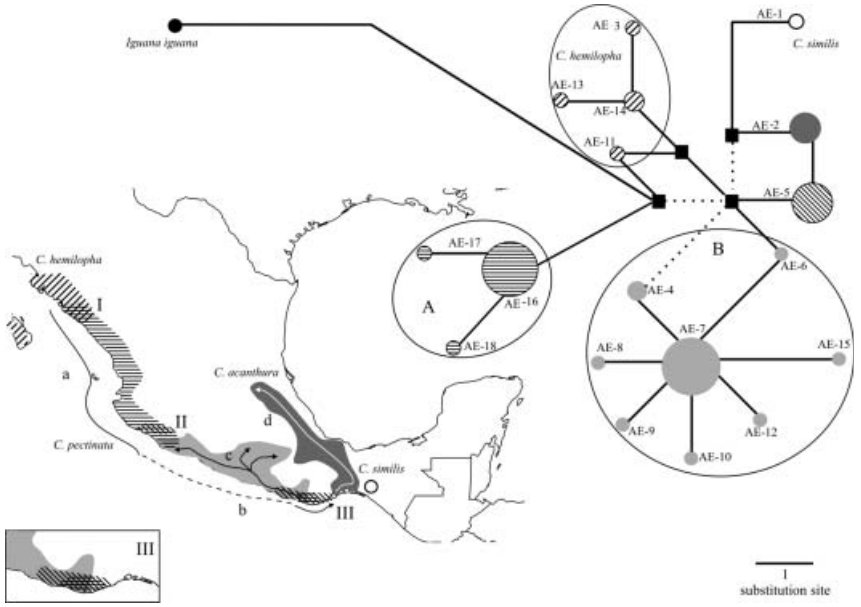

Fig. 2 Median-joining network, geographical distribution and inferred demographic patterns of α-enolase haplotypes (AE). Circles represent haplotypes and their area is proportional to the number of sampling sites where a haplotype was found. Circle shading and hatching corresponds to geography as indicated on the map. Line length in the network is proportional to genetic distance. Dotted lines represent connections not found in the parsimony network. Black squares are median vectors (interpreted as missing haplotypes). A and B indicate genetic groupings. *Ctenosaura hemilopha* and *C. similis* haplotypes are also indicated. Demographic patterns inferred from the median-joining network are shown with arrows on the map: (a) the oldest haplotypes in the network, as inferred by the root, indicate a northern origin (b) southern colonization by haplotypes AE-16 and AE-5 followed by extinction (dashed line) between the southern and northern extremes; (c) colonization and expansion from south population into central Mexico and along the Pacific coast; (d) colonization of Gulf coast by AE-2 haplotype derived from south populations. Roman numerals on the map indicate areas of secondary contact. Inset highlights the area where AE-5, AE-7 and AE-16 co-occur.

standard deviation values were set following the results obtained in the first analysis. Two runs consisting of two million generations each and sampling every 200 generations were performed. Burn-in was set to 10% of the samples. Results were combined and stationarity was evaluated. Tree parameters were combined in LOGCOMBINER version 1.4.2 (Rambaut & Drummond 2007b) and a consensus tree was calculated with TREEANNOTATOR version 1.4.2 (Rambaut & Drummond 2007c).

Results

Sampling localities can be seen in Fig. 1. Overall, 239 individuals of *Ctenosaura pectinata*, 16 *C. acanthura*, 15 *C. hemilopha*, three *C. similis* and one *I. iguana* were collected. All individuals were sequenced for the mtDNA fragment. A subsample of 83 individuals was sequenced for the nDNA gene fragment. This subset included the five aforementioned species and is geographically representative.

Nuclear DNA data

Within the 231 bp fragments of the α-enolase gene, 26 sites were variable, of which 12 sites are parsimony informative. Sequences are available from GenBank under the accession numbers EU250310–EU250328 and EU445366–EU445367. Recombination was not detected by any of the tests applied to the 19 different haplotypes identified among the *C. pectinata, C. acanthura, C. hemilopha, C. similis* and *I. iguana* samples.

The low genetic variation within the α-enolase sequence data set, both within *C. pectinata* and between this taxon and the other *Ctenosaura* species is clearly inappropriate for the estimation of a phylogenetic tree. Relationships were therefore inferred with methods for phylogenetic network construction (Posada & Crandall 2001b; Cassens *et al*. 2005). Both network estimation methods found essentially the same associations between α-enolase (AE) haplotypes. No reticulations were found among haplotypes in the parsimony network, however, in the median-joining network several ambiguous connections were detected (Fig. 2). Both *C. hemilopha* and *C. similis* are distinct both geographically and genetically. Within *C. pectinata* four α-enolase haplotypes, or genealogical groups of haplotypes, are geographically distinct: (i) group A comprises three haplotypes, with the most geographically widespread of these, AE-16, being disjunctly distributed in both coastal northwest and southwest Mexico; (ii) similar to haplotype AE-16, haplotype AE-5 is also disjunctly distributed in both coastal northwest and

