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Mitochondrial DNA phylogeography of the Mesoamerican spiny-tailed lizards (*Ctenosaura quinquecarinata* complex): historical biogeography, species status and conservation

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Abstract

Through the examination of past and present distributions of plants and animals, historical biogeographers have provided many insights on the dynamics of the massive organismal exchange between North and South America. However, relatively few phylogeographic studies have been attempted in the land bridge of Mesoamerica despite its importance to better understand the evolutionary forces influencing this biodiversity 'hotspot'. Here we use mitochondrial DNA sequence data from fresh samples and formalin-fixed museum specimens to investigate the genetic and biogeographic diversity of the threatened Mesoamerican spiny-tailed lizards of the *Ctenosaura quinquecarinata* complex. Species boundaries and their phylogeographic patterns are examined to better understand their disjunct distribution. Three monophyletic, allopatric lineages are established using mtDNA phylogenetic and nested clade analyses in (i) northern: México, (ii) central: Guatemala, El Salvador and Honduras, and (iii) southern: Nicaragua and Costa Rica. The average sequence divergence observed between lineages varied between 2.0% and 3.7% indicating that they do not represent a very recent split and the patterns of divergence support the recently established nomenclature of *C. quinquecarinata*, *Ctenosaura flavidorsalis* and *Ctenosaura oaxacana*. Considering the geological history of Mesoamerica and the observed phylogeographic patterns of these lizards, major evolutionary episodes of their radiation in Mesoamerica are postulated and are indicative of the regions' geological complexity. The implications of these findings for the historical biogeography, taxonomy and conservation of these lizards are discussed.

Keywords: *flavidorsalis*, formalin, museum, nested clade analysis, *oaxacana*, Pleistocene

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Introduction

Mesoamerica has been identified as one of Earth's biodiversity 'hotspots' (Myers *et al*. 2000). Its biological diversity is due partially to its geographical position between the Nearctic realm of North America and the Neotropics of South America, and to its highly broken topography and diverse ecosystems (Coates & Obando 1996). This region is also considered one of the most challenging and exciting areas for the study of biogeography and evolution due to the mass dispersal of organisms between North and South America. This dispersal was facilitated by the closure of the Panamanian isthmus approximately 3 million years ago (Ma), an event which also shaped this region's diversity (Webb 1991).

Through the examination of past and present distributions of plants and animals, historical biogeographers have provided many insights on the dynamics of the massive organismal exchange between the American continents at large (Briggs 1984; Webb 1991). Despite this, relatively few phylogeographic studies have been attempted

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within Mesoamerica in order to test the degree of association between specific gene genealogies and their corresponding geographical distribution. This association could be informative in understanding the forces shaping the rich biodiversity in this region and provide the required information for the development of sound conservation strategies.

Recently, a series of studies on diverse organisms (amphibians: García-París *et al*. 2000; reptiles: Zamudio & Greene 1997; Parkinson *et al*. 2000; freshwater fish: Bermingham & Martin 1998; Martin & Bermingham 2000; mammals: Demastes *et al*. 1996; Sullivan *et al*. 1997; Cropp & Boinski 2000; Harris *et al*. 2000; trees: Cavers *et al*. 2003; Novick *et al*. 2003) have taken a phylogeographic approach either within or partially within Mesoamerica. Taken together these have begun to indicate that the phylogeographic patterns within Mesoamerica may have been strongly influenced by the complexity of this region's geological history and by the cyclic changes in the climate, vegetation, and sea levels, coupled with the more constant orogenic processes. Sister species currently distributed on opposite versants of Mesoamerica (Pacific vs. Caribbean/Gulf of Mexico) may have diverged either through transcontinental organismal dispersals (east–west) due to climatic changes in the Miocene (e.g. Mexican pit viper, Parkinson *et al*. 2000) or became sundered by vicariant events occurring parallel to the Isthmus (north–south) such as the rise of the extremely mountainous Talamanca Cordillera in Costa Rica (e.g. the Neotropical bushmaster, Zamudio & Greene 1997). This dynamic orogeny has promoted extensive adaptive radiations of salamanders within mountain ranges considered one of the most speciose areas in the world (García-París *et al*. 2000). The complexity of this region's biogeographic history has also been suggested through Novick *et al*.'s (2003) work with the Mesoamerican mahogany trees, which show a greater phylogeographic structure than has been found across the Amazon Basin. For species such as freshwater fishes, phylogeographic patterns in this region are unclear, most likely due to the several colonization and extinction events provided by the cyclic rise and fall of sea levels during the Pleistocene (Bermingham & Martin 1998, 2000). Similarly, Cavers *et al*.'s (2003) work with Spanish cedar trees in Mesoamerica suggests repeated colonizations from South America.

It is evident that a much greater number of studies are needed to understand the high biodiversity and complex phylogeographic patterns of this region. Iguanid lizards of the *Ctenosaura quinquecarinata* complex are candidates to make good models for Mesoamerican phylogeography. Lizards have low vagility (see Savage 1982) and therefore may exhibit a pronounced phylogeographic structuring, whereas more mobile organisms, which occupy large extensions of continuous habitat, can exhibit less spatial differentiation (Avise 1994). Additionally, these reptiles are endemic to Mesoamerica, and occur in disjunct populations currently separated by a series of mountain ranges and lowlands of unsuitable habitat (Köhler 1993).

Lizard populations of the *C. quinquecarinata* complex are considered either mainly arboreal or mainly terrestrial (Hasbún 2001) and inhabit different life zones including the tropical and subtropical dry forests and the subtropical moist forests (*sensu* Holdridge 1957), corresponding to the dry forest and pine and oak forests of the Central American ecoregions (*sensu* Olson *et al*. 1999). Marked shifts between these life zones are due to the rugged topography of the region. In addition, habitats are increasingly highly fragmented due to current agricultural practices and human developments. These factors, coupled with an unregulated exploitation for the pet trade and local consumption (Hasbún 2001), are the most probable causes for their endangered status (see UICN–WWF 1999).

