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FILOGENIA MOLECULAR DEL GRUPO *Sceloporus spinosus* (SQUAMATA: PHRYNOSOMATIDAE) Y EVOLUCIÓN DE LA RETENCIÓN UTERINA DE LOS HUEVOS: EVALUACIÓN DE FACTORES RELACIONADOS CON EL SURGIMIENTO DE LA VIVIPARIDAD

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P R E S E N T A

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ÍNDICE GENERAL

RESUMEN

El presente trabajo se enmarca en el contexto del estudio de la evolución de la viviparidad en escamados. En este caso evaluamos la evolución de la capacidad de retención y avance embrionario en hembras de distintas poblaciones del grupo *Sceloporus spinosus*. La variación de esta característica así como su relación con algunos factores ambientales se analiza en un contexto filogenético.

Para esto obtuvimos una hipótesis de filogenia derivada de secuencias de dos genes mitocondriales, ND4 y 12S, obtenidas de 66 individuos de 53 localidades representativas de las seis subespecies del grupo. La exclusividad de las especies y subespecies no se recupera en esta hipótesis. A partir de la relación entre los haplotipos y su distribución geográfica sugerimos que el grupo está constituido por siete especies.

Colectamos hembras de siete localidades representativas de distinto linajes mitocondriales. Estos individuos se mantuvieron en cautiverio y bajo dos condiciones experimentales que permitieron detectar la presencia de capacidad de retener los huevos y su efecto sobre el desarrollo de los embriones. Se evaluó la relación entre el avance embrionario y el grosor de la membrana de la cáscara, masa relativa de la nidada y frecuencia de nidadas. Igualmente, se analizó la relación entre la capacidad de avance y algunos factores climáticos como temperatura y precipitación, todo esto en un contexto filogenético.

Nuestros resultados indican que la capacidad de retención y avance embrionario en las hembras de este grupo no está relacionada con ninguno de los factores evaluados. No obstante, el patrón filogenético de la variación interpoblacional del estado máximo de desarrollo embrionario (32.5, 34/35) observado durante la retención, sugiere una relación entre esta variación y una modificación ancestral en la distribución altitudinal del grupo.

ABSTRACT

This work is focused on the study of the evolution of squamate viviparity. Particularly, we evaluated the evolution of egg retention and its effect on intrauterine embryonic development in females of the *Sceloporus spinosus* group. Variation of these traits as well as their relationship with physiological and environmental factors was evaluated in a phylogenetic context.

We obtained a phylogenetic hypothesis for this species group from the mitochondrial genes ND4 and 12S sequenced from sixty six individuals from fifty three localities, which represent the taxonomic subspecies of the *S. spinosus* group. The exclusivity of species and subspecies was not recovered in this hypothesis. We recognized seven species based on haplotypes relationship as well as their geographic distribution and taxonomic composition of clades.

We collected females from seven localities representative of different mitochondrial lineages. Individuals were maintained in captivity and under experimental conditions in order to detect egg retention ability and its effect on embryonic development *in utero*. Relationship of the embryonic development stage with eggshell membrane thickness, relative clutch mass and clutch frequency was estimated. Similarly, we contrasted the stage of embryonic development with climatic parameters such as temperature and precipitation; all this, under a phylogenetic context.

Our results indicate that the ability of females to retain eggs, which exhibit further embryonic development *in utero*, is not related to any of these factors. Nonetheless, the phylogenetic pattern of variation among localities in the maximum stage of development (32.5, 34/35) observed during egg retention, suggests a relationship between this variation and an ancestral shift in altitudinal distribution within this species group.

1. INTRODUCCIÓN

La evolución del modo reproductor en reptiles escamados se ha abordado de forma separada desde dos perspectivas complementarias que incluyen el contexto filogenético y el ecológico. La información generada de estos trabajos sugiere una transición de ovíparo a vivíparo[1] estimulada principalmente por el clima frío, cuyo efecto negativo sobre los embriones en los nidos estimuló su retención y avance en el útero[2]. Esta observación se deriva de la relación existente entre la altitud y el modo reproductor en escamados en general y en especies cercanas que difieren en esta característica reproductiva, así como de estudios experimentales en los que se evalúa el efecto de distintos regímenes de temperatura sobre la supervivencia y el desempeño de las crías en distintas especies de lagartijas[2,3]. Se ha propuesto además que esta transición en el modo reproductor requirió de cambios a nivel morfofisiológico que involucran tanto al oviducto como al huevo, así como cambios en el número y tamaño de nidadas[4], entre otros. El adelgazamiento del cascarón es una modificación asociada a un incremento en el avance de los embriones en tres especies de lagartijas con reproducción bimodal, esto es, especies en las que unas poblaciones son ovíparas y otras son vivíparas[5,6,7,8], mientras que el número de nidadas parece estar relacionada con la capacidad de avance embrionario en algunas especies ovíparas de *Sceloporus*[9]. En general se considera que estas modificaciones y por lo tanto, la transición de oviparidad a viviparidad ocurrieron de manera gradual. La existencia de especies bimodales con variación en el grado de avance embrionario al momento de la oviposición ofrece evidencia a favor de esta idea. Una predicción de esta hipótesis es precisamente que la cercanía filogenética de especies ovíparas con especies vivíparas supone para las primeras, la existencia de capacidad de retención y avance embrionario más allá del observado comúnmente en especies ovíparas (estado 30-31) [El estado de desarrollo embrionario se establece con base en la presencia de características morfológicas que surgen en distintos momentos durante la embriogénesis[10]]. La evaluación de esta idea requiere de la comparación de

grupos cercanamente relacionados y con distinto modo reproductor. Este tipo de estudios se han realizado en el género *Sceloporus*, [el cual presenta cuatro orígenes independientes de la viviparidad[11], ver Figura 1] y los resultados obtenidos sustentan esta hipótesis[9,12,13]. Sin embargo, el grupo ovíparo *Sceloporus spinosus*, hermano del grupo vivíparo *S. formosus* no fue incluido en estos estudios. Por esta razón se propone evaluar si las hembras el grupo *S. spinosus* pueden retener los huevos en el útero y qué ocurre con el desarrollo embrionario durante este periodo.

La variación en la expresión de características fenotípicas, en este caso la capacidad de retención y avance del desarrollo embrionario, debe ser evaluada tanto en un contexto ambiental como filogenético. Esto debido a que la evolución de las características de interés puede estar respondiendo tanto al ambiente como a las restricciones impuestas por la historia de los organismos. Así por ejemplo, un alto componente ambiental en la expresión fenotípica de una característica es indicador de su naturaleza adaptativa, a partir de lo cual se infieren procesos evolutivos, selección natural en este caso, para el carácter en cuestión. Por otro lado, parte de la variación ínterespecífica en un carácter puede encontrarse determinada por las relaciones de parentesco entre especies, las cuales se reconstruyen a través de análisis filogenéticos. Por lo tanto, el fraccionamiento de la expresión de una característica en su componente ambiental y filogenético es relevante no sólo para detectar patrones evolutivos sino también para a partir de éstos inferir procesos. Así pues, la integración de la filogenia de los organismos, mediante el método comparativo filogenético, es imprescindible en estudios evolutivos[14].

En el caso del grupo *S. spinosus* no se cuenta con una hipótesis de su filogenia y dado que este estudio aborda la evolución de la capacidad de retención de huevos resulta necesario plantear un análisis filogenético. Además, este grupo de especies presenta problemas taxonómicos cuya solución requiere entre otras,

una perspectiva filogenética. La problemática taxonómica del grupo se expone a continuación.

El grupo *S. spinosus* está constituido por dos especies morfológicas, *S. horridus* y *S. spinosus*, cada una con tres subespecies[15]. En análisis filogenéticos previos, en donde se incluyen escasas muestras representativas de algunas subespecies del grupo, se sugiere que *S. spinosus* no es monofilética[15,16] y que ésta puede ser dividida en al menos tres linajes. Sin embargo, la monofilia de *S. horridus* no fue evaluada dado que sólo se emplearon muestras de una de sus tres subespecies. Por lo tanto, se propone realizar un análisis filogenético con base en caracteres derivados de dos fragmentos de genes mitocondriales (ND4 y 12S) y en un muestreo taxonómico más intenso con el fin de evaluar la monofilia de los taxones que componen este grupo de especies.

Los datos obtenidos sobre la capacidad de avance embrionario en condiciones de retención de los huevos, así como la relación entre estado embrionario y grosor de la membrana de la cáscara, masa relativa de la nidada y número de nidadas serán interpretados teniendo en cuenta esta información filogenética, así como las condiciones climáticas de las localidades de las que proceden los ejemplares. Esto con el fin de entender la evolución de la capacidad de retención y avance embrionario en *S. spinosus* y por tanto, tener una aproximación sobre la evolución de la viviparidad en su grupo hermano.

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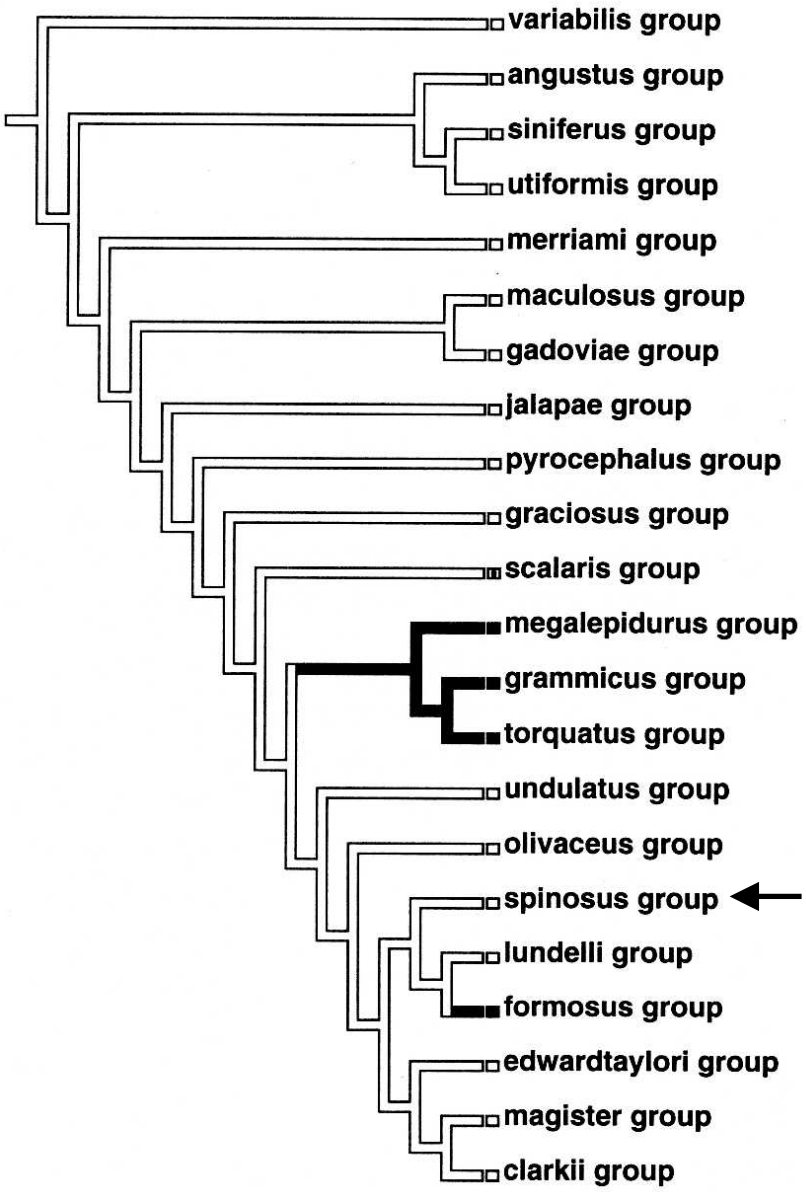

Figura 1. Filogenia del género *Sceloporus*. Tomado de Méndez De la Cruz et al. (1998), quienes a su vez se basaron en la filogenia propuesta por Wiens y Reeder (1997). Las ramas resaltadas en negro identifican a grupos vivíparos. Se señala con una flecha el grupo de estudio *S. spinosus*. El grupo *S. lundelli* es incorporado al grupo *S. formosus* en un análisis filogenético posterior (Smith, 2001).

2. OBJETIVOS

OBJETIVO GENERAL

Evaluar en un contexto filogenético la evolución de la capacidad de retención uterina de los huevos y su efecto en el desarrollo embrionario en el grupo *Sceloporus spinosus.*

OBJETIVOS ESPECÍFICOS

Capítulo 1. Molecular phylogeny of the *Sceloporus spinosus* group (Squamata: Phrynosomatidae)

1. Proponer una hipótesis de la filogenia molecular del grupo *Sceloporus spinosus* con base en caracteres derivados de los genes mitocondriales ND4 y 12S
2. Sugerir límites de especie al interior de este grupo

Capítulo 2. Evolution of egg retention in the *Sceloporus spinosus* group: exploring the role of physiological, environmental, and phylogenetic factors

1. Determinar si las hembras del grupo *Sceloporus spinosus* presentan la capacidad de retención y avance embrionario
2. Evaluar si esta característica presenta variación geográfica
3. Establecer si existe relación entre la capacidad de avance y el grosor de la membrana de la cáscara, masa relativa de la nidada y número de nidadas
4. Establecer si existe relación entre la capacidad de avance y algunos factores climáticos como temperatura y precipitación

1. MOLECULAR PHYLOGENY OF THE *SCELOPORUS SPINOSUS* GROUP (SQUAMATA: PHRYNOSOMATIDAE)

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Molecular phylogeny of the *Sceloporus spinosus* group (Squamata: Phrynosomatidae)

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Abstract

A phylogenetic hypothesis derived from 1611 bp of the mitochondrial genes ND4 (and associated tRNAs) and 12S is proposed for the *Sceloporus spinosus* group. Sampled localities (66) represent all the currently recognized species and subspecies within this group including *S. h. horridus, S. h. albiventris, S. h. oligoporus, S. s. apicalis, S. s. caeruleopunctatus* and *S. s. spinosus*. Maximum parsimony and Bayesian criteria were employed to analyze the data matrix. The hypothesis obtained strongly supports the monophyly of the group, but species and subspecies exclusivity is rejected. Thus, we suggest that seven lineages, which are incongruent with current taxonomy, constitute the *S. spinosus* group.