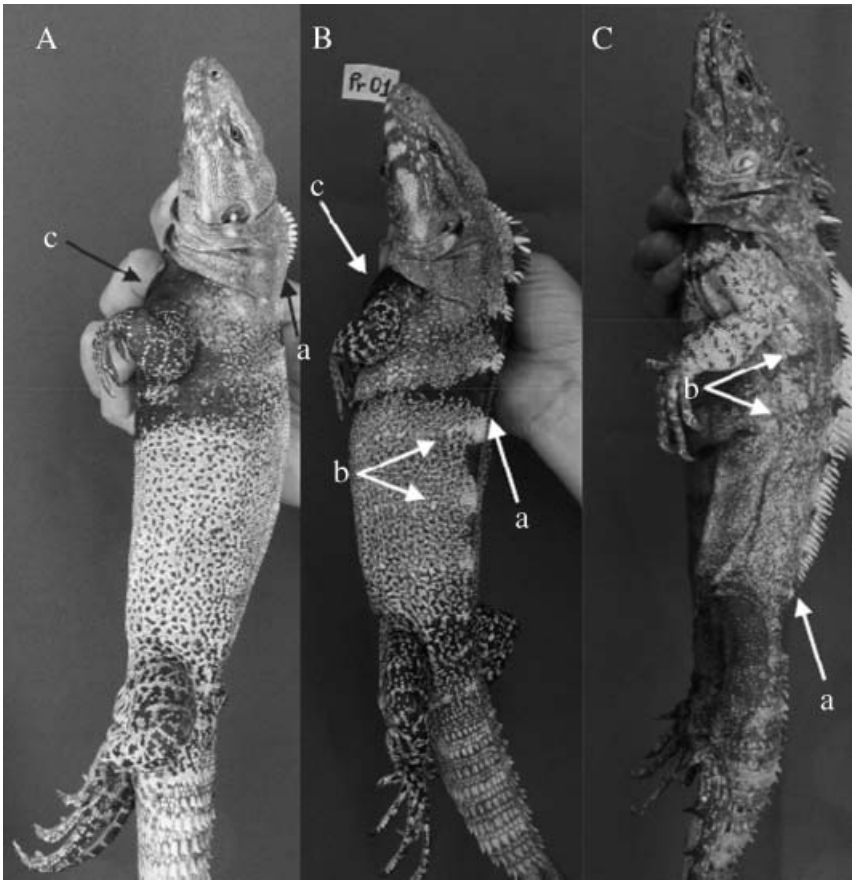

Fig. 3 Photographs of (A) *Ctenosaura hemilopha* (B) *C. hemilopha* × *C. pectinata* hybrid, and (C) *C. pectinata* male individuals, highlighting the intermediate morphological features of the hybrid. Note the enlarged but not extended dorsal crest (a) in the hybrid; the spotted colouration pattern exhibiting clear bands around the body (b); and the black colouration on the gular and pectoral region (c) in comparison to the pure individuals.

southwest Mexico; (iii) group B comprises eight haplotypes distributed coastally and further inland between the disjunct distribution of haplotypes AE-16 and AE-5; (iv) haplotype AE-2, while closely related to AE-5, is uniquely distributed along the Gulf of Mexico and the Isthmus of Tehuantepec.

One of the alternative connections between *C. pectinata* haplotype group A and the other *C. pectinata* haplotypes suggest a connection through *C. hemilopha* haplotypes (Fig. 2). Although this is not the only possible connection, as suggested by the reticulated pattern in the network, this pattern is consistent with incomplete lineage sorting between *C. pectinata* haplotype group A, *C. hemilopha* and the other *C. pectinata* haplotypes.

The median-joining algorithm that we employed to construct the network has the advantage of producing all compatible connections among possible networks and performs well when haplotypes in the data set are relatively distantly related to each other (Cassens *et al*. 2005). The distantly related *I. iguana* haplotype is unambiguously placed in the median-joining network and is most closely related to haplotypes AE-11 and AE-16 (Fig. 2). The same connection was found in the TCS network when lowering the parsimony connection limit to 88%. The topology of the network was not altered when the algorithms were run excluding *I.* iguana which reflects that, although relatively distant, the out-group does not alter relationships within the in-group. This ancestral inference for haplotypes AE-11 and AE-16 argues for a northern ancestral origin for *C. pectinata*. The haplotype AE-11 uniquely occurs in the north, and AE-16 is the most frequent haplotype in the north, with two descendant haplotypes, AE-17 and AE-18 unique to the north. Haplotypes from group B, distributed in central Mexico, form a star-like association and show significant deviation from neutrality according to the Tajima's D ($P = 0.01$) and Fu's F_S tests ($P = 0.00$), which suggests a possible past expansion event. The mismatch distribution of these haplotypes did not differ significantly from the sudden-expansion model ($P_{SDD} = 0.83$, $P_{hg} = 0.81$) nor from the spatial-expansion model ($P_{SDD} = 0.86$, $P_{hg} = 0.82$).

Among the six geographical haplotype groups, three probable contact zones can be identified. Evidence of hybridization between *C. hemilopha* and *C. pectinata* was detected in two individuals collected in northwestern Mexico (Fig. 2I). Both individuals exhibited morphological features intermediate between the two taxa (Fig. 3; see online Supplementary Material Fig. S1 for colour version). One of these individuals was heterozygous, possessing haplotypes typical of both species (AE-11 and AE-16). The other individual was homozygous for AE-16 (*C. pectinata* haplotype) but has a *C. hemilopha* mtDNA haplotype (Fig. 4,