Ctenosaura quinquecarinata lizards have been traditionally classified as a single species unit throughout their range. However, recent studies on the morphological variation from lizards belonging to several geographically disjunct populations of this complex have resulted in the following nomenclatural changes: (i) *C. quinquecarinata*-like lizards from Comayagua Valley, Honduras, were described as *Ctenosaura flavidorsalis* (Köhler & Klemmer 1994), (ii) lizards from El Salvador (once considered *C. quinquecarinata*) and newly reported specimens from Guatemala have been suggested to be conspecific to *C. flavidorsalis* (Hasbún *et al*. 2001); (iii) the *C. quinquecarinata* holotype (Gray 1842), a stuffed skin and skeleton of uncertain origin which was thought to represent Méxican populations (Bailey 1928), has recently been shown to be most likely from the southern populations of Nicaragua and Costa Rica (Hasbún & Köhler 2001); and (iv) the morphologically distinct and geographically disjunct populations of México have been renamed as *Ctenosaura oaxacana* (Köhler & Hasbún 2001). The morphological differentiation between populations, which induced the nomenclatural changes, may underlie the independent genetic histories of these lizards reflecting the unique geological history of Mesoamerica.

In this study we used mtDNA sequence data to examine the phylogeographic patterns of these reptiles throughout their distribution range. Using phylogenetic and nested clade analyses under a phylogeographic approach will add significantly to our current understanding of these reptiles and to the limited number of phylogeographic studies developed in this Neotropical realm. Results gained are interpreted in the context of the regional geological features and events, examining the plausible colonization sequence of these lizards and the implications for their taxonomy and needed conservation measures. Such information, in turn, may prove extremely valuable to better understand and protect the biological diversity of Mesoamerica as a whole.

Materials and methods

Population sampling and DNA isolation

Both freshly collected samples and formalin-fixed museum specimens from previously documented and newly recorded localities (Appendix), which together represent the entire known range of the *Ctenosaura quinquecarinata* complex, were analysed. Samples for DNA extraction were taken as blood (1 mL) from the ventral coccygeal vein following procedures recommended by Samour *et al*. (1984), or as a 10-mm fragment of tail tip, and preserved in absolute ethanol. DNA from fresh tissue was extracted by suspending a portion of the tail tip (5 mm), or blood (150 μL), in 150 μL buffer (0.05 EDTA, 0.1 M Tris pH 7.4, 0.5% SDS) and digesting overnight with 20 μL proteinase K (20 mg/mL) followed by phenol–chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989). When extracting DNA from formalin-fixed museum specimens, the integument of tail tips (10 mm long) was removed and discarded. The remaining muscle and bone tissues were coarsely chopped and placed in a 1.5-mL microcentrifuge tube containing 100 mM glycine (as a binding agent for excess formalin), 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, following Shedlock *et al*. (1997) with modifications. Microcentrifuge tubes containing the tissue homogenates were placed at room temperature in a low speed rotary shaker with the solution replaced every 24 h for 4 days. After this rinsing process, tissue samples were air-dried and DNA extracted using Chelex (Chelex® 100 Insta-Gene Matrix, Bio-Rad). Chelex was used in preference to phenol–chloroform extraction procedures in formalin-preserved samples to prevent the loss of DNA cross-linked with proteins.

ND4 gene amplification and sequencing

Published ND4 (nicotinamide adenine dinucleotide dehydrogenase subunit 4) mtDNA sequences of three *Ctenosaur* lizards (Sites *et al*. 1996) were used to design primers ND4F160 (5′-CGACAAACAGACCTAAAATCACTAATCG-3′) and ND4R623 (5′-ATGTGAAGAGCTATGATTAGATGTTCTC-3′). Due to the degraded nature of the DNA extracted from formalin-fixed tissues the following internal primers were designed to amplify shorter (~150 bp) overlapping sequences: ND4F141 (5′-CTTCCATATTATTCTGCCTAGCCA-3′); ND4R235 (5′-GA-AGTGCTATGTTGGTTAGATTGG-3′); ND4F297 5′-TCCGCACTTTTCAACTGAT-CCCAA-3′ and ND4R306 (5′-AATTGTTGGTTGGGATCAGTTGAA-3′).

Approximately 100 ng of template DNA was used in a 25-μL PCR (polymerase chain reaction) containing the following reagents: 200 μM of each nucleotide, buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20], 1.5 mM $MgCl_2$, 25 pM of each primer, 0.25 unit of *Taq* polymerase. Amplification conditions were 94 °C for 2 min; 35 cycles of 94 °C for 45 s, 64 °C for 1 min and 72 °C for 2 min; and one final extension of 72 °C for 10 min. Both strands were sequenced using a Thermo Sequenase® cycle sequencing kit (Amersham-Pharmacia Biotech) under standard conditions and resolved on an ALFexpress™ DNA sequencer (Amersham-Pharmacia Biotech). Multiple ND4 sequences were manually aligned. Relative rates of transitions (ti) and transversions (tv), together with the base composition of the sequences were obtained using PAUP* version 4.02b (Swofford 1998).

Phylogenetic analyses

The mtDNA data set was analysed under minimum-evolution (ME), maximum-parsimony (MP) and maximum-likelihood (ML) optimality criteria using PAUP* (Swofford 1998). For ML, the hierarchical likelihood-ratio test approach (Huelsenbeck & Crandall 1997) was used to select the model of DNA evolution best fitting the data set, as implemented in the program MODELTEST (Posada & Crandall 1998). MODELTEST was also used to estimate the parameters of the model of evolution for input to PAUP*. *Ctenosaura melanosterna*, a species belonging to the closest sister clade to the lizards of the *C. quinquecarinata* complex (Hasbún 2001) was used as an outgroup, although tree topologies remained constant for a range of *Ctenosaura* taxa as outgroup (*Ctenosaura alfredschmidti*, *acanthura*, *hemilopha*, *bakeri*, *similis*, data not shown).

The robustness of the results was assessed by means of bootstrap analyses (1000 pseudoreplicates from ME and MP and 100 for ML) using PAUP* (Swofford 1998).