Key words: mtDNA — ND4 — 12S — *Sceloporus spinosus* group

Introduction

The *Sceloporus spinosus* group has been regarded as monophyletic (Wiens and Reeder 1997; Smith 2001) and consists of two morphological species (*S. horridus* Wiegmann and *S. spinosus* Smith) widely distributed in México, where

they occupy mostly xeric habitats (Smith and Smith 1951; Frost 1973). These species exhibit different altitudinal distributions, with *S. horridus* ranging from sea level to approximately 1500 m, and *S. spinosus* from approximately 1500 m up to 2400 m (Smith and Smith 1951; Frost 1973; this study). Each of these species is composed of three subspecies: *S. h. albiventris* Smith, *S. h. horridus* Smith, and *S. h. oligoporus* Cope; and *S. s. apicalis* Smith and Smith, *S. s. caeruleopunctatus* Smith, and *S. s. spinosus* Wiegmann, respectively. The distribution of these taxa, based on Smith (1939), Frost (1973), Smith and Chiszar (1992), and this study (Appendix 1), is shown in Fig. 1.

Recent studies, however, suggested that *S. spinosus* is not monophyletic (Wiens and Reeder 1997; Smith 2001). In one of these studies, *Sceloporus s. spinosus* from the Tehuacán area in southern Puebla was more closely related to *S. h. horridus* than to *S. s. spinosus* from Querétaro (Smith 2001), whereas in the other *S. s. caeruleopunctatus* was more closely related to *S. h. horridus* than to *S. s. spinosus* (Wiens and Reeder 1997). Nonetheless, these findings are based on mtDNA sequences from very few individuals, and none of these studies included representatives of all of the subspecies of *S. horridus* and *S. spinosus*.

Historically, *S. horridus* and *S. spinosus* have been distinguished on the basis of two characters: femoral pore number (≤ 6 per side in *S. horridus*; ≥ 7 per side in *S. spinosus*) and frequency of supraocular-median head scales contact (almost 100% in *S. horridus*; nearly absent in *S. spinosus*; Smith 1939; Smith and Chiszar 1992). Nonetheless, this distinction has not been absolute. Variation in the above characters in a series of individuals from the vicinity of Tlaxiaco, in western Oaxaca, was interpreted as evidence of intergradation between *S. h. horridus* and *S. s. spinosus*, and these taxa were accordingly regarded as conspecific (Boyer et al. 1987). Later, the same individuals were reinterpreted as intergrades between *S. s. spinosus* and *S. s. apicalis*, despite the fact that the latter taxon has not been recorded in the region, and *S. horridus* and *S. spinosus* were returned to specific status (Smith and Chiszar 1992). Also, because of further variation found in the supraocular-median head scales contact, only the

number of femoral pores has been considered as unequivocal to separate these taxa (Smith and Chiszar 1992). Wiens and Reeder's (1997) and Smith' (2001) studies further suggest that these characters do not reflect accurately the number of species nor the species limits in the *S. spinosus* group.

Similarly, subspecies delimitation within *S. spinosus* based on external morphology has been problematical. Essentially, these subspecies differ only slightly from each other in their averages for one or two quantitative characters (numbers of femoral pores and dorsal and supraocular scales), yet their corresponding variation ranges for these characters often overlap considerably (e.g., Smith and Chiszar 1992). Also, these taxa theoretically intergrade in several localities in central and western Oaxaca (*S. s. spinosus* x *S. s. apicalis*) and central and southern Oaxaca (*S. s. apicalis* x *S. s. caeruleopunctatus* and *S. s. spinosus* x *S. s. apicalis*). As a consequence, "subspecific allocations based on series of one or two (specimens) are inevitably suspect and only series of six or more permit reasonably secure identifications" (Smith and Chiszar 1992). This is particularly aggravated in the case of *S. s. apicalis* and *S. s. caeruleopunctatus*, which have intricately interdigitated distributions in central and southern Oaxaca (Smith and Chiszar 1992). Thus, a reevaluation of the status of these taxa and their geographic distributions using molecular data seems appropriate.

On the other hand, subspecies limits within *S. horridus* are relatively well defined. Nonetheless, interaction between *S. h. horridus* and *S. h. oligoporus* appears to be restricted to a narrow area in the Balsas Basin in Guerrero (Frost 1973); also, both of these taxa have been recorded in each of three localities in this region (Saldaña and Pérez 1987). This suggests specific status for these subspecies. Similarly, specific status for *S. h. albiventris* and *S. h. oligoporus* has been suggested on the basis of their dichopatric distribution on either side of the Sierra Madre Occidental (north of 20° N), the lack of evidence of intergradation between them, and the sharp difference in male ventral coloration (Lemos-Espinal et al. 2004). However, this statement ignores most populations

of these taxa (from 20° N south to Guerrero), and no data were provided to support it. For instance, intergradation between *S. h. albiventris* and *S. h. oligoporus* in Jalisco and Nayarit has been proposed since a long time ago (Frost 1973).

Herein, we performed a phylogenetic analysis of the *S. spinosus* group with mtDNA sequence data to reevaluate the monophyly of *S. horridus* and *S. spinosus* and the status and relationships of their subspecies.

Materials and Methods

Taxon sampling

Sixty-six individuals from 53 localities representing each species and subspecies of the *S. spinosus* group were surveyed (Appendix 1). Collecting localities and the morphotectonic provinces described by Ferrusquía-Villafranca (1998) for Mexico are shown in Fig. 1. Province names are abbreviated throughout the text as per Fig. 1. The rank of specific morphotectonic units (province, subprovince, or infraprovince) is indicated in subscript (P, SP, and IP, respectively).

Sceloporus formosus, (sister taxon to the *S. spinosus* group, Wiens and Reeder 1997; Smith 2001), *S. olivaceus* and *S. torquatus* were included as outgroups. Taxonomic assignment was based on the diagnostic characters used by Smith (1939), Smith and Smith (1951), Frost (1973), and Smith and Chiszar (1992). Individual identification was generally unproblematic. Some surveyed individuals were females from the area where *S. h. horridus* and *S. h. oligoporus* intergrade in Guerrero. Females do not exhibit one of the two diagnostic characters employed to differentiate between these subspecies. Thus, in order to assign these females, additional material (including adult males) was examined from the same or nearby localities. In all of the cases, these assignments were unambiguous.

Laboratory protocol

DNA was extracted from liver tissue following the ammonium acetate precipitation method of Fetzner (1999). We amplified the following regions of the mitochondrial DNA: a portion of the gene encoding the fourth unit of the NADH dehydrogenase (ND4), the adjacent genes encoding $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ (part), and a portion of the ribosomal gene encoding 12S. These regions have been successfully employed to elucidate phylogenetic relationships among closely related species in the genus *Sceloporus* (Arévalo et al. 1994; Benabib et al. 1997; Flores-Villela et al. 2000; Smith 2001; Leaché and Reeder 2002; Wiens and Reeder 1997). The ND4-tRNAs region was amplified with the primers designed by Arévalo et al. (1994) (forward 5' TGACTACCAAAAGCTCATGTAGAAGC 3', and reverse 5' TRCTTTTACTTGGATTTGCACCA 3'),whereas the 12S region was obtained with the primers reported in Leaché and Reeder (2002) (forward 5' AAAGCAC (A/G) GCACTGAAGATGC 3' and reverse 5' GT(A/G)CGCTTACC(A/T)TGTTACGACT 3'). An additional primer was designed for sequencing the reverse strand of the 12S region in some samples (5' TATCCGCCAGAARACTACGA 3'). Polymerase Chain Reaction (PCR) temperature protocol was as follows: an initial denaturation at 94 ºC for 5 min, followed by 35-38 cycles at 94 ºC for 1 min, 50-52 ºC for 1 min, and 72 ºC for 1-2 min, and a final extension at 72 ºC for 5 min. PCR products were cleaned with PEG/NaCl precipitation (Leaché and Reeder 2002) and sequenced with the Big Dye Terminator v. 3.1 cycle sequencing kit and an ABI 3100 Genetic Analyzer sequencer.

Phylogenetic analysis

Sequences were edited and assembled with Sequencher™ version 4.1 (Gene Codes Corporation 2000), and aligned with Clustal W (Thompson et al. 1994) using default penalties for gap opening and gap extension. The alignment was then adjusted visually with MacClade version 4.0 PPC (Madisson and Madisson 2000). Alignment of the tRNAs and 12S regions, despite their secondary

structure, was generally unproblematic. However, a 16 bp long fragment was found in the12S region that could not be unambiguously aligned. For this fragment, alternative alignments were performed using different penalties for gap opening (10, 5) and gap extension (1, 5), and two slightly different alignments were obtained. Maximum parsimony (MP) analyses of each alignment (12S region only), with and without the ambiguously aligned fragment, were then performed to evaluate the effect of the inclusion of the fragment on tree topology. When included, gaps were treated as missing data.

Base frequencies and pair wise sequence divergences were estimated with MEGA version 2.1 (Kumar et al. 2001), and statistical tests were performed with PAUP* version 4.0b10 (Swofford 2000).

Congruence among data partitions was assessed using the incongruence length difference test (ILD) (Farris et al. 1994). The separate data sets (ND4-tRNAs and 12S) were analyzed with MP methods, whereas the combined data sets were analyzed using MP and Bayesian methods. Maximum parsimony analyses consisted of heuristic searches with tree bisection and reconnection (TBR) branch swapping and 10000 random sequence addition replicates. Characters were treated as unordered and equally weighted and gaps were treated as missing data. Branch support was evaluated with 1000 non-parametric bootstrap pseudoreplicates (Felsenstein 1985). Each pseudoreplicate consisted of a heuristic searches with 10 random taxon addition replicates and TBR branch swapping. Branches recovered with a frequency higher than 70% are presented and considered as strongly supported (Hillis and Bull 1993).

Substitution models were estimated with Modeltest version 3.7 (Posada and Crandall 1998). Models were obtained for five data partitions: the first, second and third codon positions of ND4, the tRNAs and 12S. For each data partition, the two models with the highest likelihood values were selected from the uncertainty table of models generated with Bayesian criteria (Table 1). These alternative models were implemented only in the total evidence analysis.

Bayesian MCMC analyses of the complete data set were run in MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Each analysis was run for 2 x 10^6 or 5 x 10^6 generations with four chains, random starting trees, and uniform prior distribution of parameters. Trees were sampled each 100 generations. A pooled, 50% majority-rule consensus tree was computed from the sampled trees, excluding trees obtained before stationary (i.e., burn-in trees as indicated by plotting *-ln*L scores against generation time). Clades with frequencies > 95% were considered as strongly supported (Huelsenbeck and Ronquist 2001).

The Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1998) was used to compare the likelihood value for our preferred hypothesis with the values for hypotheses in which each taxon of the *S. spinosus* group was constrained as monophyletic.

Results

Sequence description

In total, 836 bp corresponding to the ND4 and tRNAs genes (681 and 155 bp, respectively) and 775–791 bp corresponding to the 12S gene (i.e., excluding and including the ambiguously-aligned fragment, respectively) were analyzed. Two hundred and thirty phylogenetically informative sites were found in the ND4-tRNAs region, and 88–94 in the 12S region (excluding and including the ambiguously-aligned fragment, respectively). A total of 59 different haplotypes corresponding to the ND4-tRNAs genes and 46 corresponding to the 12S gene were found. Base composition for both the ND4-tRNAs and 12S regions was negatively G-biased (G = 12.2%, A = 33.7%, C = 28.2%, and T = 25.9%; G = 17.8%, A = 37.2%, C = 23.1%, and T = 21.2%, respectively). Uncorrected genetic distances among ingroup and outgroup taxa ranged from 14.0% to 15.2%, whereas distances among ingroup taxa ranged from 2.0% to 9.6% (Table 2).

Phylogenetic analysis

Phylogenetic signal was highly significant for both the ND4-tRNAs and 12S regions (ND4-tRNAs: $g1 = -0.46$, $P < 0.01$; 12S: $g1 = -0.56$, $P < 0.01$). Alternative alignments of the ambiguously-aligned fragment of the 12S region had no effect on the tree topology obtained from this region. On the other hand, tree topologies from analyses of the 12S region including and excluding the ambiguously-aligned fragment were different. However, these differences involved only poorly supported clades. Because of its small size (16 bp) and negligible effect on the results, we excluded the ambiguously-aligned fragment from further analyses.

The partition homogeneity test indicated that the ND4-tRNAs and 12S data sets were combinable (P = 0.8). Overall, hypotheses obtained from this combined data set were similar to hypotheses obtained from the ND4-tRNAs data set alone, although including the 12S fragment slightly increased bootstrap branch support values.

Bayesian analyses reached stationary after 70000 to 80000 generations. Tree topologies from analyses using alternative models of substitution were only slightly different from the topology from the analysis using the best-fitting models. Differences between the former and latter analyses involved only the northern haplotypes of *S. s. spinosus*, which were recovered in two different, well supported clades in the analysis using the best-fitting models, and in a single, poorly supported clade (BPP = 60%) in analyses using alternative models. Except for a single increase in clade BPPs when a model with slightly lower likelihood value was implemented (from 90% to 96%, Fig. 2), BPP values using alternative models were not different from values using the best-fitting models.

On the other hand, the strict consensus tree from the parsimony analysis (not shown) and the pooled 50% majority rule consensus tree from the Bayesian analyses were generally similar, with the few minor differences involving relationships within some small distal clades. Most clades recovered in these hypotheses exhibited similar branch support values (Fig. 2). Thus, the pooled

50% majority-rule consensus tree from the Bayesian analyses using the best-fitting model of substitution for each data partition represents our preferred phylogenetic hypothesis.

Phylogenetic relationships

In this hypothesis (Fig. 2), the monophyly of the *Sceloporus spinosus* group was strongly supported, and five major, strongly supported clades originated sequentially from the group's basal node. Clade A contains haplotypes assigned to *S. h. albiventris* and *S. h. oligoporus* from the SMO_P, Pacific Coastal $Plain_{SP}$ (NPS_P), western end of the $TMVB_P$, and northwestern end of the Balsas $Depression_{SP}$, SMS_P, in western México from Sonora south to Michoacán. Clade B contains the northernmost haplotypes assigned to *S. s. spinosus* (from the foothills and mountain ranges of the $SMOR_P$ in San Luis Potosí and Tamaulipas). Clade C is composed of haplotypes assigned to *S. s. spinosus* from the Southern $Valleys$ and $Sierras_{SP}$ of the CP_P and the central part of the $TMVB_P$ in Jalisco, Querétaro, and Mexico. Clade D comprises the haplotypes assigned to *S. s. apicalis* and *S. s. caeruleopunctatus* from the Central $Valleys_{IP}$ and Miahuatlán $Ranges_{IP}$ in the Oaxaca-Puebla $uplands_{SP}$ of the SMS_P. [The sister groups to clades A–D also were well supported, except for the sister group to clade B, which was only well supported in the parsimony analysis (BPP = 90%; bootstrap value = 74%)].