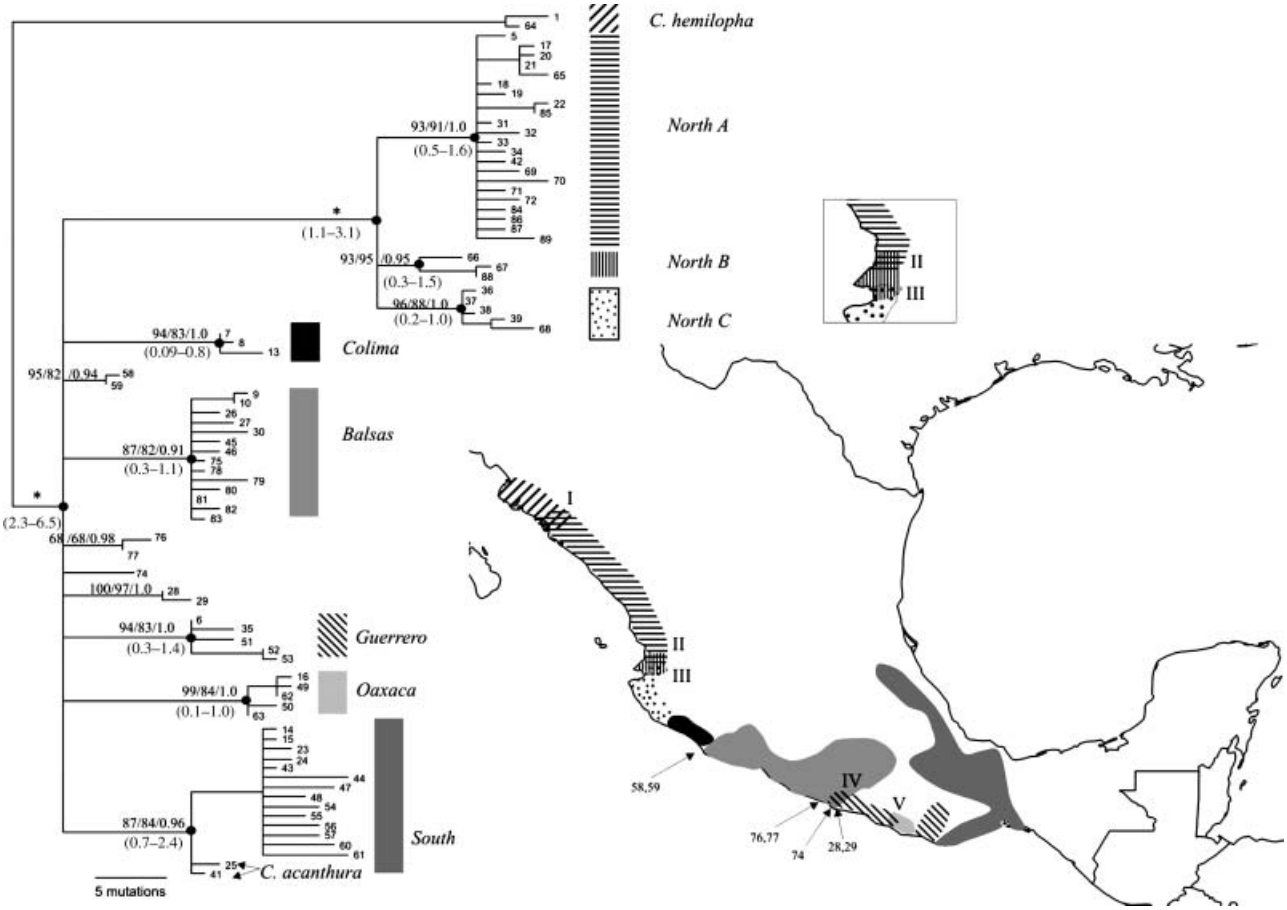

Fig. 4 ND 4 majority-consensus tree of 100 000 equally parsimonious trees (237 steps), associations with less than 50% parsimony bootstrap support were collapsed. Neighbourjoining and parsimony bootstrap values and posterior probabilities are shown. Asterisk denotes 100% bootstrap support and 1.0 posterior probability. Black circles represent nodes for which diversification times were estimated with the relaxed molecular-clock analysis. Their corresponding 95% confidence age intervals (in Ma) are shown as the respective nearest number in brackets. Branch lengths are proportional to the number of mutations between nodes or between nodes and tip haplotypes. Distribution of mtDNA clades is shown in map. Roman numerals indicate potential areas of secondary contact. Inset shows contact zones II and III. Hybrids of *C. hemilopha* and *C. pectinata* have haplotype 64 (ND4-64 in text), which is closely associated to *C. hemilopha*. Haplotypes 25 and 41 are *C. acanthura* haplotypes.

ND4-64). Group A and haplotype 5 are narrowly sympatric, with some individuals within this zone of sympatry being heterozygous (Fig. 2.II). In southeastern Mexico a region of overlap occurs for haplotype groups A and B, and haplotype AE-5, where heterozygous individuals of groups A and B, B and haplotype AE-5, and A and haplotype AE-5 were detected (Fig. 2.III). Interestingly, individuals identified as *C. acanthura* possess two haplotypes that are shared with *C. pectinata*: AE-7, AE-2, and one exclusive haplotype AE-12.

Mitochondrial DNA data

A 561 bp long fragment of the ND4 mtDNA gene was sequenced, yielding 128 variable sites of which 102 are parsimony-informative. Seventy-eight different haplotypes were identified within *C. pectinata*, two within *C. acanthura*, five within *C. hemilopha* and one for *C. similis*. Sequences are available from GenBank under the accession numbers EU246694-EU246780. Exploratory maximum-parsimony, neighbour-joining and Bayesian analyses revealed a close relationship among *C. pectinata* and *C. acanthura* haplotypes. *Ctenosaura hemilopha* haplotypes were most closely related to a monophyletic group of sequences belonging to *C. pectinata* and *C. acanthura*, with those of *C. similis* and *I. iguana* being distantly related to these three taxa. Thus, for subsequent analyses of *C. pectinata* and *C. acanthura* haplotypes, *C. hemilopha* haplotypes were used as out-group. Under the Akaike information criterion, the model of nucleotide substitution identified by MODELTEST as the best fit to the mtDNA data of these three taxa is the Tamura-Nei model with rate heterogeneity across sites ($\alpha = 0.1121$) and no

Table 3 Tamura–Nei average pairwise genetic distances between mtDNA clades (%) and standard deviation

Clade	Balsas	Colima	Guerrero	North	Oaxaca
Colima	4.11 (± 0.64)				
Guerrero	4.23 (± 1.16)	5.32 (± 0.94)			
North	10.52 (± 1.05)	11.57 (± 0.87)	9.89 (± 1.18)		
Oaxaca	5.42 (± 0.75)	7.41 (± 0.33)	4.14 (± 0.48)	8.98 (± 0.94)	
South	4.9 (± 0.8)	5.96 (± 0.83)	4.2 (± 0.98)	9.52 (±1.28)	5.23 (±0.76)

category of invariable sites. This model was employed for estimating pairwise genetic distances and a neighbour-joining tree. Given that MRBAYES does not support the Tamura-Nei model, the next more complex model supported by the program was selected (GTR with a gamma distribution of rates across sites) according to the user manual suggestions. We assumed a flat Dirichlet distribution (with all distribution parameters set to one) as the prior for both the stationary state frequencies and the substitution rates.