Nested clade analysis

To test the null hypothesis of random geographical distribution of mtDNA haplotypes, a nested clade design was constructed on the different ND4 haplotypes (Templeton *et al*. 1987; Crandall & Templeton 1996). The probabilities of the most parsimonious solution between haplotypes were calculated using the program PARSPROB 1.1 (http://bioag.byu.edu/zoology/crandall-lab/programs.htm). The hierarchical nesting design was constructed manually following rules described in Crandall (1996), Templeton *et al*. (1992) and Templeton & Sing (1993).

GEODIS 2.2 (http://darwin.uvigo.es) was used to examine the association between the haplotype's geographical location and genealogy. Exact coordinates of the collection localities in latitude and longitude were entered, and the program calculated the distance measures D_c, the clade distance, and D_n, the nested clade distance, and performed statistical tests. The clade distance $D_{c(x)}$ measures the geographical range of haplotype x, and was established by determining the geographical centre of haplotype x and

then calculating the average distance of all individuals that bore haplotype x to their corresponding geographical centre. The nested clade distance $D_{n(x)}$ measures how clade x is distributed relative to its evolutionary closest sister clades from the higher nested categories. Only those nests that reflected both genetic and geographical differences were considered as informative and therefore included in the statistical analysis. To test the null hypothesis of random geographical distribution of haplotypes, chi-squared tests were run between the probabilities of random and the observed D_c and D_n. A total of 1000 permutations were executed to achieve significance at $P < 0.05$ level. Finally, the causation for the geographical distribution of haplotypes was inferred using the inference key provided in GEODIS 2.2.

Results

mtDNA diversity

Between 1 and 11 lizards were sequenced from each sample site. Seventy-one individual ND4 sequences were generated. Sequences were collapsed into 17 unique, 377-bp mitochondrial haplotypes differing from 1 to 16 substitutions (see Table 1 for the haplotype frequencies per locality). Sequences were submitted to GenBank with Accession nos AY730644–AY730661. The average number of haplotypes per location was 1.3 (range = 1–3). From a total of 31 polymorphic sites observed 19 were parsimony informative. Sequences were A–C rich, base frequencies being similar in all haplotypes (e.g. A: 0.31425, C: 0.36059, G: 0.10548, T: 0.21969). No insertions or deletions were observed either between sequences recovered from this study or when these were compared to published *Ctenosaura* sequences (Sites *et al*. 1996). Only two transversions were observed, highly skewing the ti/tv ratio towards transitions (22:1). Twenty substitutions were at the third codon position, with none at the second and six at the first. Transitions at positions 177, 237 and 391 were in first codon positions and resulted in amino acid replacements of threonine-alanine A-G (H4), valine-methionine G-A (H12 and H15), and valine-isoleucine G-A (H3), respectively.

Phylogenetic analyses

MODELTEST selected HKY85 (Hasegawa *et al*. 1985) as the best fit model (–ln L277.3435) to describe the ND4 data set. Parameters for this model included a ti/tv ratio of 14.08 and estimated base frequencies of A (0.3602), C (0.3130), G (0.1122) and T (0.2142).

Phylogenetic analyses under all optimality criteria returned similar topologies which recovered three main monophyletic clades with a strong geographical concordance: (i) a northern lineage from México, with four haplotypes, (ii) a southern lineage from Nicaragua and Costa Rica, with four haplotypes, and (iii) a central lineage, comprising nine haplotypes from Guatemala, El Salvador and

Table 1 Frequency distribution per locality of mtDNA (ND4) haplotypes from lizards of the *Ctenosaura quinquecarinata* complex. See the Appendix for museum voucher numbers and exact localities

Haplotype and corresponding specimens (work number)	Localities																					Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
H1 — G1, G3–7, G9–11, G14, G18; ES25–29, ES32					1	4	1	6	5													17
H2 — ES11–16			6																			6
H3 — ES19–20, H6, H8–9, H11, H12		2														2	3					7
H4 — ES24		1																				1
H5 — ES21–22				2																		2
H6 — ES4	1																					1
H7 — ES1–3, ES5–7, ES18	5	2																				7
H8 — H1–2														2								2
H9 — H3–5															3							3
H10 — C4																					1	1
H11 — C1																					1	1
H12 — M18																				1		1
H13 — M1, M7–10																		5				5
H14 — M5–6																			2			2
H15 — M11–13, M16–17																				5		5
H16 — N6–9												1	3									4
H17 — N1, N3–5, N10–11										2	4											6
Total	6	5	6	2	1	4	1	6	5	2	4	1	3	2	3	2	3	5	2	6	2	71
Number of haplotypes per locality	2	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	