The last major clade is composed of three strongly supported, slightly divergent clades of unresolved relationships (E–G; genetic distances among clades = 1.9–2.4%). Clade E comprises haplotypes assigned to *S. s. spinosus* and *S. h. horridus* from the Mixteco-Zapotecan Sierras and $Highlands_{IP}$ in Oaxaca-Puebla $uplands_{SP}$ and the Tehuacán region and eastern portion of the $TMVB_P$ in Puebla. Clade F is composed exclusively of haplotypes assigned to *S. h. horridus* from the Tehuacán-Cuicatlán-Quiotepec $Depression_{IP}$ in Oaxaca-Puebla $uplands_{SP}$, and Clade G contains haplotypes assigned to *S. h. horridus* and *S. h. oligoporus* from the Pacific Coastal $Plain_{SP}$ and Pacific Ranges and

Cuestas$_{SP}$ in SMS$_{P}$ in Guerrero, southern slopes of the TMVB$_{P}$ in Michoacán and Mexico, and the Balsas Depression$_{SP}$ in Guerrero, Morelos and southwestern Puebla. All of the above clades are strongly congruent with geography, and allopatric or parapatric with regard to each others (Fig. 3).

Uncorrected genetic distances among the above clades ranged from 2.0% to 9.6% (Table 2).

The likelihood value for our preferred hypothesis was significantly higher than the values for hypotheses in which each of the species and subspecies of the *S. spinosus* group was constrained as monophyletic ($P < 0.01$). Thus, the monophyly of each of these taxa (and correspondence between current taxonomy and phylogenetic relationships within the group) was rejected with our data.

Discussion

Effect of substitution model

Bayesian analyses using alternative models of substitution generated different tree topologies. However, differences between topologies involved only the northern haplotypes of *S. spinosus* (Clades B and C), whose relationships were not well supported under any model. The well supported clades were not affected by the use of alternative models of substitution, which supports the idea that topology estimation is robust to model assumptions, and that differences in tree topology, when alternative models are implemented, usually involve weakly supported clades (Buckley et al. 2001; Bos and Posada 2005).

Phylogenetic relationships

Our data generated a nearly completely resolved phylogenetic hypothesis. Polytomies involved haplotypes from the same or very close localities within the most derived clades. In all of the phylogenetic analyses the monophyly of the *S. spinosus* group was strongly supported. Also, S. *horridus* and S. *spinosus* were not mutually exclusive. These results are congruent with those of Wiens and

Reeder (1997). Within the *S. spinosus* group, several well supported clades can be distinguished. The composition and relationships of these clades is discussed below.

Clade A was composed exclusively of haplotypes assigned to *S. h. oligoporus* and *S. h. albiventris*. Since all of the remaining haplotypes assigned to *S. h. oligoporus* were placed in the distantly related clade G, our phylogenetic hypothesis suggests that this taxon actually represents two different lineages. Although no morphological differences between these lineages are evident, the phylogenetic distinction between them is supported by their geographic separation. Whereas the haplotypes assigned to *S. h. oligoporus* in clade A were distributed from the northwestern end of the Balsas Depression$_{SP}$ in Michoacán (northwest from the Infiernillo Dam) north through Zacatecas, those assigned to *S. h. oligoporus* in clade G were distributed from the Balsas Depression$_{SP}$ in Michoacán (east from the Infiernillo Dam) south through Guerrero (Fig. 1). Thus, the name *S. oligoporus* should not be applied to populations of clade G, because the type locality for this taxon is in Colima (Frost 1973).

Within Clade A, haplotypes assigned to *S. h. oligoporus* and *S. h. albiventris* did not form mutually exclusive groups either, which seems to confirm that these taxa are conspecific. However, it has been recently affirmed that they are allospecific on the basis of their dichopatric distribution (north of 20º N) on either sides of the SMO$_P$, the absence of evidence of intergradation between them, and the sharp difference in adult male ventral coloration (Lemos-Espinal et al. 2004). Although it is true that the main character to separate *S. h. oligoporus* from *S. h. albiventris* is the presence of blue ventrolateral patches in adult males of the former taxon and their absence in males of the latter (Frost 1973), it is also true that the distribution of these taxa also extends south from 20° N, and that they have been stated to intergrade over a large area including Jalisco and Nayarit (Frost 1973), which was not mentioned by Lemos et al. (2004). Nevertheless, no intrapopulational polymorphism or intermediacy in this character have been reported in the latter region or elsewhere. This pattern of

geographic variation in adult male ventral coloration suggests that differential pressures of sexual selection exist among populations of this clade. Sexual selection is an important mechanism in the evolution of display coloration in Iguania (Cooper and Greenberg 1992). Hypotheses that involve natural selection mechanisms, like differences in predation pressure, have also been proposed to explain different degrees of sexual dimorphism (Andersson 1994). At any rate, it is not clear why any of these mechanisms should not produce different male ventral colorations throughout the geographic distribution of this clade. Wiens (1999) evaluated the phylogenetic pattern of sexual dimorphism in phrynosomatid lizards and concluded that losses of sexual dimorphism, through losses of conspicuous male coloration or gains of conspicuous female coloration, are repeated events in this family. Our results suggest that absence of adult male ventral coloration is best explained as multiple losses in clade A, which agrees with Wiens' (1999) conclusion.

Clades B and C were composed of the northernmost haplotypes assigned to *S. s. spinosus.* These haplotypes were distantly related to the remaining haplotypes assigned to *S. s. spinosus*, which were placed in clade E. This suggests that haplotypes in clades (B + C) and E actually represent different, distantly related lineages. Since some of the haplotypes in clade E were sampled near the type locality for *S. spinosus* (Cd. Puebla, Puebla), this name should not be applied to populations represented in clades B and C.

Given that populations in clades B and C appear to be morphologically indistinguishable, occur in the same or adjacent morphotectonic provinces, and there is no evident geographic barrier between them (Fig. 1), it is surprising that these clades are not sister to each other. Instead, in our preferred hypothesis clade C was closer to the southern clades, although this relationship was not strongly supported. If this relationship were supported by additional evidence, it would suggest that the morphological similarity of populations in clades B and C is the result of evolutionary stasis in clade C. However, it would be unclear what might have caused the separation of the C and southern clades from clade B.

Additional samples from the area between the geographic ranges of clades B and C are needed for a better understanding of their relationships.

Clade D was composed of haplotypes assigned to *S. s. apicalis* and *S. s. caeruleopunctatus*. Although haplotypes assigned to *S. s. caeruleopunctatus* did not form an exclusive group with regard to those assigned to *S. s. apicalis* (Fig. 2), our data can not resolve whether the opposite might be true. However, the geographic distribution that would be expected from an exclusive *S. s. apicalis* and a paraphyletic *S. s. caeruleopunctatus* (a small, peripheral range of the first with regard to that of the second) does not agree with the intricately interdigitated distribution of these taxa (Fig. 1). Thus, our data seem to confirm the conspecific status of *S. s. apicalis* and *S. s. caeruleopunctatus*.

On the other hand, the interdigitated distribution of these taxa suggests that they remain distinct (if subtly) across most of their distribution. Also, several areas of intergradation between *S. s. apicalis* and *S. s. caeruleopunctatus* have been proposed (Smith and Chiszar 1992). Interestingly, of two haplotypes (samples number 48 and 49) collected in one of these intergradation areas (between Oaxaca city and Mitla), one was assigned to *S. s. apicalis*, and the other one to *S. s. caeruleopunctatus* (Fig. 2). Thus, individuals with different morphologies also had relatively divergent haplotypes. This would suggest that this putative area of intergradation might actually represent an area of sympatry between different lineages. However, this is not supported by our limited data. More specimens from throughout the ranges of these two taxa and their putative areas of intergradation are needed to solve this issue.

The absence of morphological differences among populations of *S. h. horridus* (Frost 1973) and the low genetic distances among haplotypes assigned to this taxon (1.3–2.4%) appear to confirm that these populations represent a single lineage. However, haplotypes assigned to *S. h. horridus* did not constitute an exclusive group. Instead, they were dispersed across clades E, F, and G, and those in clades E and G were more closely related to haplotypes assigned to *S. s. spinosus* and "*S. h. oligoporus*," respectively, than to the remaining haplotypes

assigned to *S. h. horridus*. This suggests that the haplotypes assigned to *S. h. horridus* represent more than one lineage.

Within clade G, all of the haplotypes assigned to *S. h. horridus* comprised a nested, exclusive subclade. Whereas these haplotypes came from the Balsas Depression$_{SP}$ in Guerrero, Morelos, and southwestern Puebla, the remaining haplotypes, assigned to "*S. h. oligoporus*," came from the Balsas Depression$_{SP}$ in southeastern Michoacán (Fig. 1). Given that the *S. h. horridus* subclade was not well supported (BPP = 90%, bootstrap = 50%) and genetic distances among the haplotypes assigned to *S. h. horridus* and "*S. h. oligoporus*" were low (0.6–3.14%), all of these haplotypes appear to comprise a single lineage with clear-cut, congruent genetic and morphological geographic variation.

Alternatively, clade G might be composed of two different lineages. One lineage, presumably recently differentiated (genetically and morphologically), would be represented by the exclusive *S. h. horridus* subclade, and the other one by a paraphyletic "*S. h. oligoporus*". This hypothesis would be supported by the observation that interaction between the putative subspecies *S. h. horridus* and *S. h. oligoporus* seems to be restricted to a narrow contact zone (Frost 1973), which suggests limited genetic flow between them. Also, Saldaña and Pérez (1987) recorded both of these taxa in some localities in Guerrero, which would support their specific status, although these records need to be verified. Restricted genetic flow between *S. h. horridus* and "*S. h. oligoporus*" would not invalidate them as distinct species (Wiens and Penkrot 2002). Also, non-exclusive species are equivalent to exclusive species, since incomplete lineage sorting may render non-exclusive species at some point in their history (Neigel and Avise 1986).

Clade F contained all of the haplotypes assigned to *S. h. horridus* from the Tehuacán-Cuicatlán-Quiotepec Depression$_{IP}$. If all of the haplotypes in clade G represent a single lineage, haplotypes of clade F might also represent another well defined, genetically differentiated, geographic variant of this lineage. On the other hand, if the haplotypes assigned to *S. h. horridus* and "*S. h. oligoporus*" of

clade G represent distinct, sister lineages, then the former haplotypes and those of clade F would also represent different lineages. The name *S. h. horridus* would only be applied to populations represented in clade G, since the type locality of this taxon was restricted to Cuernavaca, Morelos (Frost 1973).

Haplotype relationships of clade E suggest interaction between *S. s. spinosus* and *S. h. horridus* in the northern end of the Tehuacán-Cuicatlán-Quiotepec Depression$_{IP}$ and and Mixteco-Zapotecan Sierras and Highlands$_{IP}$ in Puebla. Interestingly, interaction between different taxa of the *Sceloporus spinosus* group within this region had been previously hypothesized. Frost (1973) suggested ancient introgression of S. *horridus* with *S. spinosus* in the "upper Balsas Basin" based on the observation that some individuals of *S. horridus* exhibited no supraocular-median head scales contact, a putative diagnostic character of *S. spinosus*. Also, Boyer et al. (1987) interpreted a series of individuals from the Tlaxiaco region in northwestern Oaxaca as intergrades between *S. h. horridus* and *S. s. caeruleopunctatus*, concluding that S. *horridus* and S. *spinosus* were conspecific. Later, however, Smith and Chiszar (1992) regarded the Tlaxiaco series as the result of interaction between *S. s. spinosus* and *S. s. apicalis*, returning S. *horridus* and S. *spinosus* to specific status.

Given the traditionally recognized morphological distinctness of *S. h. horridus* and *S. s. spinosus*, their suggested broad sympatry in southern Puebla and northern Oaxaca (Smith and Chiszar 1992), and the absence of other taxa of the S. *spinosus* group from this region, it can be hypothesized that the morphological and molecular data represent evidence of hybridization between these two taxa in this region and that the individuals assigned to *S. h. horridus* in this clade reflect primary contact between females of *S. s. spinosus* and males of *S. h. horridus*, since their haplotypes are most closely related to those of *S. s. spinosus*. Neither Boyer et al.'s (1982) nor Smith and Chiszar's (1992) conclusions are supported by our results. *S. s. caeruleopuctatus* and *S. s. apicalis* do not appear to occur in the Tlaxiaco region, and haplotypes assigned to these two taxa were distantly related to those of clade E. Also, individuals

from the Tlaxiaco region included in this study were assigned straightforwardly to *S. s. spinosus*.

The identity of the individuals (heretofore labeled as *S. h. horridus*) hypothetically interacting with those of *S. s. spinosus* is uncertain. Although it is possible that all of the individuals assigned to *S. h. horridus* in clades E-G represent a single taxon, it is also possible that the haplotypes assigned to *S. h. horridus* in clades F and G represent two different lineages (see above) and, since clade E is geographically intermediate between clades F and G, the individuals of *S. h. horridus* in clade E could belong to any of these lineages, or both. However, the absence of haplotypes of the *S. h. horridus* type from clade E (i.e., haplotypes most closely related to those assigned to *S. h. horridus* of clades F or G) does not permit to know which of these lineages is involved and, in fact, it is hard to explain, since *S. h. horridus* must occur in this region. Clade F is geographically contiguous to clade E. However, it does not contain any *S. s. spinosus* haplotypes. Thus, the geographic contact between clades E and F could be interpreted as the southern end of the *S. s. spinosus* - *S. h. horridus* interaction described above.

