# Long forsaken species diversity in the Middle American lizard Holcosus undulatus (Teiidae): Species Diversity in Holcosus Undulatus

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# Long forsaken species diversity in the Middle American lizard *Holcosus undulatus* (Teiidae)

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Numerous reptile species have been divided into subspecies. Although this classification may capture the morphological variation within species, it often conceals significant species diversity because many subspecies actually represent species under lineage-based species concepts. The lizard *Holcosus undulatus* is a common, widely distributed, monotypic species in Middle America. However, 12 subspecies of this taxon were recognized until the early 1970s. We used two lineage-based methods for species delimitation to re-evaluate species limits within *H. undulatus* with DNA sequence and morphological data. We included all the previously recognized subspecies of *H. undulatus* except *H. u. miadis*. *Holcosus undulatus* was exclusive. In addition, *H. u. amphigrammus*, *H. u. gaigeae*, *H. u. hartwegi*, *H. u. parvus*, *H. u. pulcher*, *H. u. sinister*, *H. u. stuarti*, *H. u. thomasi* and *H. u. undulatus* were supported as distinct evolutionary lineages based on the molecular and morphological evidence. We therefore elevate all of these subspecies to species rank. In addition, two separate mitochondrial lineages may represent cryptic, undescribed species within *H. undulatus*. The morphological distinctness and allopatry of *H. u. miadis* and *H. u. pulcher*, as well as the high genetic divergence of the latter species, suggest that they also represent distinct evolutionary species. Our results also suggest that additional species diversity may still be hidden within the *H. u. amphigrammus*, *H. u. parvus*, *H. u. sinister* and *H. u. undulatus* lineages. This work supports resurrection of overlooked diversity within *Holcosus*, which has important implications for the conservation of this genus in Middle America.


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ADDITIONAL KEYWORDS: *Ameiva* – cryptic species – lineage-based species concept – mtDNA tree – parapatry – phylogeny – species boundaries – species delimitation criteria – species tree – subspecies.

## INTRODUCTION

A subspecies is an aggregate of populations, belonging to the same species, inhabiting a geographical subdivision of its range, and differing taxonomically from other populations of that species (Mayr, 1942, 1982). The subspecies were conceived as separate populations inbreeding freely at the zones of contact, but the category was later extended to include geographically isolated populations (Wilson & Brown, 1953). Subspecies has also been conceived as evidence of the adaptive response of species to local climatic conditions (Mayr, 1982), as incipient species (Mayr, 1982) and even as practical devices without biological meaning (Mayr, 1982; Cracraft, 1983). During the mid-20th century, the rank of subspecies became widespread with the expansion of the biological species concept and many populations previously considered as species were combined as subspecies in a single polytypic species (Mayr, 1982; Zink, 2004). In reptiles, many species were divided into subspecies on the basis of geographical variation in scalation and coloration (Wiens, 2008).

Recent studies using lineage-based species concepts (*sensu* Wiley & Lieberman, 2011) and other lines of evidence (e.g. DNA sequences) have revealed that many subspecies actually represent distinct species (e.g. Mulcahy *et al*., 2006; Schulte, Macey & Papenfuss, 2006; Pyron & Burbrink, 2009; Feria-Ortiz, Manríquez-Morán & Nieto-Montes de Oca, 2011; Glor & Laport, 2012).

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Thus, although the diversity was recognized morphologically, embracing the biological polytypic species concept resulted in its inappropriate classification, with the consequent concealment of substantial species diversity (Wiens, 2008). Because this concealment has significant negative consequences for the knowledge and conservation of biodiversity, it is important to investigate whether particular subspecies actually represent distinct, independent lineages and therefore should be recognized as full species (Frost & Hillis, 1990; Wiens, 2008).