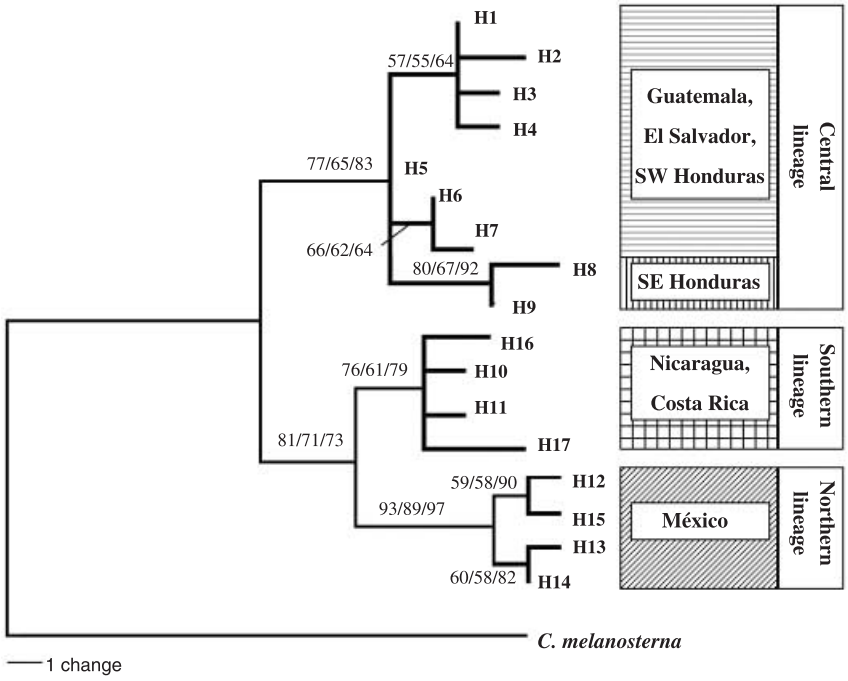

Fig. 1 Maximum-parsimony phylogram establishing three main mtDNA lineages: central populations from Guatemala, El Salvador and SW Honduras and SE Honduras; southern populations from Nicaragua and Costa Rica; and northern populations from México. Numbers over or under branches correspond to the bootstrap values under maximum parsimony (1000 pseudoreplicates), under maximum likelihood (100 pseudoreplicates) and minimum evolution (1000 pseudoreplicates), respectively. Labels at the tips refer to haplotype numbers.

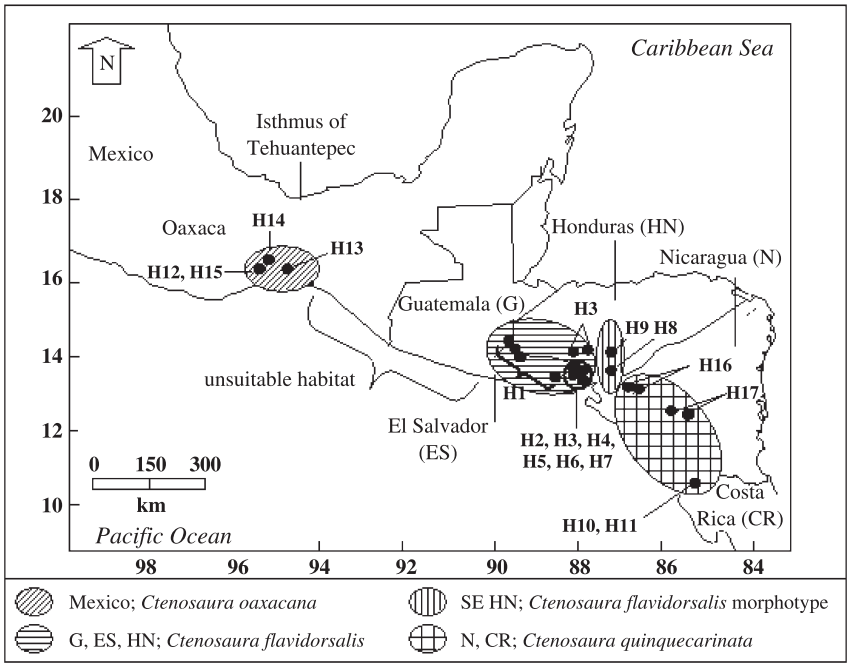

Fig. 2 Map of upper Mesoamerica showing the geographical location of the sampled haplotypes from lizards of the *Ctenosaura quinquecarinata* complex. Haplotypes H12–H15 belong to the northern lineage of México as seen in the maximum-parsimony phylogram (Fig. 1). Haplotypes H2–H7 correspond to NE El Salvador.

Honduras (Fig. 1). Refer to Fig. 2 for the geographical distribution of haplotypes. Under all optimality criteria the three main lineages are well supported with bootstrap values higher than 76% with the exception of the southern and central lineages under ML (61% and 65% bootstrap support respectively). The node joining the northern and southern lineages was well supported (> 71%) under all criteria. The average percent divergences between the three lineages are as follows: northern-central (3.7%); northern-southern (2.0%); central-southern (3.1%). We note that each of the three lineages contains the morphological type specimen for that species.

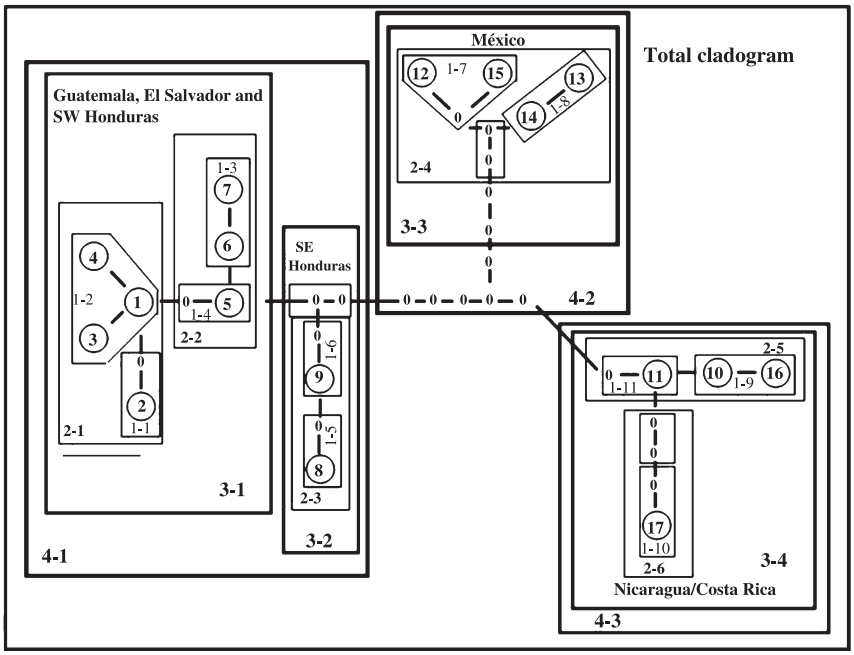

Fig. 3 NCA network of mtDNA haplotypes from *Ctenosaura quinquecarinata*-like lizards grouped into nesting clades. The haplotype number is included within a circle. Each dash represents a nucleotide substitution or step. Zeros represent ancestral or unsampled haplotypes.