The only characters that distinguish S. *horridus* from S. *spinosus* are the number of femoral pores ($\leq$ 6 for S. *horridus*, $\geq$ 7 for S. *spinosus*) and the frequency of supraocular-median head scales contact (almost 100% in S. *horridus*; rare in S. *spinosus*), and only the first character is considered as unequivocal (Smith and Chiszar 1992). However, this distinction has been made assuming that all of the populations currently assigned to each of these taxa represent a single lineage, which is not supported by our analysis. Thus, the assignment of haplotypes to *S. h. horridus* or *S. s. spinosus* in clade E based on these characters might be an artifact. In other words, it might be that all of the populations represented in clade E comprise a single lineage, with most individuals meeting the global definition of *S. s. spinosus* but a few with less femoral pores and/or supraoculars and median head scales in contact, which would be assigned to *S. h. horridus*. This would explain the absence of

haplotypes closely related to those of *S. h. horridus* in clades F or G. Variation in populations represented in this clade is indeed poorly known, given that few specimens from this region have been available until now (Smith and Chiszar 1992).

In summary, our results do not support the current taxonomy of the *Sceloporus* spinosus group. That S. *spinosus* is not monophyletic had been suggested by previous analyses (Wiens and Reeder 1997; Smith 2001). Also, that *S. s. caeruleopunctatus* is not the sister taxon to *S. s. spinosus* but to *S. horridus* had been previously suggested by morphological and molecular characters (Wiens and Reeder 1997).

The incongruence between taxonomy and phylogeny observed in this study also characterizes other species complexes of *Sceloporus*, including *S. jarrovi* (Wiens et al. 1999; Wiens and Penkrot 2002), *S. undulatus* (Leaché and Reeder 2002), and *S. magister* (Schulte et al. 2006). Like ours, these studies included only mitochondrial markers, which usefulness to elucidate lineage boundaries has been broadly controversial (Moritz 1994; Sites and Crandall 1997; Puorto et al. 2001). However, it is well known that these markers are more efficient to recover recent divergence events than nuclear markers, due to their smaller effective population size and their higher substitution rates (Moore 1995). Because it is recent divergence events that have likely created several molecularly distinct lineages within the *S. spinosus* group, mitochondrial markers seem to be useful for establishing species boundaries in this case, as have been in other species groups of *Sceloporus* (Wiens and Penkrot 2002).

Taxonomic implications

Our analysis suggests the following taxonomic implications: *Sceloporus s. spinosus* actually represents at least two evolutionary lineages. The name *S. spinosus* should be applied only to populations represented in clade E; populations represented in clades B and C likely constitute an unnamed lineage. *Sceloporus h. horridus* represents one or two evolutionary lineages. The name

S. horridus applies properly to populations previously assigned to *S. h. horridus* in clade G. Whether populations in clade F also represent *S. horridus* or an unnamed lineage is uncertain. Therefore, the identity of the populations previously assigned to *S. h. horridus* and hypothetically interacting with those of *S. s. spinosus* in the geographic range of clade E (see above) also is uncertain. *Sceloporus h. oligoporus* represents two evolutionary lineages, and *S. h. albiventris* is a synonym of *S. oligoporus*. The name *S. oligoporus* should be applied to all of the populations represented in clade A, whereas the populations previously assigned to *S. h. oligoporus* in clade G represent an unnamed lineage. *Sceloporus s. caeruleopunctatus* represents an independent lineage. The analysis also suggests that *S. s. apicalis* should be sinonymyzed with *S. s. caeruleopunctatus*; nevertheless, more data are needed to confirm this decision.

Finally, our study provides a historical framework for choosing the appropriate unities for evolutionary studies at both the macro and microevolutionary levels within the S. *spinosus* group. For example, sexual dichromatism is a feature appropriate for subsequent comparative analysis.

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APPENDIX 1

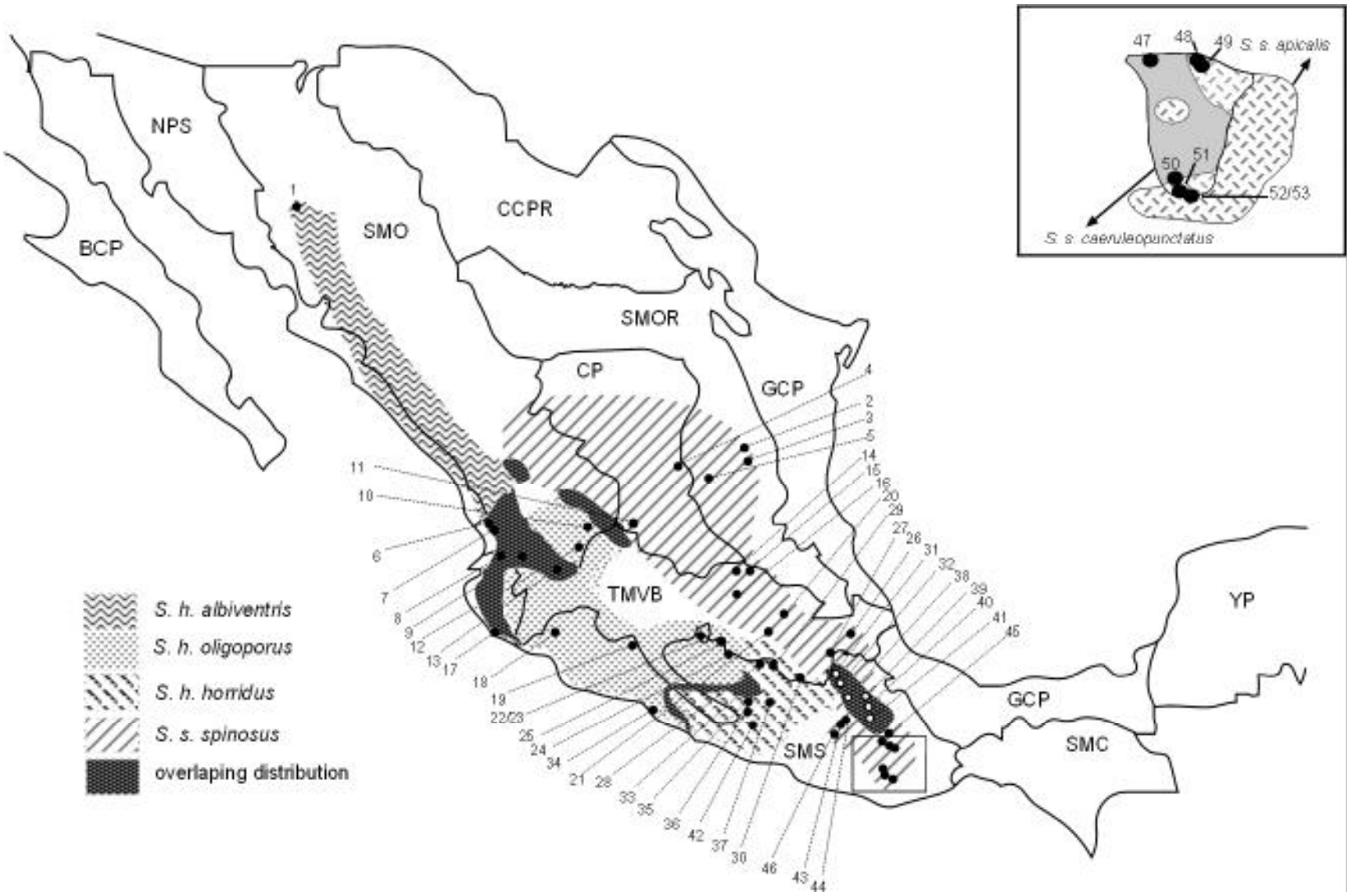
Specimens sequenced in this study. Acronyms: ANMO, A. Nieto Montes de Oca, EPR, E. Pérez Ramos; IBH, Instituto de Biología-Herpetología; JAC, J. A. Campbell; MC, M. Calderón; MZFC, Museo de Zoología Alfonso Luis Herrera; NM, N. Martínez; OFV, O. Flores-Villela; RRA, R. Reyes Avila; WSB, W. Schmidt Bayardo

Species	Locality No.	Locality	Vouchers	Accesion Number ND4+tRNAs	Accesion Number 12S
S.h.albiventris	1	Eastern San Nicolás, Sonora	MZFC 15176		
S.s.spinosus	2	33 Km NE Tula, Tamaulipas	MZFC 11216		
S.s.spinosus	3	N Ejido La Laguna, Mpio Tula, Tamaulipas	NM 134		
S.s.spinosus	4A	Alrededor pueblo El venado, San Luis Potosí	MC 043		
S.s.spinosus	4B	Alrededor pueblo El venado, San Luis Potosí	MC 046		
S.s.spinosus	5	Laguna de Gerando, San Luis Potosí	NM 112		
S.a.albiventris	6	Entronque Tepic-San Blas, Nayarit	MC 211		
S.a.albiventris	7	Caseta Santa María del Oro, Nayarit	MZFC 13081		
S.a.albiventris	8	Pueblo Francisco I. Madero, Nayarit	MC 212		
S.a.albiventris	9	Ixtlán del Río, Nayarit	MC 204		
S.a.oligoporus	10	Jalpa, Zacatecas	MC 203		
S.s.spinosus	11A	Puente Encarnación, Jalisco	MC 045		
S.s.spinosus	11B	Puente Encarnación, Jalisco	MC 202		
S.s.oligoporus	12	Puente Santa Rosa, Guadalajara-Zacatecas, Zacatecas	MC 205		
S.a.albiventris	13	Salida de Guadalajara, vía Tepic, Jalisco	MC 207		
S.s.spinosus	14A	Pueblo Nuevo, Querétaro	MZFC 9249		
S.s.spinosus	14B	Pueblo Nuevo, Querétaro	MZFC 9247		
S.s.spinosus	15	1 Km Rancho El Arbolito, Querétaro	MZFC 9764		
S.s.spinosus	16	Polotitlá, Estado de México	NM 122A		
S.a.albiventris	17 A	Chamela, Jalisco	IBH17824	EF025743	EF025751
S.a.albiventris	17 B	Chamela, Jalisco	IBH18823	EF025744	EF025752

S.h.oligoporus	18 A	Carretera 54 vía Guadalajara, Colima	MC 062		
S.h.oligoporus	18 B	Carretera 54 vía Guadalajara, Colima	MC 063		
S.h.oligoporus	18 C	Carretera 54 vía Guadalajara, Colima	MC 251		
S.h.oligoporus	19 A	Nva. Italia-Lombardía, Michoacán	MC 249		
S.h.oligoporus	19 B	Nva. Italia-Lombardía, Michoacán	MC 250		
S.s.spinosus	20 A	Cerro Gordo, Estado de México	MC 108B		
S.s.spinosus	20 B	Cerro Gordo, Estado de México	MC 124		
S.s.spinosus	21	Distrito Federal	MC 094		
S.h.oligoporus	22	Parícuaro, Michoacán	MC 246		
S.h.oligoporus	23	Timbuscatío, Michoacán	MC 226		
S.h.oligoporus	24	San Lucas, Michoacán	MC 223		
S.h.oligoporus	25	La isla, Estado de México	MC 065		
S.s.spinosus	26 A	Laguna La Preciosa, Puebla	IBH17829	EF025749	EF025756
S.s.spinosus	26 B	Laguna La Preciosa, Puebla	IBH17830	EF025750	EF025757
S.s.spinosus	27	Alrededores de Acozac, Puebla	ANMO 637		
S.h.horridus	28	Laguna El Rodeo, Morelos	IBH17825	EF025745	EF025753
S.h.horridus	28	Laguna El Rodeo, Morelos	MC 090		
S.h.horridus	29	Santa Inés, Morelos	IBH17826	EF025746	_____
S.h.horridus	30	Pueblo de Jaltianguis, Puebla	EPR 1165		
S.s.spinosus	31	3 Km S de Zamarrilla, Puebla	EPR 1173		
S.s.spinosus	32	Mpio. Zapotitlán de las Salinas, Puebla	ACS 199		
S.h.horridus	33 A	Xalitla, Guerrero	IBH17827	EF025747	EF025754
S.h.horridus	33 B	Xalitla, Guerrero	MC 230		
S.h.oligoporus	34 A	Puente Salitreras, vía Zihuatanejo-Lázaro Cárdenas, Guerrero	MC 224		
S.h.oligoporus	34 A	Puente Salitreras, vía Zihuatanejo-Lázaro Cárdenas, Guerrero	MC 225		
S.h.horridus	35	Km 179 vía México-Acapulco, Guerrero	ANMO 550		
S.h.horridus	36	25 Km N Zumpango, Guerrero	NM 54		
S.h.horridus	37	1 Km N Tlalcozotitlán	EPR 1112		

S.h.horridus	38 A	La Huerta, Mpio. Zapotitlán de las Salinas, Puebla	MC 228		
S.h.horridus	38 B	Jardín Botánico, Mpio. Zapotitlán de las Salinas, Puebla	MC 229		
S.h.horridus	39	8.1 km vía Teotitlán del Camino - Cuicatlán, Oaxaca	MZFC 15595		
S.h.horridus	41	El Venado, Oaxaca	MZFC 9075		
S.h.horridus	40	Aproximadamente 4.3 km vía Cuicatlán - Teotitlán del Camino, Oaxaca	MZFC 15598		
S.h.horridus	42	Mazatlán, Guerrero	MC 095		
S.s.spinosus	43	Santiago Yolomécatl, vía Sn. Pedro Teposcolula-Putla, Oaxaca	MZFC 15669		
S.s.spinosus	44	2.2 km vía de San Pedro Teposcolula hacia Putla, Oaxaca	MZFC 15664		
S.h.horridus	45	Agua Agria, Mpio. Santa Catarina Ixtepeji, Oaxaca	JAC 21209		
S.s.spinosus	46 A	Carretera Tlaxiaco-Putla, Oaxaca	MC 196		
S.s.spinosus	46 B	Carretera Tlaxiaco-Putla, Oaxaca	MC 199		
S.s.caeruleopunctatus	47	Parque El Tequio, Oaxaca	RRA 243		
S.s.caeruleopunctatus	48	Vía Mitla-Cd. Oaxaca, Oaxaca	IBH17828	EF025748	EF025755
S.s.apicalis	49	Vía Mitla-Cd. Oaxaca, Oaxaca	Mc 109		
S.s.caeruleopunctatus	50	San Guillermo, 15 km. E Ejutla, carr 175	RRA 335		
S.s.apicalis	51	5 Km S Miahuatlán, Oaxaca	MC 248		
S.s.caeruleopunctatus	52	Llano de Tobías, aprox 7 km. SE Mihuatlán	RRA 348		
S.s.apicalis	53	11 Km SE Miahuatlán	WSB 908		
Outgroups					
S. formosus scitulus		Omiltemi, Guerrero	OFV617		
S. f. scitulus		Chilpancingo, Guerrero	OFV626		
S. torquatus				AF154244	AF154179
S. olivaceus				AF210361	AF440095