The genus *Holcosus* Cope comprises a group of terrestrial, widely foraging teiid lizards that occurs from Mexico to trans-Andean Colombia and Ecuador and includes three species groups: the *H. orcesi*, *H. septemlineatus* and *H. undulatus* groups. The *H. undulatus* group ranges from Central Mexico to Colombia, and contains six species (Harvey, Ugueto & Gutberlet, 2012): *H. chaitzami* Stuart, 1942, *H. festivus* (Lichtenstein & Von Martens, 1856), *H. leptophrys* (Cope, 1893), *H. niceforoi* (Dunn, 1943), *H. quadrilineatus* (Hallowell, 1860) and *H. undulatus* (Wiegmann, 1834). *Holcosus undulatus* is the most extensively distributed of the six species, occurring on the Atlantic slopes from southern Tamaulipas, Mexico, to the Departamentos of Nuevo Segovia and Río San Juan, Nicaragua, and on the Pacific slopes from Nayarit, Mexico, to Puntarenas Province, Costa Rica; it is also found on Isla Mujeres, east of Quintana Roo, Mexico, and the Corn Islands, east of Nicaragua (Echternacht, 1971). The species occurs mainly in shaded habitats in forest or forest-edge areas usually below 1500 m, but it may utilize open areas in the absence of competition from other teiids such as *Aspidoscelis* (Echternacht, 1971).

*Holcosus undulatus* is currently recognized as a monotypic species (Harvey *et al*., 2012). However, until the publication of Echternacht's (1970, 1971) taxonomic works on Middle and South American *Ameiva* it was divided on the basis of geographical variation in scalation and colour pattern into 12 subspecies (see below). This suggests that adoption of a lineage-based species concept and the analysis of different lines of evidence relevant to species delimitation (e.g. external morphology and DNA sequences) may provide evidence supporting the resurrection of some or all of these subspecies and their elevation to species rank, and thus reveal the existence of more species than currently recognized in the *H. undulatus* group.

## Brief taxonomic history of *H. undulatus*

Wiegmann (1834) described *Cnemidophorus undulatus* from 'Mexico' with two varieties: alpha and beta, referred to as varieties A and B by subsequent authors. This author, however, did not provide additional information about the geographical distribution of *C. undulatus* or its varieties. Subsequently, Gray (1845) transferred *C. undulatus* to the genus *Ameiva* Cuvier.

Hallowell (1860) described *Ameiva pulchra* and *Cnemidophorus quadrilineatus* from 'Nicaragua'. However, shortly after this Cope (1862) transferred *C. quadrilineatus* to *Ameiva* (= *A. quadrilineata*). Bocourt (1874), on the basis of Wiegmann's (1834) syntypes and material collected during the labours of the 'Mission scientifique au Mexique et dans l'Amerique Centrale,' provided detailed re-descriptions of *Ameiva undulata* and both of its varieties A and B. Bocourt (1874) also stated that the National Museum of Natural History in Paris had several specimens 'identical' to the type specimens of *C. undulatus* described by Wiegmann (1834), and that all of them had been collected in diverse localities on the Pacific versant of Mexico ('Oaxaca and Tehuantepec') and Central America (scattered localities on the Pacific versant of Guatemala and El Salvador). Furthermore, Bocourt (1874) stated that Wiegmann's (1834) variety A of *A. undulata* inhabited the Atlantic versant of Mexico and Central America [the Petén, the hot lands of Vera Paz, the course of the Rio Polochic, Santa María de Pansos (=Panzós), and Isabal (=Izabal)], whereas variety B had been collected by the Mission Scientifique in the forests of Belize (British Honduras).

Barbour & Noble (1915) treated *Ameiva undulata* as a polytypic species for the first time in the early 20th century. They assigned the Mexican populations to *A. undulata undulata* (suggesting that this taxon was confined to southern Mexico), described *A. undulata parva* from 'Guatemala' and included *A. quadrilineata* in *A. undulata* (*A. u. quadrilineata*). However, Schmidt & Stuart (1941) noted that the specimens of *A. u. quadrilineata* of Barbour & Noble (1915) actually represented *A. pulchra*.

Barbour & Loveridge (1929) described *Ameiva festiva miadis* from Great Corn Island, Nicaragua. Later, Hartweg & Oliver (1937) examined a series of 30 males and 17 females from 32 km south-west of Tehuantepec in Oaxaca, Mexico, and Ranchería Lamanga, 20 km south of Tehuantepec, and concluded that *Ameiva undulata* 'as described by Wiegmann (1834: 27–28) and redescribed and figured by Bocourt (1874: 254–258)' was composed of two forms: *A. u. undulata*, from the region of Tehuantepec, and *A. u. parva*, from the Pacific slopes of Guatemala.