During the first replicate of an exploratory maximum-parsimony heuristic search more than one million equally parsimonious trees were obtained (237 steps long) and this computational demand compromised the ability to explore other islands of trees. The commands NCHUCK and CHUCKSCORE were therefore employed in order to perform a search from more than one starting point and increase the probability of exploring other islands of trees. These options allow one to effectively set a maximum number of trees (NCHUCK = 1000) of score greater than or equal to that specified by the CHUCKSCORE (= 237 in our case) per random-addition-sequence replicate (Swofford 2002). As one hundred replicates were performed, a total of 100 000 equally parsimonious trees were found. These are presented as a majorityrule consensus tree where branches with bootstrap support lower than 50% are collapsed (Fig. 4). The neighbour-joining tree resulted in similar bootstrap-supported clades as did the Bayesian inference tree. Bootstrap and posterior probability values support six geographically distinct clades (Fig. 4): North, Colima, Balsas, Guerrero, Oaxaca and South. The relationships of seven haplotypes, ND4-28, 29, 58, 59, 74, 76 and 77 are unresolved in the trees. Interestingly all these haplotypes occur within the geographical confines of the Balsas clade (Fig. 4). The inferred relationships among the geographical clades are not supported, and with the exception of the North clade, neither are the relationships among haplotypes composing them. The North clade comprises three geographically distinct subclades (Fig. 4): North A, North B and North C. The average Tamura-Nei genetic distance between the North clade and the more southerly distributed clades is approximately 10%, and genetic distances between the five southerly clades range between 4 and 7% (Table 3). Individuals identified as *C. acanthura* clustered within the South clade and showed an average divergence of 1.85% from *C. pectinata* sequences within this clade.

Geographic clades composing the statistical parsimony and median-joining networks are the same as those identified within the phylogenetic trees with one interesting difference. While the North (A, B and C), South, Guerrero, Colima and Oaxaca clades are identical among all analyses, in both the statistical parsimony and median-joining networks an apparent close relationship was found between the Balsas clade and haplotypes ND4-58, 59, 74, 76 and 77, with haplotypes 28 and 29 closely related to this clade. As these haplotypes occur within the geographical confines of the Balsas clade (Fig. 4), this grouping increases the genetic diversity of the Balsas clade. Due to the high level of divergence among haplotypes, TCS calculated six unconnected clusters (results not shown). Genetic distances between these geographical groupings, as well as the reticulated relationships within, are presented in Fig. 5. The demographic processes that possibly shaped the geographical distribution of mtDNA lineages within the different geographical clades were inferred with NCPA and are described in Table 4. Statistically significant associations between genetic variation and geographical distribution were detected only for the North A, North C, Balsas, South and Guerrero mtDNA clades. Range expansion was detected in both North clades and in two clades within the Balsas grouping. Allopatric fragmentation was suggested as the explanation for the distribution of genetic variation within the Guerrero clade, whereas long distance colonization or past fragmentation were inferred to have shaped the patterns observed within the South clade. Significant deviations from neutrality that could reflect past expansion events were only detected with Fu's test for the North A and Balsas clades (Table 5). Additionally, the distribution of pairwise differences within the Balsas clade was found to be consistent with a sudden expansion event.

Estimation of divergence times

No published fossil records for *Ctenosaura* were found that could be used as calibration points within the focal taxa of this study. However there is appropriate palaeontological information for other iguana species (Estes & Price 1973; Carroll 1988; Reynoso 2006). We identified two external calibration points that could be used as tree height priors for a Bayesian analysis of the subfamily Iguaninae, to estimate the age of the most recent common ancestor (mrca) for

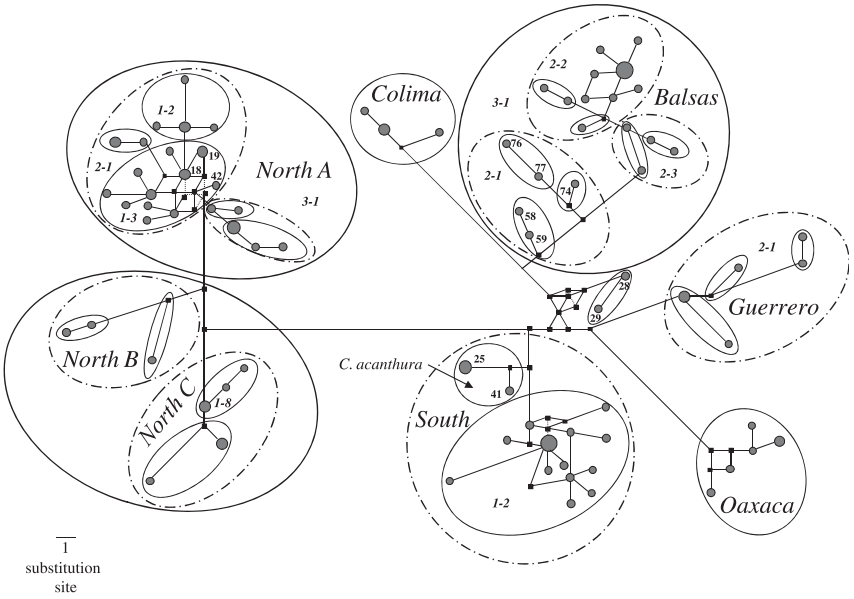

Fig. 5 Median-joining network of *C. pectinata* and *C. acanthura* (25 and 41) ND4 haplotypes and nesting clade design. Subnetworks are labelled according to their equivalent clade in the evolutionary tree (Fig. 4). For simplicity, only haplotypes and nested clades (in italics) that are mentioned in the text are labelled. Branch length is proportional to genetic distance. Black squares represent median vectors. Circles represent haplotypes and their area is proportional to the number of localities where such haplotype was found.