Fig. 1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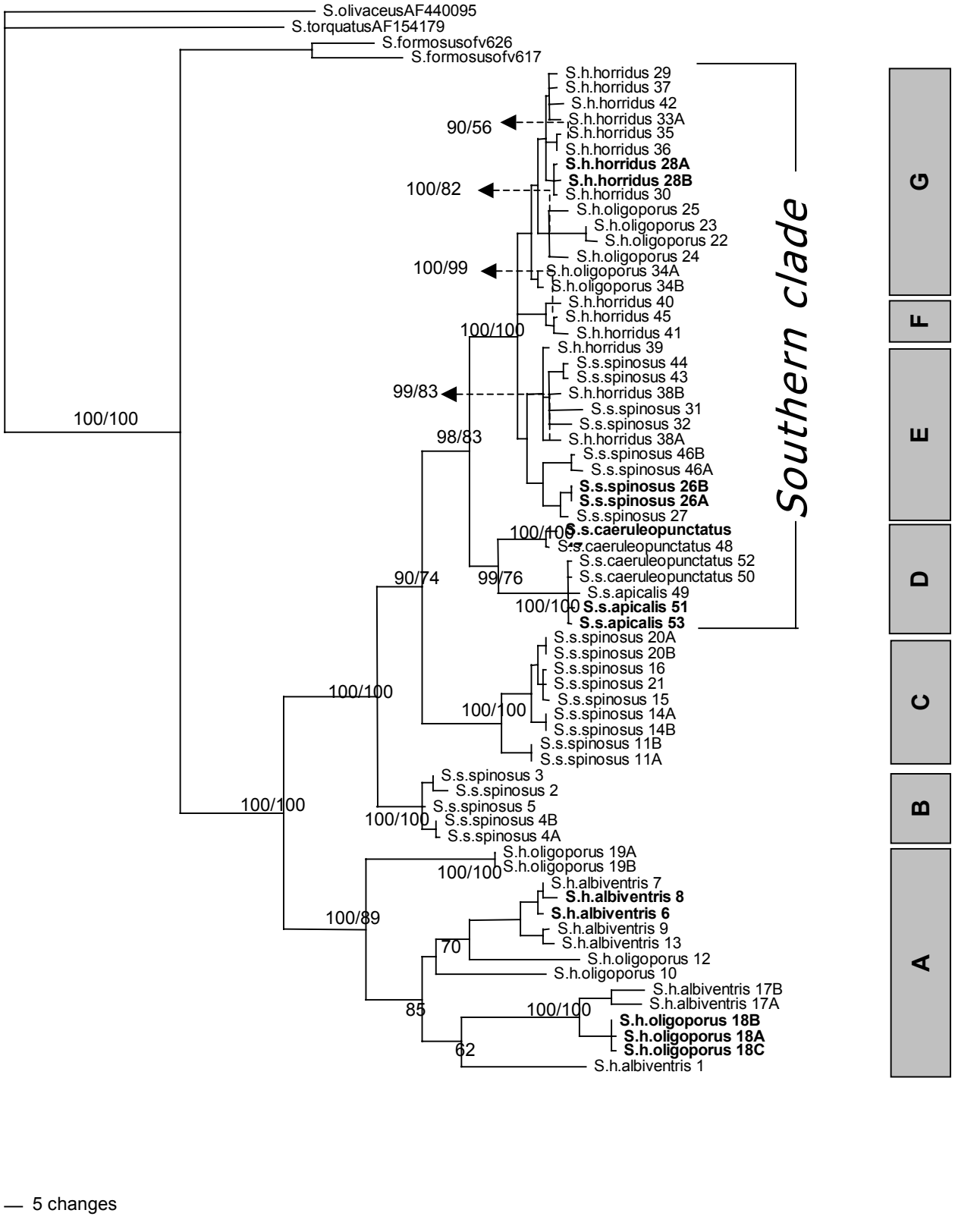
BCP
SMO
CCPR
SMOR
CP
GCP
TMVB
YP
GCP
SMC
SMS
S. h. albiventris
S. h. oligoporus
S. h. horridus
S. s. spinosus
overlaping distribution
47
48
49
S. s. apicalis
50
51
52/53
S. s. caeruleopunctatus

Fig. 2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S.torquatusAF154179
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S.formosusofv617
S.h.horridus 29
S.h.horridus 37
S.h.horridus 42
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S.h.horridus 35
S.h.horridus 36
S.h.horridus 28A
S.h.horridus 28B
S.h.horridus 30
S.h.oligoporus 25
S.h.oligoporus 23
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S.h.oligoporus 24
S.h.oligoporus 34A
S.h.oligoporus 34B
S.h.horridus 40
S.h.horridus 45
S.h.horridus 41
S.h.horridus 39
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S.s.spinosus 43
S.h.horridus 38B
S.s.spinosus 31
S.s.spinosus 32
S.h.horridus 38A
S.s.spinosus 46B
S.s.spinosus 46A
S.s.spinosus 26B
S.s.spinosus 26A
S.s.spinosus 27
S.s.caeruleopunctatus
S.s.caeruleopunctatus 48
S.s.caeruleopunctatus 52
S.s.caeruleopunctatus 50
S.s.apicalis 49
S.s.apicalis 51
S.s.apicalis 53
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S.s.spinosus 21
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S.s.spinosus 14B
S.s.spinosus 11B
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S.s.spinosus 4A
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S.h.oligoporus 19B
S.h.albiventris 7
S.h.albiventris 8
S.h.albiventris 6
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100/100
100/100
100/100
100/100
100/100
100/89
70
85
100/100
62
Southern clade
G
F
E
D
C
B
A
— 5 changes

Fig. 3

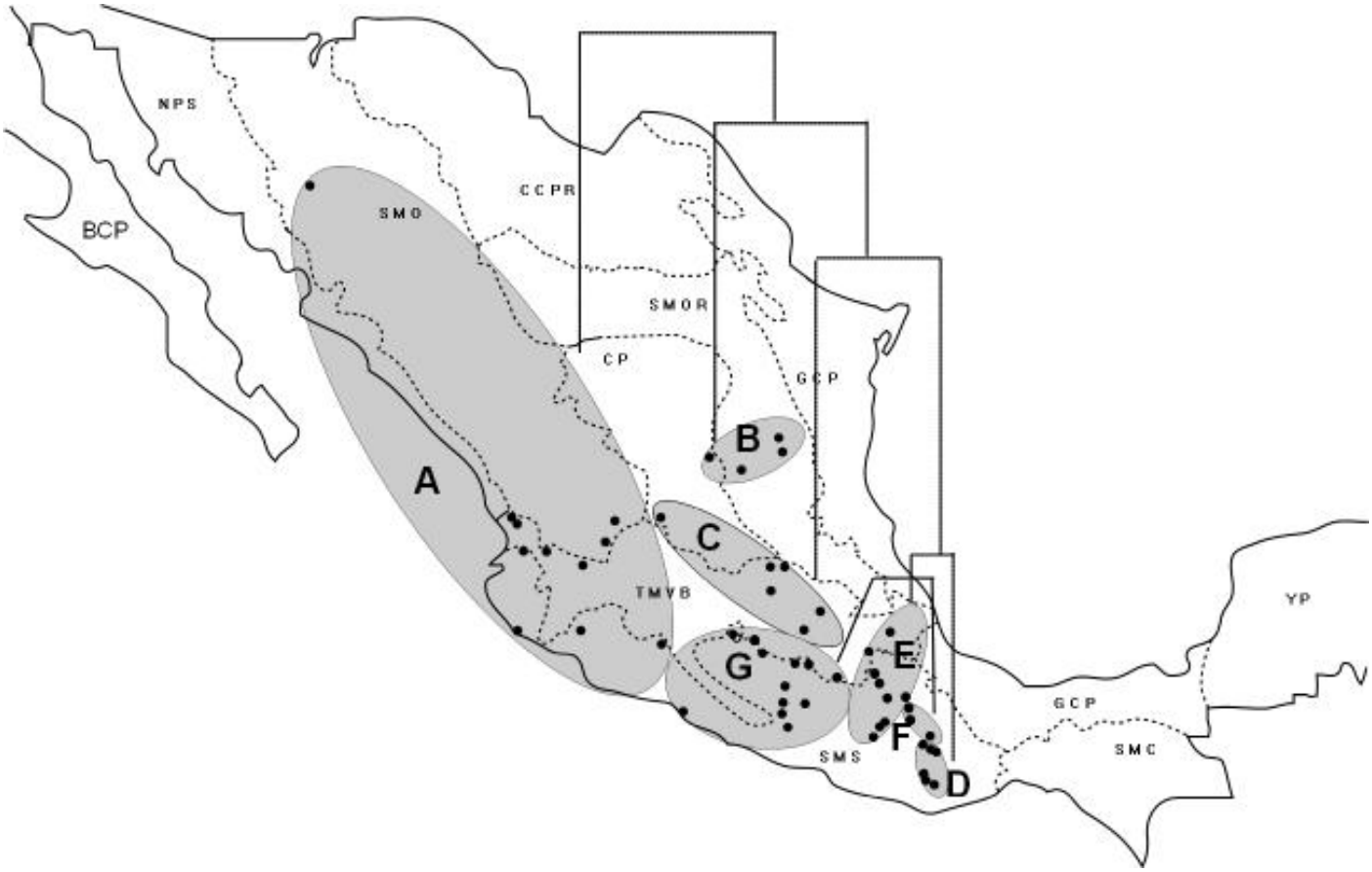

Figure headings

Figure 1. Geographical distribution of the *S. spinosus* group. Sampled localities are identified by dots and numbers (listed in appendix 1). Mexican Physiographic provinces of México: BCP, Baja California Peninsula; NPS, Norwestern Plains and Sierras; SMO, Sierra Madre Occidental; CCP, Chihuahua-Coahuila Plateaus and Ranges; PPC, Pacific Plain Coast; SMOR, Sierra Madre Oriental; GCP, Gulf Coast Plain; CP, Central Plateau; TMVB, Trans-Mexican Volcanic Belt; SMS, Sierra Madre del Sur; SMC, Sierra Madre de Chiapas; YP, Yucatan Plataform. Subspecies distribution and sampled localities from central-southern Oaxaca are detailed in the box.

Figure 2. Phylogenetic relationships among taxa of the *S. spinosus* group. Majority consensus tree obtained with Bayesian inference. Branch support values are shown as posterior probabilities/bootstrap values. Clades are identified by gray bars and capital letters. Samples collected in or next to the type locality for each taxon are indicated with bold type.

Figure 3. Geographical distribution of main clades of the *S. spinosus* group. Capital letters correspond to clade name according to the phylogram.

Table 1. Best-fitting and alternative substitution models per data partition.

Partition	Best fitting Model	Likelihood score (-*ln* L)	Alternative model	Likelihood score (-*ln* L)
12S (771 bp)	TrN + I	2405.1011	HKY + I	2408.7913
tRNA´s	HKY + G	465.9314	HKY + I	466.3222
First codon position	HKY + G	963.4197	HKY + I	964.1971
Second codon position	TrN + I	466.7155	HKY + I	469.6276
Third codon position	TrN + G	2483.8845	TIM + G	2482.7151

Table 2. Uncorrected genetic distances (below diagonal) and mean nucleotide differences (above diagonal) among main clades of the *Sceloporus spinosus* group.

	Outgroup	Clade A	Clade B	Clade C	Clade D	Clade E	Clade F	Clade G
Outgroup	—	34	17.2727	14.3523	43.2917	37.2	56.4	101.85
Clade A	0.1451	—	31.6104	16.8125	46.6263	31.3875	71.5852	98.55
Clade B	0.1404	0.0803	—	30.6339	46.5556	32.7818	67.425	107.1389
Clade C	0.1526	0.102	0.053	—	43.746	36.4	67.4848	102.2813
Clade D	0.142	0.0863	0.0462	0.0623	—	32.4	67.7111	102.7273
Clade E	0.1463	0.0961	0.0467	0.0664	0.045	—	60.5524	103.6667
Clade F	0.1477	0.0965	0.0519	0.0663	0.0484	0.0246	—	99.6786
Clade G	0.1457	0.096	0.0447	0.0617	0.0436	0.0204	0.0239	—

2. EVOLUTION OF EGG RETENTION IN THE *SCELOPORUS SPINOSUS* GROUP: EXPLORING THE ROLE OF PHYSIOLOGICAL, ENVIRONMENTAL, AND PHYLOGENETIC FACTORS

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EVOLUTION OF EGG RETENTION IN THE *SCELOPORUS SPINOSUS* GROUP: EXPLORING THE ROLE OF PHYSIOLOGICAL, ENVIRONMENTAL, AND PHYLOGENETIC FACTORS

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ABSTRACT: The evolution of viviparity in squamates has involved intermediate stages of egg retention. Reduction in the thickness of the eggshell, in relative clutch mass (RCM), and in clutch frequency would have facilitated the transition from oviparity to viviparity, while low temperatures are likely the ultimate selective force that promoted this evolutionary shift. We tested these ideas using the *Sceloporus spinosus* group. Because it is the sister clade of the viviparous *Sceloporus formosus* group, we predicted that members of the S. *spinosus* group would exhibit extended egg retention and other features associated with the evolution of viviparity. To test this idea, we examined the ability to retain eggs past the time of normal oviposition in the *Sceloporus spinosus* group and evaluated the association between egg retention and physiological and environmental factors in a historical context. Gravid females were collected from seven localities at a wide range of altitudes. We estimated the normal stage of embryos at oviposition and the stage at oviposition when females were induced to retain eggs under captive conditions. Stages of embryos varied within clutches; less developed embryos were usually dead and the most advanced embryos were usually alive. The maximum stage observed was therefore used as an index of egg retention for each clutch. The maximum embryonic stage at oviposition was

contrasted with RCM, egg membrane thickness, and several climatic variables (temperature and precipitation) in a phylogenetic framework. Females exhibited the ability to retain eggs as predicted. Maximum stage at oviposition varied within same clutch, same locality, and among localities. Variation observed in the maximum stage at oviposition was not related to egg membrane thickness, RCM, or clutch frequency or to environmental temperature and precipitation. Instead, mapping the maximum stage at oviposition on a phylogeny of the *S. spinosus* group suggested that the invasion of high elevations was associated with an enhanced potential for longer periods of egg retention.