Dunn (1940) suggested that both *Ameiva pulchra* and *A. festiva miadis* were 'a form of *A. undulata* Wiegmann'. In the same year, Smith (1940) described variety A of Wiegmann (1834) and Bocourt (1874) as *A. u. hartwegi* from 'Chiapas, Mexico, across the Usumacinta River from Piedras Negras, Guatemala', and also *A. u. stuarti* from Palenque, Chiapas.

Schmidt & Stuart (1941) recognized four subspecies of *Ameiva undulata*: *A. u. undulata*, ranging along the Pacific coast of Mexico north-westward from Tehuantepec; *A. u. parva*, ranging along the Pacific coast of Mexico and well into that of Guatemala south-eastward from Tehuantepec; *A. u. hartwegi*, from Yucatán, Mexico, and El Petén, Guatemala; and *A. u. stuarti*, from Veracruz, Tabasco and Chiapas in the Atlantic versant of Mexico. Schmidt & Stuart (1941) suggested that both *A. leptophrys* and *A. pulchra* also were forms or races 'of the *undulata* group'. Shortly thereafter, Stuart (1942) described *A. chaitzami* from Alta Verapaz, Guatemala, and Smith & Laufe (1945) described *A. u. amphigramma* from San Andrés Tuxtla, Veracruz.

Smith & Laufe (1946), in their taxonomic review of Mexican *Ameiva*, continued to recognize the previously described subspecies of *A. undulata* and described five more: *A. u. dextra*, *A. u. gaigeae*, *A. u. podarga*, *A. u. sinistra* and *A. u. thomasi*. Also, following Bocourt's (1874) assignment of populations of *Ameiva* to Wiegmann's (1834) varieties A and B of *A. undulata*, Smith & Laufe (1946) concluded that both of these varieties corresponded to *A. u. hartwegi*. Because *A. u. pulchra* and *A. u. miadis* do not occur in Mexico, Smith & Laufe (1946) did not include them in their study. The ten subspecies of *A. undulata* from Mexico recognized by Smith & Laufe (1946), along with the references of their original descriptions, type localities and distributions, are listed in Table 1. Smith & Laufe (1946) also indicated the existence of several putative areas of intergradation among the subspecies (Fig. 1). Smith & Laufe's (1946) taxonomic arrangement of Mexican *Ameiva* was maintained by Smith & Taylor (1950) and Stuart (1963).

Echternacht (1970) discussed the taxonomy of two Middle American *Ameiva*. *Ameiva festiva miadis* was formally designated a subspecies of *A. undulata*, and *A. undulata thomasi* was placed in the synonymy of *A. chaitzami*. Subsequently, Echternacht (1971) performed a detailed analysis of the geographical variation in Middle American *Ameiva* and placed all the other subspecies of *A. undulata* into the synonymy of *A. undulata*. Also, he recognized five other species of *Ameiva* in Middle America: *A. ameiva*, *A. chaitzami*, *A. festiva*, *A. leptophrys* and *A. quadrilineata*. Finally, in a recent, detailed morphological study of the Teiidae, all Mexican and Central American species of *Ameiva* were transferred to the genus *Holcosus* (Harvey *et al*., 2012).

Herein, we use molecular and morphological data to investigate the potential existence of multiple species within *H. undulatus* and whether their limits correspond to those of the formerly recognized subspecies.

## METHODS

### Taxon sampling

We sampled broadly from the geographical distribution of *H. undulatus* in Mexico and Central America (Fig. 1, Table S1), including multiple samples from the geographical distribution of each subspecies of *H. undulatus* except for *H. u. pulcher* and *H. u. miadis*, of which only one and no samples were available, respectively. Acronyms for museums and collections follow Sabaj-Pérez (2014). We followed Hallowell (1860), Cope (1862) and Smith & Laufe (1946) to evaluate the subspecific identity of the sampled individuals of *H. undulatus*. When single individuals from particular localities could not be easily identified (e.g. female or juvenile specimens), we relied on the examination of other specimens from the same or nearby localities. Although we tried to include samples from the type locality of each subspecies (e.g. *H. u. amphigrammus* and *H. u. sinister*), this was sometimes not possible, either because the type locality was imprecise (e.g. *H. u. parvus* and *H. pulcher*, from 'Guatemala' and 'Nicaragua', respectively), or because no specimens were found at the type-locality. In such cases we included all available samples from the subspecies reported distribution.