Subnetwork	Clade	Conclusion
North A	A1-2	Restricted gene flow with isolation by distance
	A1-3	Past fragmentation followed by range expansion
	A3-1	Contiguous range expansion
North C	NC1-8	Contiguous range expansion
Balsas	B1-6	Contiguous range expansion
	B2-1	Past gradual range expansion followed by fragmentation
	B3-1	Restricted gene flow with isolation by distance
South	S1-2	Long distance colonization and/or Past fragmentation
Guerrero	G2-1	Allopatric fragmentation

Table 4 Conclusions derived from nested-clade phylogeographical analysis for mtDNA clades. Nested design is shown in Fig. 5. Only clades where significant associations between geography and genetic variation were found are shown in the table

Table 5 *P*-values from mismatch distribution and neutrality tests for mtDNA clades. Statistics suggesting range expansion are shown in bold font. SDD = sum of square deviations; hg = Harpending's raggedness index. Dashes indicate that there was not enough variation within a population to perform the test

	Sudden-expansion model		Spatial-expansion model			
Mitochondrial DNA clade	*P* (SDD)	*P* (hg)	*P* (SDD)	*P* (hg)	Tajima's D	Fu's F_S test
North A	0.000	0.000	**0.116**	**0.10**	–0.52 (0.34)	**–8.01 (0.005)**
North B	**0.75**	**0.94**	**0.59**	**0.94**	0.57 (0.70)	0.82 (0.70)
North C	**0.34**	**0.58**	0.02	**0.40**	–1.28 (0.12)	1.91 (0.85)
Colima	—	—	—	—	–0.76 (0.27)	0.78 (0.66)
Balsas	**0.354**	**0.511**	**0.288**	**0.684**	–0.5697 (0.30)	**–4.66 (0.031)**
Guerrero	0.047	0.034	**0.53**	**0.46**	–0.31(0.42)	1.67 (0.80)
South	**0.052**	0.011	**0.35**	**0.30**	–0.13 (0.51)	–0.62 (0.46)
Oaxaca	**0.79**	**0.865**	**0.70**	**0.87**	–0.88 (0.22)	–0.69 (0.29)

C. pectinata, *C. acanthura* and *C. hemilopha*. For this analysis we used published sequences for 12 species representing all the extant genera of the Iguaninae and the genus *Enyalioides* as the out-group (on line Supplementary Material, Table S1). The tree was calibrated using, as prior information (i) an upper boundary of 93 Myr (million years) for the oldest record for an iguanid lizard (Pristiguana, 93 Myr, Estes & Price 1973); and (ii) a lower boundary for the minimum age for the appearance of the genus *Dipsosaurus* within the subfamily Iguaninae (Dipsosaurus, 24 Myr;

Carroll 1988) (on line Supplementary Material, Fig. S2). Implementing these calibrations constrains the subfamily Iguaninae to have originated at least 24 million years ago (Ma) but to be no older than the emergence of the family Iguanidae, 93 Ma. From this analysis we obtained a mean value of 9.24 Ma for the age of the mrca for *C. hemilopha, C. pectinata* and *C. acanthura* with a 95% confidence interval of 13.99–5.29 Ma. The mean, its standard deviation (2.47 Myr) and the estimated mutation rate values (mean = 0.0078 mutations per site per million year, standard deviation = 0.0018) were then used as prior information for dating divergence times among and within the *C. pectinata* mtDNA clades. According to this analysis, divergence within *C. pectinata* began between 2.3 and 6.5 Ma. The deepest divergences within mtDNA lineages occur within the North (3.1–1.1 Ma) and South (2.4–0.7 Ma). Mean ages, and 95% confidence intervals and posterior probabilities of mtDNA clades are shown in Fig. 4.

Discussion

The geographical distribution and genealogical relationships of genetic variation detected with nDNA and mtDNA markers provide a powerful data set for the inference of the probable evolutionary history of *Ctenosaura pectinata* and related species. Nuclear DNA markers are typically expected to record older demographic events than mtDNA markers (Avise 2004), and that would clearly seem to be the case in this study. Compared to the ND4 network (Fig. 5) the α-enolase network (Fig. 2) has fewer long branches and missing nodes, even more so when one excludes *I. iguana* and *C. similis* haplotypes. Thus the α-enolase network, while containing fewer haplotypes, represents a more complete sampling of genetic variation within this locus. While the ND4 phylogenetic tree and network (Figs 4 and 5) clearly define geographical clades, the relationships among these are poorly resolved. The long branches of Figs 4 and 5 are consistent with the faster mutational rate of ND4, and the absence of genealogically older haplotypes through natural population genetic processes. Together, these two data sets provide different but complimentary depths of resolution for phylogeographical inference.