Key words: Egg Retention; Evolution of viviparity; *Sceloporus spinosus* group

THE evolution of viviparity in squamate reptiles has long been interpreted as an adaptive strategy that enhances the survival of embryos (Shine, 1985; Tinkle and Gibbons, 1977). This evolutionary phenomenon has occurred more than 100 times in the Squamata (Blackburn, 1992, 2000; Shine, 1985) and reversal to oviparity is rare, if it occurs at all (Lee and Shine, 1998; Smith et al., 2001). Factors that could be detrimental to embryos in a nest (i.e. extreme temperatures, environmental unpredictability, dry environments, predation, and microbial attack) have been suggested as selective forces that stimulated the evolution of this reproductive mode (Shine, 1985; Tinkle and Gibbons, 1977). Of these environmental factors, the most widely accepted on the basis of comparative and experimental studies is low temperature (Shine, 1985, 2002). According to the cold climate model, low temperature promotes the evolution of viviparity because egg retention provides the embryos a warmer or more stable environment than they would experience in a nest (Shine, 1985, 2004). In contrast, predictions of an unpredictability hypothesis were falsified for some Australian oviparous scincid species suggesting that at least in that system, female inability to predict climatic environmental conditions did not promote the origin of viviparity (Shine, 2002).

Viviparity has evolved from oviparity (Lee and Shine, 1998). Observations on reproductively bimodal species like *Lacerta vivipara*, *Lerista bougainvilli*, and *Saiphos equalis* suggest that this involved a gradual increase in the length of egg retention (Andrews and Mathies, 2000; Heulin et al., 2002; Shine, 1985; Smith and Shine, 1997). The scarcity of species that lay eggs when embryos are at intermediate stages, (between stage 33 and 40), suggests that this transition occurs rapidly or that intermediate forms are not adaptive (Blackburn, 1995, 1998). Some oviparous populations of bimodal species lay eggs with embryos well beyond the modal stage at oviposition observed in squamates (stage 30, Andrews and Mathies, 2000). In *L. vivipara*, for example, females of oviparous populations closely related to one of the viviparous clades, lay their eggs in a greater range of stages (30-35) than those from the basal oviparous clade (stages 30-32) (Heulin et al., 2002). In reproductively bimodal species, extension of intrauterine development of embryos is negatively correlated with eggshell thickness (Heulin et al., 2002; Smith and Shine, 1997; Qualls, 1996; Qualls et al., 1995). Reduction in eggshell thickness, increased vascularization of the oviduct, and more extensive development of the chorioallantois membrane are suggested physiological and morphological modifications associated with the evolutionary transition from oviparity towards viviparity (Andrews, 1997; Guillette, 1993; Heulin et al., 2002).

To assess the phylogenetic context of the evolution of viviparity in reptiles, Lee and Shine (1998) mapped reproductive mode on reptile phylogeny. They concluded that the evolution of viviparity in this group has been under phylogenetic constraint, as it has evolved in some squamates but not in archosaurs, turtles and sphenodontid reptiles. The hypothesis that the evolution of viviparity is physiologically constrained was explored in the genus *Sceloporus* by contrasting a clade with both oviparous and viviparous species (*S. scalaris* group) and a clade in which all species are oviparous (*S. undulatus* group) (Andrews, 2002; Andrews and Rose, 1994; Andrews and Mathies, 2000). The capacity to support embryonic development *in utero* under conditions that inhibit oviposition was assessed for oviparous species of these species groups. The evolution of viviparity in the *S. undulatus* species group is constrained due to the inability of embryos of most species to continue development beyond the

normal stage at oviposition (Andrews and Mathies, 2000). *Sceloporus virgatus*, however, is an exception; its embryos continue development until stage 37 (Andrews, 1997; Andrews and Rose, 1994). In contrast to most species in the *S. undulatus* group, however, *S. scalaris* and *S. aeneus*, close relatives of viviparous species, have the ability to support embryogenesis in utero to advanced stages - at least stage 36 in *S. aeneus* (Andrews, unpublished data), and 39.5 in *S. scalaris*, (Mathies and Andrews, 1996).

The genus *Sceloporus* exhibits four different independent origins of viviparity, one for the *S. grammicus*, *S. megalepidurus* and *S. torquatus* groups, one for the *S. formosus* group and two for the *S. scalaris* group (Méndez de la Cruz et al., 1998). We chose the oviparous *Sceloporus spinosus* group as a model to evaluate the evolution of egg retention. We evaluated the effect of egg retention on embryonic development and tested the general hypothesis that members of the *S. spinosus* group should exhibit a greater capacity to retain eggs beyond the normal time of oviposition than 'typical' oviparous *Sceloporus* (e. g. about stage 30) because the clade shares a recent common ancestor with its entirely viviparous sister clade, the *S. formosus* group (Smith, 2001). We also tested the hypotheses that: 1) the maximum stage at oviposition is negatively related to several factors associated with the evolution of viviparity in other studies [relative clutch mass (RCM), eggshell thickness, and environmental temperature], and 2) the maximum stage at oviposition is associated with the phylogenetic history of the *Sceloporus spinosus* group.

MATERIALS AND METHODS

The *Sceloporus spinosus* group is monophyletic (Wiens and Reeder, 1997) and all populations involved in this study are closely related (see below). The group is distributed along the Pacific coast of Mexico from Guerrero to southern Sonora and

Chihuahua, and from Oaxaca to southern Tamaulipas, Nuevo León, and Durango (Sites et al., 1992; Smith, 1939). Their altitudinal distribution extends from sea level to about 2400 m; xeric habitats are the most typical (Smith, 1939). Mean size of sexually mature females varies among populations (69-106 mm SVL) in parallel with mean clutch size (7-25 eggs) (Calderón, unpublished). Courtship occurs in early spring and eggs are laid mainly during middle or late summer. Females of some populations lay a single clutch per season while females in other populations may lay more than one (Castro-Franco, 2002; Valdéz-Gonzáles and Ramirez-Bautista, 2002). Oviposition is asynchronous within populations as females at all stages of gravidity are found at any one time during the reproductive season.

Gravid females were captured between May 2001 and July 2004 from seven localities: La Preciosa, Puebla, N 19º 22' W 97º 23' 2", 2400 m, *n* = 10; Huahutla, Morelos, N 18º 25' 58" W 99º 02' 01", 1040 m, *n* =11; El Rodeo, Morelos, N 18º 44' 8", W 99º 20' 06", 1100 m, *n* = 28; Cerro Gordo, Mexico, N 17º 0' 45.66", W 96º 33' 17.34", 2000 m, *n* =14; Cd. Oaxaca-Mitla, Oaxaca, N 17º 0' 44.76", W 96º 33' 21.96", 1665 m, *n* = 17; Chamela, Jalisco, N 19º 23' 42", W 104º 57' 39.66", 30-70 m, *n* = 24; and Xalitla, Guerrero, N 18º 00´ 13.9", W 99º 32´ 28.8", 538 m, *n* = 8. Females were transported to the laboratory and housed individually in plastic terraria (51 x 35 x 21 cm). Terraria were provided with a 40W light bulb at one end to allow females to thermoregulate during the day. Room temperature fluctuated between 25 and 30°C during the observation period. Vegetation, woody debris, and rocks were provided as shelter. Females were fed wax worms and *Tenebrio* larvae three times per week and water was sprinkled on the vegetation and terraria walls daily. Most females were maintained on a dry substrate to promote egg retention (Andrews and Rose, 1994; Andrews and Mathies, 2000; Mathies and Andrews, 1996). The dry substrate consisted of a 5-7 cm deep layer of dry soil, and we were careful not to let water drip on the soil when lizards were watered. In 2001 and 2002 some females from La Preciosa (*n* = 2), Huahutla (*n* = 3), El Rodeo (*n* = 14) and Chamela (*n* = 9), were also maintained under control conditions (wet substrate) to establish the stage of the embryo when eggs would normally be laid. The wet substrate consisted of a 5-7 cm deep layer of soil that was mixed with sufficient water to make it suitable as a

successful oviposition site. The soil was checked daily and water added when needed to keep it moist.

We inspected the terraria three times per day. When clutches were laid, we recorded egg mass (0.01 g). Relative clutch mass (RCM) was calculated by dividing the mass of the clutch by the non-gravid mass of the female. Non-gravid mass of females was estimated from snout-vent length using the formula Mass = c(Length)^{d}, where c = 3.5 x 10^{-5}, and d = 3.01 (based on data for *Sceloporus occidentalis*, Andrews, 1982), because females were not weighed after oviposition. Embryos were staged according to the normal table of development of Dufaure and Hubert (1961). Half stages were used to indicate intermediate stages (i.e., an embryo between 30 and 31 was described as 30.5). Embryos were scored as alive or dead. Live embryos exhibited a heart beat at the time of dissection. In those few cases where eggs desiccated because they were laid on the surface before they were recovered, we assumed that the embryo that exhibited the most advanced stage in a clutch was alive at oviposition. While the tissues of dead embryos had lost some integrity, staging was still possible. The relative position of eggs in oviducts and their corresponding embryonic stages were recorded for nine gravid females that died or were euthanized in the laboratory.

To estimate the length of egg retention, we contrasted the mean date of oviposition between females maintained under wet conditions and females maintained under dry conditions. This estimate was made only for two localities, La Preciosa and El Rodeo, because control and experimental females were collected during the same reproductive season only at these localities.

We measured the thickness of the shell membrane of one egg per clutch for four to six clutches per locality. Shells were stored in 70% alcohol prior to processing for Scanning Electron Microscopy (SEM). Small pieces of eggshell were excised from the embryonic pole, submerged in dilute (1N) HCL overnight in order to remove the mineral layer, dried to the critical point, coated with gold-paladium and observed with SEM model S-2460N Hitachi. Five measurements per shell were taken on printed images using a caliper (0.01 mm) and averaged to obtain mean shell membrane thickness. The mineral layer was removed from eggshells because its highly irregular

distribution made obtaining consistent measures of thickness difficult. Clutch means were averaged for each site for subsequent correlation analyses.

Environmental data for each locality were obtained from Sistema Metereológico Nacional de México**.** Historical records (11 - 48 years) were used to calculate means for monthly precipitation, minimum and maximum temperature for the three months during which oviposition occurs (June – August). The difference between the monthly means of minimum and maximum temperature was used as an index of temperature fluctuation. For each variable, we averaged the three monthly means to obtain an annual value that was used in subsequent analyses. We also used the coefficient of variation (variance/mean) as an index of variability in rainfall for June, the first month of egg-laying season.

All statistical analyses were conducted with Statistica vers. 4.5. We report mean values $\pm$ their standard errors. All analyses were tested for statistical significance at the $P < 0.05$ level. We evaluated the correlation of maximum stage at oviposition with eggshell membrane thickness, relative clutch mass, minimum temperature, fluctuation between minimum and maximum temperature and variation of minimum, maximum temperature and precipitation within a phylogenetic framework using site means for each variable. For this, we calculated independent contrasts with COMPARE software (Comparative Method Analysis, Martins, 2001). We evaluated correct standardization of branch lengths and then we used standardized contrasts to perform regression analysis with regression lines forced through the origin (Garland et al., 1992).

We conducted a phylogenetic analysis that included all localities involved in this study. We sampled one or two individuals per locality (Appendix I) and obtained approximately 1637 base pairs of mitochondrial genes ND4 and associated $tRNA^{Hist}$, $tRNA^{Ser}$ and $tRNA^{Leu}$ as well as partial gene sequence of the 12S rRNA. Amplification cycles followed this PCR protocol: denaturation temperature: $94^{o}C$/3 and 1 minute, annealing at 50-$53^{o}C$/30 s, and extension at $72^{o}C$/1:00 and 2:30 min., respectively (Arévalo et al., 1994; Leache and Reeder, 2002). Amplification products were cleaned with PEG/NaCl precipitation. Sequences of primers employed are given in Appendix II. *Sceloporus formosus*, *S. olivaceus* and *S. torquatus* were employed as outgroups.

Sequences were aligned by eye. We performed maximum parsimony and bayesian analyses in PAUP v. 4.01b10 (Swofford, 1998) and Mr. Bayes vr. 3.0 (Huelsenbeck and Ronquist, 2001). For maximum parsimony analysis we implemented branch and bound searches of unordered characters with equal weights, addition sequence "as is" and tree bisection reconnection options. Clade support was evaluated with 1000 replicates of non-parametric bootstrap analyses. A nucleotid substitution model was estimated with Modeltest vr. 3.06 (Posada and Crandall, 1998). Bayesian analysis was run for five million generations.

RESULTS

Oviposition and Egg Retention Under Control (wet) and Experimental (dry) Conditions

Most females maintained under wet conditions excavated nests in the substrate and laid their entire clutch. All embryos in these eggs were alive. Their stages ranged from 29.5 to 30.5 (mode 30). In contrast, females maintained under dry conditions retained eggs past the time of normal oviposition and they usually oviposited on the surface of the substrate and produced eggs over a period of several days. Egg retention was stressful to females; individuals that retained eggs for long periods of time were emaciated and less active than females that retained for short periods of time. Eight retaining individuals died prior to oviposition and three were euthanized because they were in such poor health. Control females from La Preciosa oviposited from June 6 until June 21 2001, and experimental females from June 22 until July 21 2001 while control females from El Rodeo oviposited from 6 until 7 June 2002, and experimental females from 21 June until 24 July 2002. Modal lengths of egg retention were thus 21 and 30 days, respectively, for females from La Preciosa and El Rodeo.

Within Clutch Variation in Development of Embryos from Retained Eggs

Clutches laid by females maintained under dry conditions typically contained embryos that ranged widely in stage. For example, clutches with advanced embryos (>30.5) usually contained early embryos ($\leq$30.5) as well (Table 1). Variance in embryo stage within the same clutch was the result of death of embryos at different times

during retention. Live embryos were always those at the maximum stage found in a clutch, while dead embryos were usually at earlier stages. Only a few embryos per clutch reached late stages at most localities, and clutches with most advanced stages observed per locality were found at low frequencies at all localities (Fig. 1). However, at Chamela, Oaxaca and Xalitla, some clutches contained embryos all at the same advanced stage and most of these embryos were all alive at oviposition.
Variation in embryonic stage within clutches was not related to the relative position of eggs in the oviduct; early and late embryos were intermixed in sequence in nine clutches of females that died and were necropsied. Similarly, the physical crowding of eggs in the oviduct as indexed by RCM was not related to either the degree of variation in stage (as assessed by the coefficient of variation) or to the maximum stage observed within each clutch at any locality (*P's* > 0.05, Spearman correlation analyses).