The locality of Finca El Carmen on the Pluma Hidalgo-Huatulco road lies in the putative area of intergradation between *H. u. dexter* and *H. u. undulatus* (Smith & Laufe, 1946). We tentatively assigned our sample of *H. undulatus* from this locality to *H. u. undulatus* because the only male available from this population exhibited the dorsolateral colour pattern described by Smith & Laufe (1946) for this subspecies. Similarly, although our specimens of *H. undulatus* from Honduras fell within the variation range described for *H. u. parvus* by Smith & Laufe (1946), they are from the interior highlands of Honduras, outside of the known distribution of this subspecies (Table 1). Thus, assignment of those samples to *H. u. parvus* was only provisional. Finally, although Echternacht (1971) synonymized *H. u. thomasi* with *H. chaitzami*, examination of most of the types of *H. chaitzami* (the holotype and three paratypes), as well as the holotype, four topotypes and nine additional specimens from the general region of the type locality of *H. u. thomasi*, suggests that these two taxa actually represent distinct species (our pers. observ.). In addition, *H. u. thomasi* and *H. chaitzami* appear to be allopatric and separated by the intervening highlands of the Sierra de los Cuchumatanes. Thus, we consider as an incontrovertible population of *H. chaitzami* only that from its type locality, and tentatively assigned the specimens from Comitán, Chiapas and Huehuetenango, Guatemala, to *H. u. thomasi* following Smith & Laufe (1946). A summary of the

**Table 1.** Original descriptions, type-localities, and geographic distributions of *H. chaitzami* and the formerly recognized subspecies of *H. undulatus*. Except as otherwise noted, the geographic distributions are taken from Smith & Laufe (1946)

| Taxon | Original description | Type locality | Geographical distribution |
|---|---|---|---|
| *H. chaitzami* | Stuart (1942) | Along Cahabón–Lanquín Trail about 2 km north of Finca Canihor (about 38km ENE [straight line] of Cobán, Alta Verapaz, Guatemala). | Probably distributed thoughout the savanna area of the semi-arid lower Cahabón Valley bellow about 1000 m from Lanquín (30 km [straight line] ENE from Cobán) to Taquincó (56 km [straight line] E from Cobán) (Stuart, 1942) and Poptún (Echternacht, 1971) |
| *H. u. amphigrammus* | Smith & Laufe (1945) | San Andrés Tuxtla, Veracruz | Northern Veracruz (exact area uncertain, perhaps in the vicinity of Laguna Tamiahua), southward through most of Veracruz to the Isthmus of Tehuantepec; westward into valleys extending into extreme eastern Oaxaca and probably north-eastern Puebla |
| *H. u. dexter* | Smith & Laufe (1946) | Near Rincón, Guerrero | Southern slopes of the Sierra Madre del Sur of Guerrero and perhaps extreme western Oaxaca |
| *H. u. gaigeae* | Smith & Laufe (1946) | Progreso, Yucatán | Northern half of the Yucatán Peninsula, and southward to the Island of Carmen along the extreme eastern coast |
| *H. u. hartwegi* | Smith (1940) | Chiapas, Mexico, across the Rio Usumacinta from Piedras Negras, Petén, Guatemala | Atlantic slopes of Mexico and Guatemala from the vicinity of the south-eastern end of Laguna de Términos south and eastward across the base of the Yucatán Peninsula to north-western Honduras |
| *H. u. miadis* | Barbour & Loveridge (1929) | Great Corn Island, 40 miles off Nicaraguan coast | Known only from Islas del Maíz (Corn Islands), Depto. Zelaya, Nicaragua |
| *H. u. parvus* | Barbour & Noble (1915) | Guatemala; restricted to Mazatenango by Smith & Laufe (1946) | Pacific slopes from the Isthmus of Tehuantepec in Oaxaca, near Niltepec, south-eastward to Costa Rica |
| *H. u. podargus* | Smith & Laufe (1946) | 7 miles west of Victoria, Tamaulipas | Atlantic coast from the latitude of Victoria, Tamaulipas, southward into northern Veracruz |
| *H. u. pulcher* | Hallowell (1860) | Nicaragua | Pacific coast of Nicaragua (Echternacht, 1971) |
| *H. u. sinister* | Smith & Laufe (1946) | Manzanillo, Colima | Pacific coastal drainage from the arid Balsas Basin at the border of Guerrero and Michoacán north-westward at least to Jalisco, and perhaps farther; the northern drainage of the Río Balsas, at lower elevations and in humid localities, from Michoacán to Puebla |
| *H. u. stuarti* | Smith (1940) | Palenque, Chiapas | Atlantic slopes of Mexico from the middle of the Isthmus of Tehuantepec eastward in the lowlands to the southern borders of Laguna de Términos and to Tenosique, Tabasco; southward up the valley of the Río Grijalva at least as far as Tuxtla Gutiérrez, Chiapas |
| *H. u. thomasi* | Smith & Laufe (1946) | La Libertad, Chiapas, near Río Cuilco where it crosses the Guatemalan border | Probably in all the dry, hot valleys of the upper tributaries of the Río Grijalva in the interior of Chiapas and of western central Guatemala |
| *H. u. undulatus* | Wiegmann (1834) | Mexico (by inference); restricted to Tehuantepec by Smith (1940) | The Pacific slopes of the Isthmus of Tehuantepec in Oaxaca, as far west as Puerto Angel and eastward about to Niltepec |