Table 2 Inference chain for the results of the *Ctenosaura quinquecarinata*-like lizards phylogeography provided by NCA

Nested clade	Chain of inference	Inferred outcome
Total cladogram	1, 19, 20, 2, 3, 5, 15 — No	Past fragmentation
Clade 4-1		
Four step nested in 4-1	1, 2, 3, 5, 15, 16, 18 — Yes	Fragmentation or isolation by distance
Three step nested in 3-1	1, 2, 11, 12 — No	Contiguous range expansion
Two step nested in 2-3	1, 19, 20 — No	Inconclusive
Two step nested in 2-2	1, 2, 3, 5, 15, 16, 18 — No	Fragmentation, range expansion or isolation by distance
Two step nested in 2-1	1, 2, 3, 4, 9 — No	Allopatric fragmentation
Haplotypes nested in 1-3	1, 2, 11, 17 — No	Inconclusive
Haplotypes nested in 1-2	1, 2, 11, 12 — No	Contiguous range expansion
Clade 4-2		
Two step nest in 2-4	1, 2, 3, 5, 15, 16, 18 — No	Fragmentation, range expansion or isolation by distance
Haplotypes nested in 1-8	1, 19, 20 — No	Inconclusive
Clade 4-3		
Three step nest in 3-4	1, 2, 3, 5, 15, 16, 18 — Yes	Fragmentation or isolation by distance
Two step nest in 2-5	1, 19, 20 — No	Inconclusive
Haplotypes nested in 1-9	1, 19, 20 — No	Inconclusive

Nested clade analysis

The NCA nested design of the ND4 haplotypes is provided in Fig. 3. A minimum of nine and a maximum of 13 mutational steps separate the basal haplotypes between the three main networks, 4-1 (*Ctenosaura flavidorsalis*), 4-2 (*Ctenosaura oaxacana*) and 4-3 (*Ctenosaura quinquecarinata*). A lower level of subdivision separates the populations from Guatemala, El Salvador and SW Honduras (3-1) from SE Honduras (3-2) within clade 4-1. Connections of more than eight steps have less than 95% probability of being parsimonious. Thirteen out of 30 nested clades were informative as they contain both the geographical and haplotype differences required for statistical analyses (Fig. 3).

The chain of inference and inferred results is shown in Table 2 in regards to population structure and history when the inference key is applied to the significant results. Four

(1-3, 1-9, 2-3, 2-5) out of the 17 clades were not significant at a $P < 0.05$ level and clades 2-5, 2-3, 1-9, 1-8, 1-3 where inconclusive, therefore these will not be discussed further. The rest provided information on the possible evolutionary history of the studied haplotypes. Inferential results are provided below and follow an interior to tip explanation.

Total cladogram. The total cladogram contains nested clades 4-1 (*C. flavidorsalis*), 4-2 (*C. oaxacana*) and 4-3 (*C. quinquecarinata*), and the statistical test indicated a significant correlation between clades and geography. The inference key points to past fragmentation for the observed geographical distribution of clades.

Nested clade 4-1. Clade 4-1 includes all haplotypes from Guatemala, El Salvador and Honduras, and corresponds to populations described as *C. flavidorsalis*. This clade is subdivided into the nested clades 3-1 (Guatemala, El Salvador and SW Honduras) and 3-2 (SE Honduras). Results from the inference key indicate that fragmentation or isolation by distance may explain the observed pattern in this clade. Heading inwards, clade 3-1 includes clade 2-1 and clade 2-2. Results reject the null hypothesis of no geographical association with haplotypes and the inference key indicates a contiguous range expansion of the species in this area. Clade 2-2 includes clade 1-3 and an internal, basal, haplotype H5, which originates in El Salvador. Further geographical sampling is required to indicate if fragmentation, range expansion or isolation by distance between these two clades is causal for the documented phylogeographic pattern. Clade 2-1 covers clade 1-2 and haplotype H2, this latter originating in NE El Salvador, a population close to some haplotypes from clade 1-2. The inference key points to allopatric fragmentation of *C. flavidorsalis* populations in this area.

Clade 1-2 is the first-level nest with the greatest haplotype diversity and geographical range. Haplotypes included in this clade are H1 from Guatemala, NW and central El Salvador; H3 from NE El Salvador and SW Honduras, and H4 from NE El Salvador. The inference key suggests contiguous range expansion as a possible cause for the observed geographical distribution of haplotypes, which have ranges that are mostly nonoverlapping with other haplotypes within the nest.

Nested clade 4-2. The second major clade, clade 4-2, contains all haplotypes from México, which correspond to *C. oaxacana*. For statistical analyses, clade 1-8 was considered as the interior clade as haplotype H14 nested within this clade is the most basal when compared to a close sister taxa, *Ctenosaura melanosterna*. Results from the inference key suggest that a more intensive sampling program may be required to discriminate between fragmentation, range expansion or isolation by distance.

Nested clade 4-3. The third major nested clade, clade 4-3 contains all haplotypes from Nicaragua and Costa Rica, and corresponds to all described *C. quinquecarinata* populations. The inference key indicates that fragmentation or isolation by distance may account for the geographical distribution of haplotypes.

Discussion

In this study we have used both phylogenetic and nested clade analyses on mtDNA sequence variation in lizard populations of the *Ctenosaura quinquecarinata* complex. The results of this study were consistent in showing a significant geographical structuring of mtDNA haplotypes grouped into three major lineages, clades 4-1, 4-2 and 4-3 with evidence of past fragmentations (*sensu* Templeton 1998). The average sequence divergences between these three lineages ranges from 2% to 3.7%; however, since no mtDNA molecular clock for Iguanidae is available, any estimates for times of divergence must be taken with a degree of caution. Several molecular clock calibrations are available in squamates and Wüster *et al.* (2002) suggest 1.36–1.44% per million years (Myr) as an estimate for ND4 and cyt *b* genes in snakes. Using this calibration the cladogenesis of the three lineages of lizards of the *C. quinquecarinata* complex (northern, central, southern) may have occurred in the Pliocene or Pleistocene. Around this period the Mesoamerican mountain ranges experienced an uplift associated with the closure of the Panamanian isthmus (Ferrusquia-Villafranca 1978). In addition the Pleistocene climatic oscillations (Hays *et al.* 1976) that had profound effects in the amount of emergent terrain and vegetation formations in the area (MAG/IGN 1985; Coates & Obando 1996) are likely to have further shaped the phylogeography of these lizards.