Nuclear DNA data

The root location of the network (Fig. 2), defined by the placement of the *I. iguana* sequence, provides a temporal framework for interpreting the origin of *Ctenosaura* haplotypes. Populations of northwest Mexico appear to be the oldest (Fig. 2), as indicated by the close genetic relationship of the *I. iguana* out-group sequence to two haplotypes occurring in the north. One of these haplotypes, AE-11, is restricted to the north, while the other, AE-16 is the most widely distributed α-enolase haplotype with a disjunct distribution among populations of the Pacific coast of Mexico. A similar disjunction was detected in the distribution of AE-5 (Fig. 2). This congruent pattern can be interpreted as the imprint left by a past extinction event of a formerly continuous distribution of both haplotypes along the Pacific coast. The cause of such extinction cannot be determined easily. There is no direct evidence available of changes in sea levels for the Pacific Coast of Mexico (Pirazzoli 1991). However it has been speculated that, given its low altitude, the Río Balsas Basin might have been affected by marine transgressions (Marshall & Liebherr 2000). However, it may simply have been the consequence of other climatically mediated unfavourable conditions within the history of this region.

Group B of α-enolase haplotypes occurs between the disjunct distributions of AE-5 and AE-16. Both the star-like arrangement of group B, and the results of the mismatch distribution test, suggest a demographic and spatial expansion within this group. It is not clear whether this haplotype group is derived from southern or northern populations but, given the distribution of AE-6 (southeastern Mexico) that connects group B to the remainder of the network, it probably originated from southern populations and extended along the Pacific coast and into central Mexico (Fig. 2). The haplotype AE-2 is also probably derived from southern populations and from there colonized the Gulf coast (Fig. 2).

While some AE haplotypes are shared between *C. pectinata* and *C. acanthura*, no mtDNA genetic variation is shared between the two species. Rather than hybridization between these two species, this pattern would seem more consistent with incomplete lineage sorting of the more slowly evolving nuclear gene between *C. pectinata* and the recently derived form described as *C. acanthura* (see South clade section).

Mitochondrial DNA data

We estimate *C. pectinata* matrilines (including *C. acanthura*) started to diverge approximately 4.3 Ma (6.5–2.3 Ma) during the Pliocene. This clearly establishes the species itself originated prior to the Pleistocene, which is in general agreement with the idea that lowland tropical species are generally pre-Pleistocene in origin (Fjeldsa & Lovett 1997; Pennington *et al*. 2000; Pennington *et al*. 2004; Weir 2006). However, it is important to recognize the role of Pleistocene events within the evolution of this species.

Within several geographically defined mitochondrial lineages, haplotype diversity is estimated to have originated within the Pleistocene (e.g. Colima, Oaxaca, Guerrero, Balsas; Fig. 4). Thus although divergences between lineages appear to have Pliocene origins, diversity within lineages appears to have been shaped to a large extent by Pleistocene events. The distribution of mtDNA and nDNA lineages agrees to some point with the boundaries of the biogeographical

provinces (Fig. 1). Such areas are regarded as natural biotic regions resulting from a combination of both historical and ecological processes affecting a range of taxa. The Pacific Coast province is described as a continuous entity, however, several phylogroups within *C. pectinata*, and indeed other species (Zaldivar-Riverón *et al*. 2004; Mateos 2005; Devitt 2006), are found within this province. Thus, rather than being homogenous, biotas along the Mexican western coast have been greatly influenced by past events that have led to patterns of strong genetic differentiation within species.

North and Colima clades

Obvious geographical structure is present within the northern clade, with three geographically distinct subclades (North A, North B and North C) exhibiting partially overlapping ranges, and genetic distances between them of up to 4%. We estimate divergence within the North clade to have commenced during the Late Pliocene (LPl)–Early Pleistocene (EP) approximately 2 Ma (3.1–1.1 Ma). The most northerly distributed clade, North A, extends from Sinaloa to Nayarit (Fig. 4) and has probably undergone recent range expansion, as indicated by both the mismatch distribution and NCPA analyses, with diversification within this lineage estimated to have commenced approximately 1 Ma (1.6–0.5 Ma; EP). The interior placement of haplotypes ND4-18 and ND4-19 in the North A subclade (Fig. 5) is consistent with their being among the older extant haplotypes within this subclade. Both are found only in Culiacán (Fig. 1), and surrounding areas, suggesting that from here expansion towards the north and south has occurred.

At its northern boundary the North A subclade forms a contact zone with *C. hemilopha* (Fig. 4.I). Five individuals were collected in this area of overlap, with two of them having an mtDNA haplotype characteristic of *C. hemilopha* (Fig. 4, ND4-64). Of these two individuals, one is heterozygous at the α-enolase locus, possessing both *C. hemilopha* (AE-11) and *C. pectinata* (AE-16) type alleles. The other individual is homozygous for *C. pectinata* AE-16 allele. These individuals are consistent with a hybrid origin because they exhibit alleles typical for two different recognized species, and this is only observed where their distribution ranges come into contact. These hybrid individuals also exhibit a combination of morphological features that distinguish the two species. For example, the dorsal crest of the male hybrid individual we collected (Fig. 3.B) has large scales, as *C. pectinata* (Fig. 3.C), but this does not extend beyond the middle part of the body, as in *C. hemilopha* (Fig. 3.A). The colour pattern greatly resembles *C. hemilopha* (light grey body with black spots and black gular and pectoral regions) however, it also exhibits yellow spots forming bands surrounding the body typical of *C. pectinata* (see online Supplementary Material Fig. S1 for colour version). Given that sample size in the contact zone is small (five), the finding of two hybrid individuals suggests that hybridization may be fairly extensive within this region.