Within Locality Variation in Development of Embryos from Retained Eggs

The maximum stage per clutch varied considerably among females at each locality (Fig. 1). Maximum stage per clutch was not related to crowding in the oviduct as judged by female mass after oviposition or to relative clutch mass at any locality (all *P*'s > 0.05, regression analyses). Moreover, the proportion of embryos within clutches that reached the maximum stage was not related to RCM at any locality (*P*'s > 0.05, regression analyses).

Inter Locality Variation in Development of Embryos from Retained Clutches

Mean maximum stage at oviposition did not vary among localities (*K-W*, $H_{6,82}$= 8.47, *P* = 0.21). This result may reflect high variability of maximum stage at oviposition and the relatively small number of clutches at each locality (Fig. 1). For example, no clutch from Chamela contained embryos beyond stage 32.5 while at least a few clutches from all other localities had embryos at stages up to 34-35. We therefore related mean locality values of maximum embryonic stage per clutch to other

variables using analyses of standardized contrasts to examine biological associations of this variable.

Standardized contrasts of mean maximum stage at oviposition were not related to mean shell membrane thickness (P = 0.21, $F_{1,5}$ =2.11, R = -0.54) or to mean RCM (P = 0.84, $F_{1,5}$ = 0.04, R = 0.09). Mean shell membrane thickness varied among localities (P = 0.03, $H_{6,25}$ = 13.9), however, with Chamela and Oaxaca having the highest and the lowest values, respectively. Shell membrane thickness variation was negatively related to precipitation during egg laying season (P = 0.04, $F_{1,4}$ = 7.85, R = -0.81). Mean relative clutch mass did not vary among localities (P = 0.11, $F_{6,61}$ = 1.83) (Table 2).

Standardized contrasts of mean maximum stage at oviposition were not related to mean minimum or maximum temperature during the egg laying season (P = 0.7, $F_{1,5}$= 0.39, R = -0.24 and P = 0.98, $F_{1,5}$ = 0.5, R = 0.008) or to temperature fluctuation (difference between maximum and minimum temperature) (P = 0.19, $F_{1,5}$= 2.22, R = 0.55). Similarly, mean maximum stage at oviposition was not related to variation in maximum and minimum temperature or precipitation during June, as assessed by the coefficient of variation (P > 0.05, $F_{1,5}$=0.95, R = -0.4; P > 0.05, $F_{1,5}$=0.37, R = 0.26 and P > 0.05, $F_{1,5}$ = 0.17, R = 0.18 respectively). None of these environmental variables were associated with the altitude of the study localities (P's > 0.05 in all cases).

Phylogenetic Component of Patterns of Egg Retention

Phylogenetic relationships of the *S. spinosus* and *S. formosus* species groups were strongly supported judging by high bootstrap and posterior probability scores (Fig. 2). Within the *S. spinosus* group, the first divergence is between Chamela and the other six localities. Chamela is a low elevation site and the maximum stage at oviposition was correspondingly low. In contrast, Chamela's sister clade was associated basally with high elevations and with advanced maximum stages at oviposition. Three low elevation sites (El Rodeo, Huahutla, and Xalitla) were nested with the 'high elevation' clade and presumably represent a reinvasion of low elevations without a corresponding reduction in the maximum stage at oviposition.

DISCUSSION

Females from the *Sceloporus spinosus* group exhibited the ability to retain eggs with intrauterine embryogenesis continuing beyond that of the normal stage at oviposition. However, the consequences of egg retention were quite different from those observed in other *Sceloporus* species. Moreover, the specific hypothesis of an association among biological and environmental variables was rejected. Our data did, however, support the hypothesis that the ability to retain eggs is associated with the evolutionary and geographic history of the group.

Within Clutch and Within Locality Variation in Embryonic Development

The high degree of variation in the stage at oviposition that we observed within clutches was unexpected. Such intra-clutch variation has not been reported for any other oviparous lizard species under natural conditions or under laboratory conditions where egg retention was induced by not providing a nesting substrate (Andrews, 1997; Andrews and Rose, 1994; Andrews and Mathies, 2000; Heulin et al., 2002). An even more unexpected observation was that the extent of embryogenesis varied within clutches as a result of embryonic death during the period of retention. Within clutch variation in the degree of embryogenesis in *S. spinosus* group generates a number of questions about possible physiological and morphological differences along oviducts or among eggs or embryos that influences their survival and development. We were able to determine that the position or degree of crowding in the oviduct was not related to embryo survival. Other possible factors related to variation in stage when embryos died could be heterogeneity in the degree of vascularization of the oviduct, the permeability of eggshells to oxygen, or that embryos may differ in their ability to develop or survive under low oxygen. Moreover, biochemical signals produced by embryos could differ. Embryos of non-mammalian viviparous vertebrates, like amphibians, secrete steroids and prostaglandins as pregnancy recognition systems (Guillette, 1989); these embryonic signals may influence development of maternal uterine tissue to benefit of the embryo (Crespi and Semeniuk, 2004). At this

time, however, the reason why embryonic survival varied within clutches of *S. spinosus* is unknown. Nonetheless, the high mortality of retained embryos and gravid females suggests that egg retention reduce fitness and that selection would not normally favor this trait.

Maximum stage at oviposition also varied within localities when females were forced to retain eggs. Maximum stage ranged from 31– 34 at Cerro Gordo, 31 - 32.5 at Chamela, 30 - 34 at El Rodeo, 30 - 35 at Huahutla, 29.5 – 34.5 at Oaxaca, 29 – 35 at La Preciosa, and 31 – 34.5 at Xalitla. Variation of stages of development under wet conditions was only 0.5-1.0 stages. While the stage at ovipostion varies little within clutches of lizards, variation among females also occurs among non-egg retainers in the genus *Scleoporus* (Andrews, unpublished data) as well as in species that retain eggs (Andrews, 1997; DeMarco, 1993; Mathies and Andrews, 1996; Smith and Shine, 1997). In both situations differences observed are of two to four or to five stages, respectively. For instance, females of *S. scalaris* from low and high elevations normally laid their eggs at stages 31-33.5 and 33-38, respectively, (Andrews, 1997; DeMarco, 1993; Mathies and Andrews, 1996). The variation in maximum stage at oviposition that we documented among females of *S. spinosus* group as a consequence of extended egg retention parallels variation in the stage at oviposition among females of other oviparous lizards under natural conditions.

Association Between Physiological and Demographic Traits and the Maximum Stage of Development During Egg Retention

In contrast to our prediction, differences in RCM among females at the same locality were small and did not account for differences in the maximum stage at oviposition. In fact, RCM exhibited little variation among females or among localities. This result suggests that changes in RCM are not related to the evolution of extended egg retention in *S. spinosus*, at least. The effect of RCM on embryonic development has only been evaluated for two other species. Half of the clutch (that is, one oviduct) of retaining females of *S. scalaris* and *S. virgatus* was removed; decreasing clutch

volume increased water uptake by eggs *in utero*, but did not affect embryonic stage (Andrews, 1997).

Shell membrane thickness was also not related to the maximum stage at oviposition that occurred during egg retention in the *S. spinosus* species group. It was, however, related to rainfall with thinner egg membranes associated with localities with higher rainfall and thicker membranes were associated with drier localities. Thicker shells may be adaptive in drier soils because they retard water loss (Andrews and Sexton, 1981). In parallel with our observations on *S. spinosus*, variation in the structure and thickness of eggshells of several other species of *Sceloporus* was not related to the capacity to retain eggs with developing embryos (Andrews and Mathies, 2000). The lack of association between embryonic development *in utero* and shell membrane or shell thickness in the *S. spinosus* and *S. undulatus* groups does not support the prediction that reduction in eggshell thickness is associated with extended egg retention. In contrast, eggshell thickness is reduced in populations with longer periods of egg retention in intra-specific comparisons of *Sceloporus scalaris* (Mathies and Andrews, 1995) and of reproductively bimodal species like *Lacerta vivipara* (Heulin et al., 2002, 2005), *Saiphos equalis* (Smith and Shine, 1997) and *Lerista bougainvilli* (Qualls, 1996; Qualls et al., 1995). These contrasting results imply that the evolution of egg retention and ultimately viviparity may involve different modifications that enhance embryonic development in utero, at least initially, among different taxa. Accordingly, a single species or clade cannot be used as a general model to explain evolution of viviparity (Blackburn, 2000).

Differences in clutch frequency also did not explain the variation in maximum stage at oviposition observed among females at different localities. Females that laid one clutch per season (La Preciosa, Cerro Gordo) exhibited the same ability to extend intrauterine embryogenesis as those that laid more that one clutch (El Rodeo, Huahutla, Xalitla). Clutch frequency had been suggested as a limiting factor of egg retention evolution in lizards (Andrews and Mathies, 2000; Shine, 1985); females that produce one clutch per year could retain eggs in utero, as this would not reduce their fitness (Shine, 1985). Our observations in the *S. spinosus* group suggest that

extended egg retention could evolve in multiple clutch layers at first, but long periods of egg retention would require a reduction in clutch frequency to one per season.

Environmental and Historical Factors in the Evolution of Egg Retention

The origin of viviparity in the *S. spinosus* and *S. formosus* clade is a comparatively ancient event. By assuming a divergence rate of 2% per mya for our haplotype data set we calculated that these species groups diverged 5.7 – 7.1 mya and the two main clades within the *S. spinosus* group diverged 4.2 – 6.5 mya. In contrast, the most recent evolution of viviparity in *Sceloporus* is within the *S. scalaris* group (Benabib et al., 1997). Using haplotype data generated by these authors, we estimated that the oviparous *S. aeneus* and viviparous *S. bicanthalis* diverged 3.2 - 4.9 mya. The origins of viparous clades of *Lacerta vivipara* are even more recent with splits from oviparous clades during the Pleistocene (1.0 – 2.6 mya) (Surget-Groba et al., 2001). While members of the *S. spinosus* group appear to have had enough time to respond to selective pressures associated with changes in elevation, changes in the ability to retain eggs with developing embryos are limited in efficacy and appear to have been slow. For example, while embryos can be retained to stages as advanced as 34 or 35, retention is associated with high embryonic and female mortality. We observed a shift in the maximum stage at oviposition associated with the invasion of high elevations by the ancestor of the high elevation clade, that is, populations R, H, X, P, O, and CG (Fig. 2). Nonetheless, females from relatively low elevation localities (R, H, and X), which are nested within the high elevation clade, still are capable of retaining eggs to embryonic stages 34-35 which suggests that the maximum stage at oviposition was not modified when *S. spinosus* re-invaded low elevation localities 0.5 mya. Possible reasons to explain why more substantial changes in egg retention have not occurred in the *S. spinosus* group are physiological constraints on the ability to support embryonic development in utero, the absence of appropriate selective forces, or that oviparity may continue to have adaptive value in arid environments.

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APPENDIX I

Species used in the phylogenetic annalyses, their museum numbers, Gen Bank accession numbers and localities. Acronym: IBH = Instituto de Biología-Herpetología

Taxon	Sample	ND4 and tRNAs	12S	Locality
Outgroup				
Sceloporus formosus		AF210346	L40455	
S. olivaceus		AF210361	AF440095	
S. torquatus		AF154244	AF154179	
Ingroup				
S. horridus albiventris	IBH17824	EF025743	EF025751	Chamela, Jalisco
	IBH18823	EF025744	EF025752	Chamela, Jalisco
S. h. horridus	IBH17825	EF025745	EF025753	El Rodeo, Morelos
	IBH17826	EF025746	_____	Santa Inés, Morelos
	IBH17827	EF025747	EF025754	Xalitla, Guerrero
S. spinosus caeruleopunctatus	IBH17828	EF025748	EF025755	Cd. Oaxaca-Mitla, Oaxaca
S. s. spinosus	IBH17829	EF025749	EF025756	La Preciosa, Municipio Las Minas, Puebla
	IBH17830	EF025750	EF025757	La Preciosa, Municipio Las Minas, Puebla

APPENDIX II

Primers used in this study

Primer name	Sequence (5´→ 3´)	Source
Nd4	TGACTACCAAAAGCTCATGTAGAAGC	Arévalo et al., 1994
Leu	TRCTTTTACTTGGATTTGCACCA	Arévalo et al., 1994
12e	GT(A/G)CGCTTACC(A/T)TGTTACGACT	Leache and Reeder, 2002
tPhe	AAAGCAC(A/G)GCACTGAAGATGC	Leache and Reeder, 2002