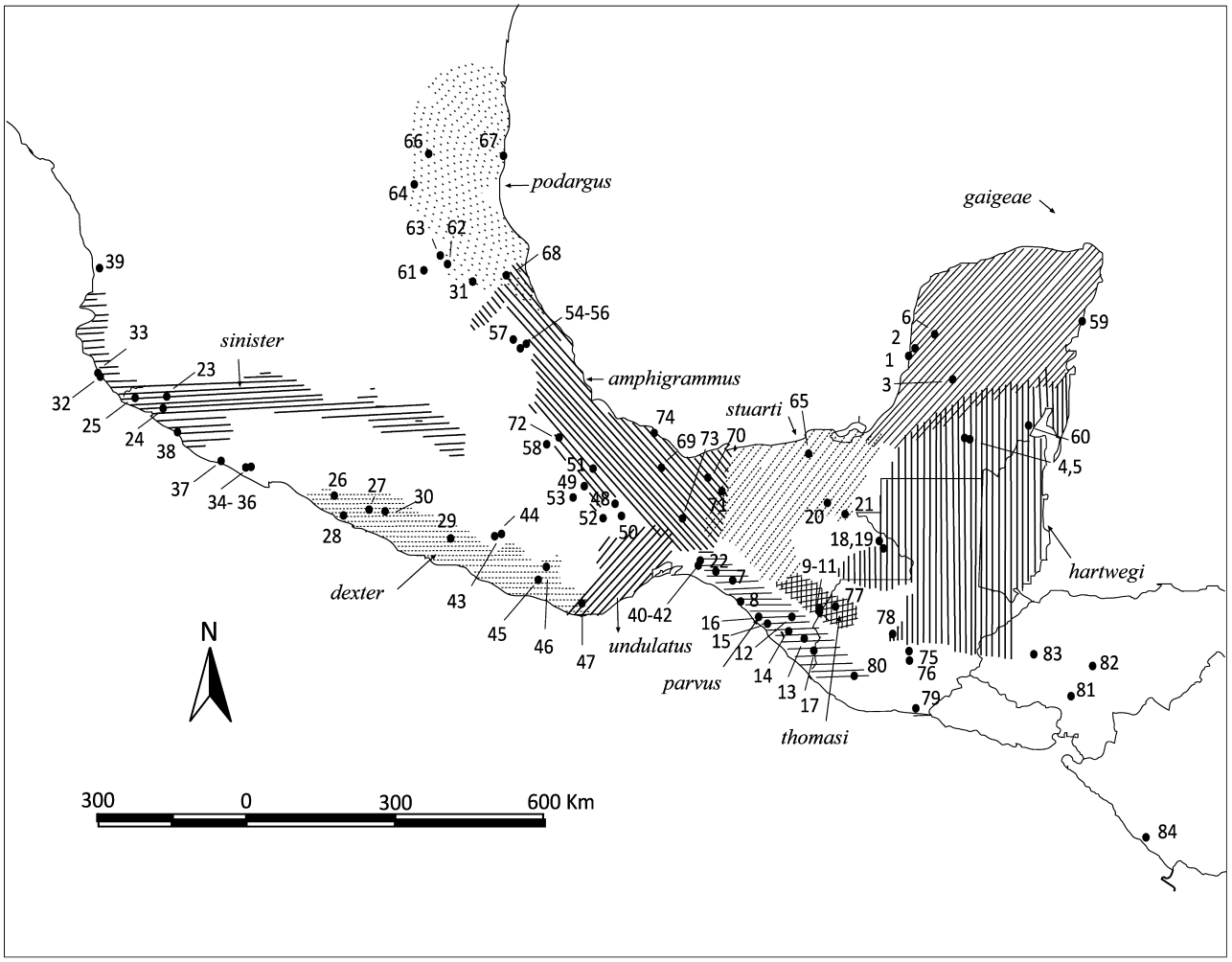



**Figure 1.** Geographical distribution of the formerly recognized subspecies of *H. undulatus* in Mexico (reproduced from Smith & Laufe, 1946); and sampling localities for *H. undulatus*. Numbers at dots refer to specific sample numbers of *H. undulatus* specimens used in this study. Locality data for these specimens are given in Table S1.

diagnostic characters of the subspecies of *H. undulatus* is given in Table 2.

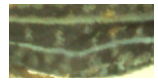
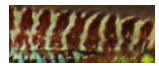
To evaluate the exclusivity of *H. undulatus*, we included as outgroups representatives of other species of *Holcosus* in Mexico and Central America (*H. festivus* and *H. quadrilineatus*). Unfortunately, no samples of either true *H. chaitzami* (i.e. from its type locality) or of *A. leptophrys* were available. Finally, we included *Aspidoscelis deppii*, *Ameiva ameiva*, *A. auberi* and *A. chrysolaema* (Teiidae) and *Leposoma parietale* (Gymnophtalmidae) in the analysis as more distant outgroups to root the tree.