The southern limit of the North A clade is in the state of Nayarit, where it overlaps with North B, a possible area of secondary contact following preceding population isolation (Fig. 4.II). North B is restricted to an area between the volcanic mountains of Nayarit, an active branch of the MVB. Only three haplotypes were found in this area. They are approximately 3% divergent from other North clade haplotypes. Divergence within this subclade is estimated to have commenced approximately 0.8 Ma during the Early Middle Pleistocene (MP) 1.5–0.3 Ma. Haplotypes within the North C subclade are distributed further south. A past range expansion was detected with the NCPA. This is further supported by the results of the mismatch distribution test that suggest a sudden demographic expansion. We estimate this to have commenced approximately 0.6 Ma (1.0–0.2Ma; MP). North B and North C haplotypes are found together around the area of Puerto Vallarta, indicating another probable zone of secondary contact (Fig. 4.III).

There are a large number of mutations separating the mtDNA North clade from the remaining clades, as shown by the long branch between these clades in both the median-joining network and the phylogenetic tree. Despite their close geographical proximity (< 70 km), the greatest genetic distance between mtDNA lineages is between the Colima and North clades (11%). Haplotype differentiation within the Colima clade, is estimated to have been initiated approximately 0.4 Ma (0.8–0.09 Ma; MP).

Interestingly, the southern limits of the mtDNA Colima clade, and both the α-enolase groups A and B, coincide with the southwest extreme of the MVB. This geographical feature originated in the Miocene and is considered to be an important geographical barrier for a number of taxa (Zaldivar-Riverón *et al*. 2004; Devitt 2006) and is also recognized as the boundary between the Nearctic and Neotropical biogeographical regions (Marshall & Liebherr 2000). Volcanic activity has decreased since its formation, however, there is evidence of volcanism in the area during the late Tertiary and Quaternary (Johnson & Harrison 1989), and this may have contributed to vicariance within *C. pectinata* and other similarly distributed taxa. Phylogeographical analyses of freshwater fish in this region have suggested that tectonic events during the Plio-Pleistocene impacted upon their distribution (Mateos 2005). Vicariant events have also been reported at the eastern end of this volcanic range (Mulcahy *et al*. 2006). We speculate that vicariance, associated with geological activity of the MVB around the Plio-Pleistocene period, impacted upon *C. pectinata*. Consistent with such a scenario would be the monophyly of the mtDNA clades distributed south the MVB resulting in two fundamental lineages, one to the north and one to the south of the MVB. To test the mtDNA data for compatibility with such a topology we performed a corrected two-tailed Kishino-

Hasegawa test with PAUP*. Enforcing this constraint resulted in a tree 238 steps long, and not significantly different to the MP tree of 237 steps (two-tailed $P = 0.38165$), consistent with our speculation that the separation of the North clade from the more southern clades is probably the result of vicariance mediated by the geological activity of the MVB.

Balsas clade

Both the mismatch distribution test and NCPA support the conclusion of population range expansion in central Mexico (Tables 4 and 5). We estimate this to have been initiated approximately 0.7 Ma (0.3–1.1 Ma) during the Middle Pleistocene. α-Enolase sequence data also reveals signatures of population expansion within this region. Haplotype AE-7 and derived haplotypes (AE-4, AE-6, AE-8, AE-9, AE-10, AE-12, AE-15) are found within the geographical range of the mtDNA Balsas clade and are also frequent within the ranges of the Guerrero and Oaxaca mtDNA clades. Taken together, with their different temporal utilities, the nDNA and mtDNA markers suggest that the Balsas, Guerrero and Oaxaca mtDNA lineages differentiated, probably as a consequence of range fragmentation, after an earlier range expansion within central and southeastern Mexico. ND4-28, ND4-29 and Guerrero haplotypes co-occur in one of the sampled localities, paralleling the overlap of Guerreo and Oaxaca haplotypes distributed further south (Fig. 4.IV, V).

α-Enolase group B and the Balsas mtDNA clades are found concordantly within the Balsas Depression biotic province. This means that the patterns of distribution of genetic variation of *C. pectinata* in this area could be the result of past events that also shaped the evolution of this biogeographical area. Other codistributed species may exhibit a similar demographic pattern.

Guerrero and Oaxaca clades

The NCPA detected allopatric fragmentation between haplotypes forming the Guerrero cluster, and this is fairly intuitive when looking at the geographical disjunction this clade presents (Fig. 4). Haplotypes within the Oaxaca clade are distributed entirely within the Guerrero disjunction. According to the estimated divergence times, Oaxaca haplotypes started to differentiate approximately 0.6 Ma (0.1–1.0 Ma), slightly later than the estimated age for the Guerrero clade (0.8 Ma, 0.3–1.4). The geographical pattern and general timing of these events is most easily reconciled with a once continuous distribution of the Guerrero clade that underwent fragmentation, followed by re-colonization of the unoccupied area by the Oaxaca matriline. This would imply that both lineages were already differentiated from each other when the fragmentation of the Guerrero clade range occurred. This area is particularly interesting for the genetic diversity it contains because three nDNA haplotypes (AE-5, AE-7, AE-16; Fig. 2.III) and three mtDNA lineages (Oaxaca, Guerrero, South; Fig. 4.IV,V) can be found in a relatively small geographical area.

South clade

The distribution of the South mtDNA lineage follows a bifurcated biogeographical pattern that has been observed in other taxa (Huidobro *et al*. 2006), extending along the Pacific and Atlantic coasts, crossing the Isthmus of Tehuantepec. There is substructure within this clade with haplotypes ND4-25 and ND4-41, corresponding to samples of *C. acanthura*, distributed along the Gulf of Mexico biotic province, while the remaining haplotypes are distributed along the Pacific Coast biotic province. Boundaries of these provinces meet in the Isthmus of Tehuantepec (Fig. 1, 'c'), a feature that has been regarded as an important barrier for highland taxa (Marshall & Liebherr 2000), but as a connection between both Mexican coasts for lowland taxa (Huidobro *et al*. 2006). Although the two mtDNA groups within the South clade have diverged within two different provinces, there are signs of a past origin on the Pacific coast side of Isthmus of Tehuantepec. The geographical distribution of α-enolase genetic variation (Fig. 2) suggests that the *C. acanthura* populations on the Atlantic coast are derived from populations from the Pacific coast. The three Atlantic coast *C. acanthura* α-enolase haplotypes are either derived from haplotypes restricted to the Pacific coast (AE-12 from AE-7) or identical to the Pacific coast haplotypes (AE-2 and AE-7). Although morphological studies have described *C. acanthura* and *C. pectinata* as different species (Köhler *et al*. 2000), our data do not support this classification. Rather, it would appear *C. acanthura* is a geographically distinct, and relatively recently derived, form of *C. pectinata*.