Phylogeographic patterns and postulated events

As discussed previously, the deepest genetic subdivisions observed are those between clades 4-1, 4-2 and 4-3, resulting from past fragmentations. The northern clade 4-2 and the southern clade 4-3 are the geographically most distant, yet genetically most similar. This interesting, nonlinear pattern is similar to the phylogeographic patterns of Spanish cedar trees in Mesoamerica where the geographically distant northern and southern cpDNA lineages were genetically the least differentiated (Cavers *et al.* 2003). These authors suggest that the observed phylogeographic pattern is most likely due to repeated colonizations of Mesoamerica from source populations in South America during the fluctuations in vegetation assemblages associated with the Pleistocene climatic oscillations.

Bermingham & Martin (1998) have also indicated the above-described pattern for freshwater fishes, originating

from divergent South American sources and dispersing into lower Mesoamerica. As forests and associated habitats in the Mesoamerican region were repeatedly fragmented into refugial areas in the Pleistocene (Toledo 1982; Coates & Obando 1996), it seems probable that repeated colonizations by lizard populations also contributed towards the establishment of the observed nonlinear north–south phylogeographic pattern.

However, the pattern of divergence in ND4 sequences, tree topologies and NCA suggests that the lineage of lizards of the *C. quinquecarinata* complex underwent several distinct episodes associated mainly with population fragmentation and range expansion, associated with both an east–west and north–south colonization. The split between this lizard complex and its closest sister clade (*Ctenosaura bakeri*, *Ctenosaura melanosterna*, *Ctenosaura oedirhina*, and *Ctenosaura palearis*, all endemic to the Caribbean versant of Honduras and Guatemala; Hasbún 2001) may have occurred as early as the Middle Miocene (12.5 Ma). Considering these patterns and dates, we postulate that the ancestral forms of the *C. quinquecarinata* complex originated in the Caribbean versant of northern Mesoamerica, dispersing towards its Pacific versant before vicariant events such as the SHN mountain rise sundered the extant Pacific and Caribbean taxa. During the Early Pliocene, the central Salvadoran/Honduran forms (clade 4-1) split from the northern Méxican (clade 4-2) and southern Nicaraguan/Costa Rican (clade 4-3) forms. These latter clades remained connected possibly through a southern coastal or a northern mountainous Pleiocene–Pleistocene corridor, until becoming sundered by the continuous mountain rise or climatic changes of the Pleistocene. Finally, populations in NE El Salvador expanded into Guatemala and Honduras (clade 4-1).

Clade 4-1 seems to have undergone a more complex phylogeographic history. Lizard populations belonging to the inner clade 3-1 (Fig. 3, Table 2) occur in fragmented habitats on eastern Guatemala, northern El Salvador and western Honduras. The highest number of haplotypes from this clade occurs in the northern section of Morazán, El Salvador (H2–H7, Fig. 2). Terrains here are mostly broken with sharp contrasts in altitude, sometimes well over 1000 m high, the elevational limit for ctenosaur lizards (Köhler 1993). Some populations may remain disjunct, even though geographically proximate, due to the unsuitability of habitats between populations. This is the case for clade 2-1 where the inference key indicates allopatric fragmentation to explain the observed genetic subdivision between closely located lizard populations (i.e. cerro El Junco — H2 vs. cerro El Aguacate — H3, H4, located 3 km apart, Fig. 3).

On the other hand, the distribution of a single *C. flavidorsalis* haplotype from northern El Salvador, towards Guatemala (H1) and Honduras (H3; see Fig. 2 and clade 2-1 in Fig. 3), is interpreted as contiguous range expansion. This agrees well with the availability of suitable habitat throughout the sampled location sites (personal observation) and is consistent with the commonly observed loss of genetic diversity in colonizing populations (Avise 2000).

The inferred phylogeographic patterns are indicative of the complexity of the geological history of the Mesoamerican region. Thin strips of land such as this region can be more affected by the cyclical changes of climate and water levels of the Pleistocene than larger, more stable landmasses. Phylogeographic concordant patterns in North America (Avise 2000) and Europe (Hewitt 2000) have been documented considering a broad range of land and aquatic species. For Mesoamerica, however, many more phylogeographic studies are needed to determine concordance patterns.

Implications for taxonomy

The range of mtDNA sequence divergence between the three distinct lineages uncovered in this study (Fig. 1; 2.0–3.7%) are in the magnitude of the divergences documented on ND4 and cyt *b* sequences (JC corrected) from other close iguanid species [i.e. *Cyclura nubila nubila–C. cyclura cyclura*: 1.8%; *Cyclura carinata–C. ricordi*: 5.4% (Malone *et al*. 2000) and *Sauromalus hispidus–S. varius*: 2.3% (Petren & Case 1997)]. These authors consider their levels of sequence divergences to agree well with the species boundaries defined by traditional taxonomy.

Lizards from México have been recently described as *C. oaxacana* (Köhler & Klemmer 1994) and those from Nicaragua and Costa Rica are referred to as *C. quinquecarinata* (Gicca 1983). *Ctenosaura flavidorsalis* lizards, on the other hand, are known from the Comayagua Valley, La Paz, Honduras (Köhler & Klemmer 1994) and from El Salvador, Guatemala and Honduras (Hasbún *et al*. 2001). This nomenclature is consistent with the main division in lineages in our mtDNA phylogenetic analyses.