## DATA

Mitochondrial DNA (mtDNA) has well-known advantages and limitations for species delimitation. Because of its low effective population size (one-quarter the size of a given nuclear gene) newly formed species become exclusive in their mtDNA haplotype phylogenies in a quarter of the time they become distinct in nuclear-based markers (Moore, 1995). Therefore, incomplete lineage sorting is less of a concern for mitochondrial than for nuclear loci. Nonetheless, mitochondrial gene trees can be particularly susceptible to the effects of introgressive hybridization, male-biased dispersal (female philopatry) and the development of strong geographical patterns produced by temporary isolation (Palumbi & Baker, 1994; Thorpe, Black & Malhotra, 1996; Wiens & Penkrot, 2002; Funk & Omland, 2003).

It has been suggested that the above potential problems of mtDNA gene trees may be common, and thus the species limits inferred must be corroborated by other evidence (i.e. Funk & Omland, 2003; Bond & Stockman, 2008; Zink & Barrowclough, 2008). Nuclear DNA gene trees can be used to corroborate mtDNA gene trees

**Table 2.** Summary of the diagnostic characters of *H. chaitzami* and the subspecies of *H. undulatus*. Data for *H. chaitzami*, the Mexican subspecies and *H. u. pulcher* taken from Stuart (1942), Smith & Laufe (1946), and Hallowell (1860) and Cope (1862), respectively