Mitochondrial DNA diversification within the South clade is estimated to have been initiated approximately 1.5 Ma (0.7–2.4 Ma; LPl-MP), and this would appear to reflect differentiation of the Atlantic and Pacific lineages. According to the results of the NCPA, populations within the Pacific coast are inferred to have experienced long distance colonization and/or past fragmentation. Given the unlikely scenario of long distance terrestrial colonization for an iguana, past fragmentation seems a more plausible history.

Taxonomic and conservation implications

Regarding the relationships of *C. pectinata* with other species of the genus *Ctenosaura*, our results question the taxonomic status of *C. acanthura*. Both data sets suggest that this southern Mexican taxon is a recently derived and morphologically distinct population of *C. pectinata*. We did not find support for the generally suggested sister relationship between *C. similis* and *C. pectinata*/*C. acanthura*.

Contrary, our data suggest a closer relationship between *C. hemilopha* and *C. pectinata/C. acanthura*.

Ctenosaura pectinata has differentiated into eight geographically distinct mtDNA lineages. Genetic differences among these lineages range from 4% to 11%. Similarly, high levels of divergence have been detected among species of other *Ctenosaura* (Hasbún *et al*. 2005) and among species of Caribbean rock iguanas (Malone *et al*. 2000). At the broadest level, conservation efforts for *C. pectinata* should aim to recognize these fundamental genetic divisions and the genetic diversity within them. Our analyses have identified geographical regions within the range of *C. pectinata* that encompass particularly high genetic diversity within the species. For example, in southeastern Mexico several mtDNA lineages and nDNA groups occur within a narrow geographical region (Figs 2.III and 4). Our results suggest this region is the location for multiple refugia during cooler periods from where previously restricted populations have expanded to recolonize central and southern Mexico, and the Gulf coast. This high genetic diversity, and the high levels of vertebrate species richness detected within this region (García 2006), suggest that southeastern Mexico probably serves as a refugia for other species too. Paradoxically populations of *C. pectinata* in the region are greatly affected by illegal hunting.

Using both mtDNA and nDNA data we have detected several definite, and a number of probable, zones of secondary contact among geographically distinct mtDNA lineages. These zones represent a range of relative divergence times between the contacting lineages. North subclades represent contact between evolutionary young lineages, with greater divergence between the contacting clades of Balsas and Colima, and the deepest divergence between the hybridizing North clade and *C. hemilopha*. It has not escaped our attention that this system provides a useful model to investigate the significance of evolutionary divergence for gene flow. The use of microsatellite markers for the analysis of gene flow in areas of secondary contact would provide useful information for quantifying evolutionary significant units and for the development of conservation and management plans.

Conclusion

The nDNA and mtDNA phylogeography of *C. pectinata* provides a detailed view of evolutionary history within the northern neotropics through the Pliocene and Pleistocene. We conclude from our results that the *C. pectinata* evolutionary lineage originated in the Pliocene, as has been inferred to be probable for other lowland neotropical species (Pennington *et al*. 2000; Pennington *et al*. 2004; Weir 2006). *Ctenosaura pectinata* contains cryptic and geographically distinct mtDNA lineages, some of which originated at the end of the Pliocene and early Pleistocene, with the remainder originating during the last 1.5 Myr of the Pleistocene. Differentiated phylogroups located north and south of the MVB, support the idea that the geological activity associated with this mountain range has acted as a barrier to gene flow for species within this region (Zaldivar-Riverón *et al*. 2004; Mateos 2005; Devitt 2006). Several mtDNA lineages within *C. pectinata* show signs of recent Pleistocene associated demographic events of fragmentation and range expansion, possibly associated with changes in the distribution of the SDTF. Zones of secondary contact between lineages of differing evolutionary divergence offer an exciting opportunity to explore the possible mechanisms leading to speciation within this lizard group. Geographic patterns of genetic variation described in this study provide for the identification of regions of high genetic diversity, and probable refugia during cooler periods. It will be interesting to look at similarly widespread species to evaluate the generality of these patterns.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1 Photographs of (A) *Ctenosaura hemilopha* (B) *C. hemilopha* × *C. pectinata* hybrid and (C) *C. pectinata* male individuals, highlighting the intermediate morphological features of the hybrid. Note the enlarged but not extended dorsal crest (a) in the hybrid; the spotted colouration pattern exhibiting clear bands around the body (b); and the black colouration on the gular and pectoral region (c) in comparison to the pure individuals.

Fig. S2 Majority-rule consensus tree of 1802 trees sampled during two independent runs of a Bayesian analysis implementing a lognormal relaxed molecular clock. Assigned ages for external calibration points (black circle and square) are denoted in italics and correspond to fossil records of *Pristiguana* (93 Ma) and *Dipsosaurus* (24 Ma). Estimated 95% confidence age intervals and posterior probabilities for nodes of interest are shown under branch. Gene bank accession numbers are indicated in Table S1.

Table S1 GenBank accession numbers of taxa included in the Bayesian analysis for estimation of divergence times

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