However, NCA clade 4-1 (Fig. 3) contains lizards with two different morphologies (Hasbún 2001) corresponding to the lower level nested clades contained within it (3-1 and 3-2). Lizards of the *C. flavidorsalis* form occur in Guatemala, El Salvador, SW Honduras and the type locality in La Paz, Honduras (Hasbún *et al*. 2001), and their mtDNA haplotypes are in nested clade 3-1. These lizards are mainly terrestrial, seek refuge in ground burrows and have a yellow dorsal colouration (Hasbún 2001). In contrast, lizards from SE Honduras, representing a new locality record of this species complex, are morphologically distinct (very closely resembling the *C. quinquecarinata* forms from Nicaragua and Costa Rica) despite their strong mtDNA affinity to the *C. flavidorsalis* lineage. SE Honduran lizards, as well as those from Nicaragua, usually exhibit arboreal habits, seek refuge in tree hollows, have green colour, and their tails are not as spiny as the true *C. flavidorsalis* forms. Observing the

geographical location of morphologically distinct SE Honduras lizards (between the distribution range of *C. flavidorsalis* and *C. quinquecarinata*, haplotypes H8–H9, Fig. 2), a contact zone between the two species could possibly explain the above-described inconsistency. However, only SE Honduran haplotypes (and their characteristic morphology) were found in this area, contrary to expectations if hybridization between these two species occurred (Hewitt 1988). Similarly, the null hypothesis of no hybridization may be accepted when all haplotypes from one species (in our case the morphologically distinct SE Honduran lizards) are nested together before they are nested with haplotypes from another species (Crandall 1996). This is shown in our NCA design (Fig. 3). Considering that SE Honduran lizards (*C. flavidorsalis*) are morphologically and ecologically more similar to *C. quinquecarinata* forms, ecological convergence could most likely explain this scenario. The evolution of morphological characters on independent lineages as a result of the selective pressures has been well illustrated in *Anolis* lizards, where similar morphological characteristics have evolved independently in different species from different Caribbean islands due to similar ecological environments (Losos 1990). For our case however, a larger geographical sampling between the two *C. flavidorsalis* forms, together with the use of nuclear markers and further ecological data, may shed more light onto this phenomenon.

Conservation implications and final considerations

For effective conservation of biodiversity, the identification of populations and phylogeographic groups with independent evolutionary histories such as evolutionarily significant units (ESUs *sensu* Moritz 1994) and/or that have unique adaptive characteristics (Crandall *et al*. 2000) has been strongly recommended. The importance of the identification of these units prior to the development of conservation programs is evident. Species survival plans often depend on *ex situ* conservation measures such as captive breeding and reintroduction or the translocation of specimens from one population to another in order to increase its population size or heterozygosity (Marshall & Spalton 2000). Most efforts in conservation genetics therefore have been aimed at describing the genetic processes in endangered populations and at developing guidelines for the optimal conservation of the genetic variation within these populations (see Loeschcke *et al*. 1994; Smith & Wayne 1996). This provides a sound genetic basis for making various difficult decisions on how to maintain the genetic variability of phylogeographic groups or populations and on how to manage gene flow between conspecific populations. With the identification of ESUs the consequences of practicing translocations or other 'genetic manipulations' of genetically distinct populations can be analysed and if considered inappropriate, prevented (Hansen & Loeschcke 1994).

Considering mtDNA divergences, the three main monophyletic clades of lizards of the *C. quinquecarinata* complex fit the description of ESUs (Crandall *et al*. 2000) and their suggested treatment as distinct species, this being congruent to the current established nomenclature. Additionally, the *C. flavidorsalis* distinct populations from SE Honduras also warrant classification as an ESU under the criteria of Crandall *et al*. (2000), since these lizard populations are morphologically and ecologically differentiated from other *C. flavidorsalis* populations from SW Honduras, El Salvador and Guatemala (Hasbún 2001). As seen in the NCA, the haplotypes of the morphologically distinct SE Honduran lizards are monophyletic and no haplotypes are shared with western *C. flavidorsalis* populations, indicating that, despite being closely related, current gene flow is restricted. Therefore, translocations between populations originating from SE Honduras and those from other sources should be avoided.

Another widely recognized criterion for the identification of priority areas for conservation is determining those areas that maintain high genetic diversity (Ehrlich & Wilson 1991; Wilson 1992). As observed in Fig. 2, populations of *C. flavidorsalis* located on NE El Salvador possess a variety of haplotypes (H2–H7) as compared to other sampled regions. If conservation strategies should be geared to the preservation of their genetic diversity, the populations from this area of El Salvador should be considered a priority.

Finally, this study has shown that the use of a modern phylogeographic approach can be an extremely powerful and effective method to contribute to the recognition and conservation of biodiversity. It remains to be seen however, the extent to which other phylogeographic studies in Mesoamerica show concordance with this study. Only through the analysis of various taxa in this region may we have clearer insights to the underlying forces that have shaped biodiversity and hence enlighten future conservation strategies for this regional biota.

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CRH is a conservation biologist with longstanding interest in the biodiversity of Mesoamerica. GK is a herpetologist who has worked extensively with the genus *Ctenosaura* in Mesoamerica. AG and DHL have a range of interests in population subdivision, gene flow and the interpretation of phylogeographic patterns. This work constituted part of CRH's PhD in DHL's molecular ecology lab at the University of Hull (UK).

Appendix

Samples used for nested clade analysis and their corresponding locations. Museum abbreviations: Senckenberg Museum of Natural History (SMF); Museo de Historia Natural de El Salvador (MUHNES); The Natural History Museum, London (BMNH); Museo de la Universidad de San Carlos, Guatemala (USAC); Museo de Zoología 'Alfonso Herrera', UNAM, México (MZFC); and Museo de Zoología de la Universidad de Costa Rica (MZUCR)