FIG. 1

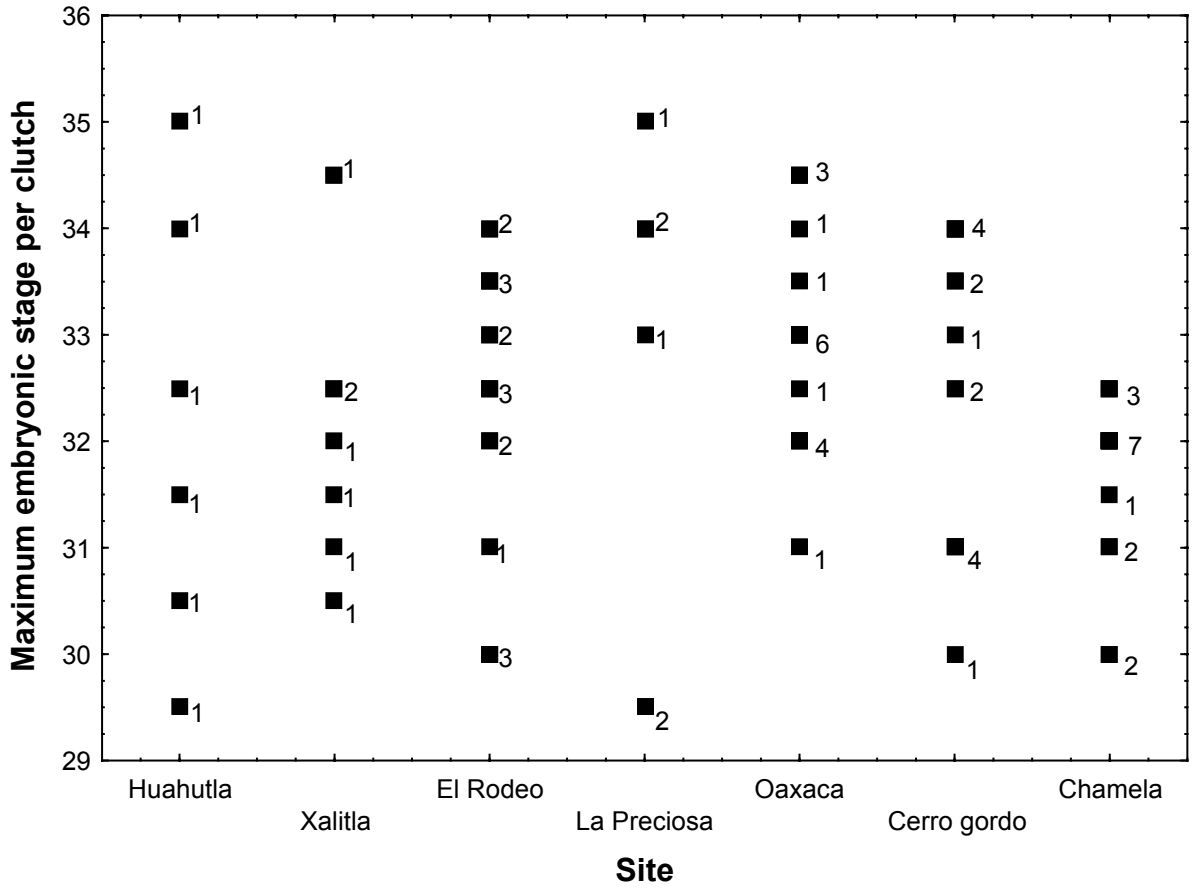
Maximum embryonic stage per clutch
36
35
34
33
32
31
30
29
Huahutla
Xalitla
El Rodeo
La Preciosa
Oaxaca
Cerro gordo
Chamela
Site

FIG. 2

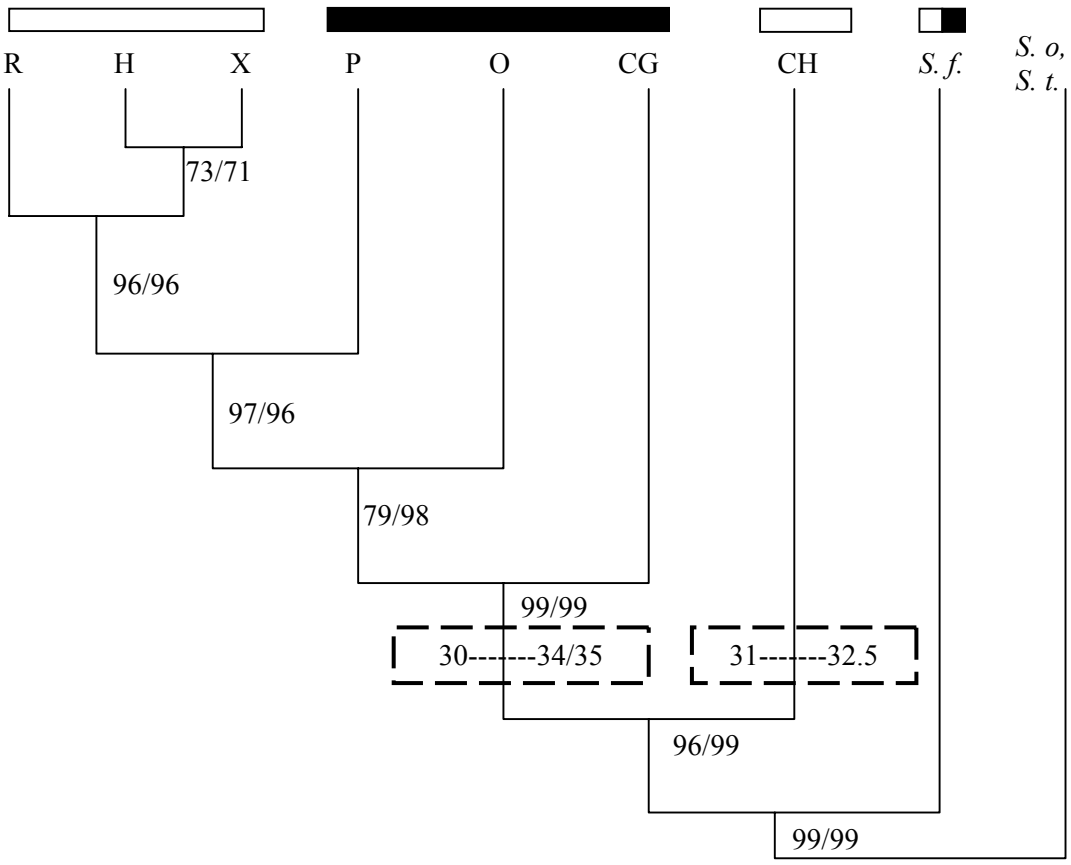
R
H
X
P
O
CG
CH
S. f.
S. o,
S. t.
73/71
96/96
97/96
79/98
99/99
30-------34/35
31-------32.5
96/99
99/99

FIG. 1. —Within and inter-locality variation in maximum stage at oviposition observed per clutch. Numbers represent the number of clutches.

FIG. 2. — Phylogenetic pattern of maximum stage at oviposition during egg retention. Altitudinal distribution is indicated by open (low elevation) and black (high elevation) bars. The taxon with open and black bars occupies a broad altitudinal distribution. Range of stages at oviposition is shown at the base of clades. Letters identify different localities (R = El Rodeo, H = Huahutla, X = Xalitla, P = La Preciosa, O = Oaxaca, C = Cerro Gordo and CH = Chamela) and outgroups (*S.f.* = *Sceloporus formosus*, *S. o*. = *S. olivaceus* and *S. t.* = *S. torquatus*). The single most parsimonious tree (length = 539, C.I.= 0.7699, R. I. = 0.7459) and majority consensus tree obtained from Bayesian analyses of mtND4, RNAt $^{His,\ Ser,\ Leu}$ and 12S. Substitution model selected by LRT´s criteria: TrN + I + Γ. Numbers below each clade represent clade support (bootstraps and posterior probabilities respectively).

TABLE 1. —Data collected on retained clutches. The range of embryonic stages is the minimum and maximum stage of development observed within each clutch, within clutch variation in embryonic stage was measured as the coefficient of variation (C. V.), and the fate of embryos in each clutch is indicated by the numbers of live and dead embryos. X indicates data that are missing because eggs were desiccated when found. * indicates six clutches from Huahutla and La Preciosa that were not staged but were incubated at 30-33 °C. Hatchlings document the presence of living embryos from these clutches.

Locality	*Clutch N°. Clutch size (n).*	*Range of embryonic stages*	*Within clutch variation (C. V.)*	*Embryos Live, Dead*
Cerro Gordo	1 (16)	30	0	X
	2 (17)	30-32.5	2.68	0,17
	3 (14)	30-31	0.99	0,14
	4 (23)	32.5-34	1.23	0,23
	5 (20)	30-33.5	3.94	0,20
	6(14)	30-34	5.20	2,12
	7(23)	31-34	3.07	3,20
	8(17)	30-33.5	3.24	0,17
	9(17)	30-31	1.34	0,17
	10(13)	31-34	2.8	X
	11(18)	29.5-32.5	2.38	0,18
	12(14)	31.5-33	1.77	1,13
	13(11)	30-31	1.17	0,11
	14(15)	30-31	1.80	1,14
Chamela	1(10)	30.5-31	0.84	10,0
	2(11)	32	0	7,4
	3(7)	30-32	3.72	2,5
	4(13)	31-32.5	1.40	8,5
	5(10)	31	0	X
	6(11)	32	0	X
	7(11)	30-32	2.08	X
	8(9)	30-32	2.86	X
	9(9)	32-32.5	0.68	0,9
	10(8)	31-32	1.32	3,5
	11(8)	32	0	8,0
	12(8)	32	0	8,0
	13(7)	31.5	0	X
	14(9)	30	0	9,0
	15(7)	30	0	X
El Rodeo	1(9)	30	0	X
	2(17)	30	0	1,16
	3(23)	30-32.5	2.17	X
	4(22)	30-32.5	2.6	X
	5(11)	30-32	2.44	0,11
	6(18)	30-32	3.14	13,5

	7(21)	32-33	1.46	X
	8(14)	28-30	2.78	0,14
	9(17)	32-33.5	1.33	2,15
	10(14)	30-32.5	2.27	0,14
	11(18)	33-34	0.80	X
	12(16)	31-33.5	3.13	1,15
	13(12)	30-31	1.43	0,12
	14(11)	30.5-33.5	1.43	0,11
	15(20)	30-33.5	3.19	0,20
	16(20)	31-33	1.95	0,20
Huahutla	1(17)	32.5 (staged 2)	No data	1,16*
	2(18)	28.5-30.5 (staged 2)	No data	0,18
	3(11)	31-32 (staged 2)	No data	3,8*
	4(10)	35 (staged 6)	No data	3,7*
	5(14)	29.5-30 (staged 6)	No data	X
	6(9)	32.5-34 (staged 3)	No data	0,9
La Preciosa	1(10)	32-34 (staged 4)	No data	0,10
	2(10)	29-30 (staged 2)	No data	1,9*
	3(10)	34 (staged 2)	No data	1,9*
	4(11)	34 (staged 1)	No data	1,10*
	5(10)	30-33 (staged 4)	No data	0,10
	6(9)	31.5-35 (staged 8)	No data	0,9
Oaxaca	1(14)	29.5-33.5	4.92	0,14
	2(17)	30-31	1.57	2,15
	3(12)	33	0	X
	4(19)	33-34.5	1.12	X
	5(9)	32-34	1.75	0,9
	6(10)	30-33	4.02	X
	7(12)	32	0	0,12
	8(12)	34.5	0	5,7
	9(13)	33	0	0,13
	10(9)	31-32	0.95	0,9
	11(12)	33	0	3,9
	12(13)	30-32.5	2.66	X
	13(14)	33-34.5	1.60	4,10
	14(14)	30-33	2.85	X
	15(16)	30-32	2.43	0,16
	16(14)	31-32	0.90	11,3
	17(9)	32-33	1.50	4,5
Xalitla	1(15)	32-32.5	0.80	4,11
	2(13)	31.5	0	0,13
	3(22)	32-34.5	1.97	1,21
	4(10)	30.5	0	0,10
	5(21)	32-32.5	0.79	0,21
	6(17)	32	0	0,17
	7(14)	31	0	1,13
	8(13)	34.5	0	0,13

TABLE 2. — Mean values of maximum embryonic stage at oviposition, eggshell membrane thickness, and relative clutch mass for each locality.

Locality	**Maximum embryonic stage** (X± S. D.)	**Eggshell membrane thickness in microns** (X± S. D.)	**Relative clutch mass** (X± S. D.)
Chamela	31.7 ± 0.8	76.60 ± 9.14	0.31± 0.11
Cerro Gordo	32.5 ± 1.5	65.69 ± 7.19	0.32 ± 0.085
El Rodeo	32.3 ± 1.7	69.64 ± 7.92	0.30 ± 0.09
Huahutla	32.2 ± 2.8	72.42 ± 12.75	0.31 ± 0.12
La Preciosa	32.5 ± 2.5	77.23 ± 11.89	0.27 ± 0.12
Oaxaca	32.9 ± 1.6	57.04 ± 8.36	0.28 ± 0.06
Xalitla	32.4 ± 2.1	78.77 ± 4.5	0.31 ± 0.11

CONCLUSIONES GENERALES

1. El grupo *Sceloporus spinosus* es monofilético, no así las distintas especies y subespecies reconocidas para el grupo según la taxonomía tradicional
2. Este grupo de especies está constituido por al menos siete especies.
3. Los haplotipos representativos de las poblaciones distribuidas sobre la Sierra Madre Oriental, la parte oeste del Eje Neovolcánico Transversal y hasta el norte de la presa del Infiernillo en la depresión del Balsas, constituyen un solo linaje y el nombre de *S. oligoporus* debería aplicarse exclusivamente a estas poblaciones.
4. Las poblaciones de *S. s. spinosus* distribuidos hacia el norte de México, sobre la Planicie Central y las poblaciones de *S. s. spinosus* distribuidas hacia el sur no constituyen un mismo linaje.
5. Los individuos catalogados como *S. s. apicalis* y *S. s. caeruleopunctatus* constituyen un único clado. La exclusividad de *S. s. apicalis* respecto a *S. s. caeruleopunctatus* no se resuelve con nuestros datos. Se propone que estas poblaciones representan al menos una especie.
6. Los haplotipos muestreados hacia el sur de la distribución y catalogados como *S. h. horridus*, "*S. h. oligoporus*" y *S. s. spinosus* se reúnen en tres clados cuya relación aparece como no resuelta en nuestra hipótesis. Sin embargo, según la relación entre estos haplotipos, la composición taxonómica de cada clado, así como su distribución geográfica se sugiere que las poblaciones representadas por estos clados constituyen tres o cuatro especies distintas.
7. Los límites de especie propuestos al interior del grupo *Sceloporus spinosus* requieren ser evaluados con información adicional, derivada por ejemplo de marcadores nucleares.

Con base en la hipótesis de filogenia obtenida para este grupo y en la evaluación de la capacidad de retención de huevos y avance embrionario concluimos lo siguiente:

8. Las hembras del grupo *Sceloporus spinosus* presentan la capacidad de retención y avance embrionario según lo esperado dado su parentesco con un grupo de especies vivíparas
9. El avance embrionario observado durante el periodo de retención alcanza un estado máximo de 32.5—34/35 según la población.
10. Esta capacidad de avance no está relacionada con características tales como el grosor de la membrana de la cáscara, la masa relativa de la nidada ó el número de nidadas.
11. Igualmente, factores climáticos como la temperatura y la precipitación no influyen en el grado de avance embrionario durante la retención.
12. La variación interpoblacional de la máxima capacidad de avance refleja un patrón filogenético que sugiere que esta característica se modificó debido a cambios en la distribución altitudinal ancestral del grupo.
13. Estos resultados apoyan parcialmente la hipótesis de que la evolución de esta característica ha sido influenciada por factores relacionados con la altitud.