Museum	Voucher no.	Work no.	GPS	Number and locality
SMF	79506	ES1	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
MUHNES	301223	ES2	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
SMF	79507	ES3	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
SMF	79508	ES4	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
SMF	79509	ES5	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
BMNH	2000.5	ES6	13°49.31′N, 87°57.6′W	1) Corinto, Morazán, El Salvador
SMF	79511	ES7	13°49.45′N, 87°57.15′W	2) Aguacate, Morazán, El Salvador
MUHNES	301226	ES11	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
MUHNES	301227	ES12	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
SMF	79513	ES13	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
SMF	79512	ES14	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
BMNH	2000.6	ES15	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
BMNH	2001.4	ES16	13°49.87′N, 87°58.02′W	3) El Junco, Morazán, El Salvador
SMF	79515	ES18	13°49.45′N, 87°57.15′W	2) Aguacate, Morazán, El Salvador
SMF	79514	ES19	13°49.45′N, 87°57.15′W	2) Aguacate, Morazán, El Salvador
BMNH	2000.7	ES20	13°49.45′N, 87°57.15′W	2) Aguacate, Morazán, El Salvador
released	—	ES21	13°40.15′N, 87°47.15′W	4) El Sauce, La Unión, El Salvador
MUHNES	301229	ES22	13°40.15′N, 87°47.15′W	4) El Sauce, La Unión, El Salvador
MUHNES	301230	ES24	13°49.45′N, 87°57.15′W	2) Aguacate, Morazán, El Salvador
released	—	ES25	14°20.30′N, 89°22.05′W	5) Santa Rita, Metapan, El Salvador
released	—	ES26	14°23.14′N, 89°24.08′W	6) Casa de Tejas, Metapan, El Salvador
released	—	ES27	14°23.14′N, 89°24.08′W	6) Casa de Tejas, Metapan, El Salvador
released	—	ES28	14°23.14′N, 89°24.08′W	6) Casa de Tejas, Metapan, El Salvador
released	—	ES29	14°23.14′N, 89°24.08′W	6) Casa de Tejas, Metapan, El Salvador
MUHNES	301231	ES32	13°42.31′N, 88°34.11′W	7) San Ildefonso, San Vicente, El Salvador
USAC	559	G1	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
SMF	79418	G3	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
BMNH	2000.2	G4	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
SMF	79415	G5	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
BMNH	2000.3	G9	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
SMF	79417	G7	14°25.11′N, 89°35.02′W	8) SE El Rincón, Jutiapa, Guatemala
SMF	79505	G11	14°25.51′N, 89°35.52′W	9) NW El Rincón, Jutiapa, Guatemala
SMF	79416	G10	14°25.51′N, 89°35.52′W	9) NW El Rincón, Jutiapa, Guatemala
BMNH	2000.1	G6	14°25.51′N, 89°35.52′W	9) NW El Rincón, Jutiapa, Guatemala
USAC	561	G14	14°25.51′N, 89°35.52′W	9) NW El Rincón, Jutiapa, Guatemala
released	—	G18	14°25.51′N, 89°35.52′W	9) NW El Rincón, Jutiapa, Guatemala
SMF	79521	N1	12°25.65′N, 85°53.5′W	10) Haciento Viejo, Teustepe, Nicaragua
SMF	79522	N3	12°25.05′N, 85°52.13′W	11) Teustepe, Boaco, Nicaragua
SMF	79523	N4	12°25.05′N, 85°52.13′W	11) Teustepe, Boaco, Nicaragua
SMF	79524	N5	12°25.05′N, 85°52.13′W	11) Teustepe, Boaco, Nicaragua
SMF	79526	N6	13°14.23′N, 86°30.02′W	12) 1 km San Fco. Norte, Esteli, Nicaragua
SMF	79529	N7	13°14.31′N, 86°30.56′W	13) 6 km San Fco. Norte, Esteli, Nicaragua
SMF	79528	N8	13°14.31′N, 86°30.56′W	13) 6 km San Fco. Norte, Esteli, Nicaragua
SMF	79527	N9	13°14.31′N, 86°30.56′W	13) 6 km San Fco. Norte, Esteli, Nicaragua
SMF	79530	N10	12°25.05′N, 85°52.13′W	11) Teustepe, Boaco, Nicaragua
SMF	79532	N11	12°25.05′N, 85°52.13′W	11) Teustepe, Boaco, Nicaragua
SMF	79516	H1	13°28.43′N, 86°08.25′W	14) Orocuina, Choluteca, Honduras
SMF	79517	H2	13°28.43′N, 86°08.25′W	14) Orocuina, Choluteca, Honduras
SMF	79519	H3	13°46.43′N, 86°11.83′W	15) Montegrande, Fco. Morazán, Honduras
SMF	79520	H4	13°46.43′N, 86°11.83′W	15) Montegrande, Fco. Morazán, Honduras
SMF	79518	H5	13°46.43′N, 86°11.83′W	15) Montegrande, Fco. Morazán, Honduras
MZFC	12435	M1	16°31.05′N, 94°27.12′W	18) Nisanda, Oaxaca, México
MZFC	12439	M5	16°34.14′N, 94°36.32′W	19) Niltepec, Oaxaca, México

Appendix *Continued*

Museum	Voucher no.	Work no.	GPS	Number and locality
MZFC	12440	M6	16°34.14′N, 94°36.32′W	19) Niltepec, Oaxaca, México
MZFC	12469	M7	16°31.05′N, 94°27.12′W	18) Nisanda, Oaxaca, México
MZFC	12470	M8	16°31.05′N, 94°27.12′W	18) Nisanda, Oaxaca, México
MZFC	12443	M9	16°31.05′N, 94°27.12′W	18) Nisanda, Oaxaca, México
MZFC	12444	M10	16°31.05′N, 94°27.12′W	18) Nisanda, Oaxaca, México
MZFC	12445	M11	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
MZFC	12446	M12	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
MZFC	12447	M13	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
MZFC	12441	M16	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
MZFC	12442	M17	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
released	—	M18	16°30.45′N, 95°04.02′W	20) Mixtequilla, Oaxaca, México
SMF	79129	H11	13°55.17′N, 88°23.43′W	16) Santa Lucia, Intibucá, Honduras
SMF	79127	H12	13°55.17′N, 88°23.43′W	16) Santa Lucia, Intibucá, Honduras
SMF	79128	H6	13°55.17′N, 88°23.43′W	16) Santa Lucia, Intibucá, Honduras
SMF	77084	H8	14°16.23′N, 87°40.02′W	17) La Paz, Honduras
SMF	80897	H9	14°16.23′N, 87°40.02′W	17) La Paz, Honduras
MZUCR	12677	C1	10°52.02′N, 85°40.23′W	21) Guajiniquil, Costa Rica
MZUCR	13625	C4	10°52.02′N, 85°40.23′W	21) Guajiniquil, Costa Rica