| | | Median gular scales | | | | | | | Colour pattern | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxon/ character | SVL (mm) | Size | Enlarge-ment | Arrangement | Scale rows between 3rd supraocular and superciliaries | Preanals arrangement | Femoral pores‡ | Lamellae under 4th toe | Dorsolateral dark line | Dorsolateral light line | Upper lateral light stripe | Upper lateral dark stripe | Lateral vertical bands** | Male lateral pattern |
| *chaitzami* | ♂ 70 ♀ 66 | Enlarged | Abrupt | One median row | One | Paired | 16 (15–17), $N$ = 4 (this study) | 24.6 (22–26), $N$ = 4 (this study) | Continuous | Distinct | Absent | Split into transverse spots, separated by light spots | Few, irregular |  |
| *amphigra-mmus* | ♂ 105 ♀ 101 | Enlarged | Abrupt | Usually one median row (87%); ≤ two of the largest scales divided | One | Paired | 17.0 (14–23), $N$ = 180; usually ≥ 16 in ♀ (75%) | 27.7 (24–33), $N$ = 187 | Broken into speckles | Absent | Broad, continuous, or broken into blotches§ | Split into dorsal and ventral narrow lines by the upper lateral light line | Poorly developed (this study) |  |
| *dexter* | ♂ 113 ♀ 80 | Enlarged | Abrupt | One median row | One | One row†, posterior-most usually paired (85%) | 18.2 (15–21), $N$ = 24 | 28.9 (27–31), $N$ = 20 | Broken into speckles in ♂, continuous in ♀ | Dim in ♂, distinct in ♀ | Broad, continuous, or broken into blotches in ♂ | Split into dorsal and ventral narrow lines by the upper lateral light line | Poorly developed, particularly in ♀ |  |
| *gaigeae* | ♂ 125 ♀ 107 | Small | Usually not abrupt | Irregular | One | Paired | 18.5 (15–22), $N$ = 138 | 30.4 (26–36), $N$ = 132 | Broken into speckles | Absent in ♂, distinct in ♀ | Absent | Split into transverse spots, separated by vertical light lines | Well developed; 13.1 (9–18), $N$ = 57; usually ≥ 12 (89.5%) |  |
| *hartwegi* | ♂ 138 ♀ 115 | Small | Not abrupt | Irregular | Two* | Paired | 20.5 (16–23), $N$ = 100; usually ≥ 18 (98%) | 31.8 (29–36), $N$ = 97; ≥ 29 (100%) | Broken into speckles (100%; this study) | Absent or dim (this study) | Absent (this study) | Split into transverse spots, separated by vertical light lines | Well developed; (8–12), $N$ = 55; usually ≤ 11 (98.2%) |  |
| *parvus* | ♂ 109 ♀ 95 | Enlarged | Abrupt | One median row | Usually two | Paired | 16.4 (13–21), $N$ = 98 | 29.1 (26–33), $N$ = 97 | Broken into speckles | Distinct | Absent | Split into transverse spots, separated by vertical light lines | Well developed |  |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *podargus* | ♂ 116<br>♀ 96 | Enlarged | Abrupt | Completely irregular or ≤ two scales forming a row (84.6%) | One | Paired | 15.8 (13–18), *N* = 31; usually ≤ 15 in ♀ (64.7%) | 29.4 (28–31), *N* = 30; ≥ 28 (100%) | Broken into speckles | Dim | Broad, broken into large blotches¶ | Split into dorsal and ventral narrow lines by the upper lateral light line | Present, usually dim |
| *pulcher* | No data | Enlarged | Abrupt | Irregular | No data | Paired | 20 (holotype) | No data | Continuous (Hallowell, 1860) | No data | Absent | Split into transverse spots, separated by vertical light lines | Numerous, black (Hallowell, 1860) |
| *sinister* | ♂ 109<br>♀ 95 | Enlarged | Abrupt | One median row | One | One row†, posterior-most rarely paired (37.7%) | 18.1 (15–22), *N* = 121 | 29.6 (26–32), *N* = 111 | Broken into speckles | Absent or dim | Absent | Split into transverse spots, separated by light spots | Well developed in adult ♂ |
| *stuarti* | ♂ 90*<br>♀ 81* | Enlarged | Abrupt | One median row | No data | Paired | 15.5 (13–18), *N* = 73; usually ≤ 17 (97.3%) | 25.5 (22–30), *N* = 72; usually ≤ 27 (87.5%) | Usually continuous | No data | Absent | Split into transverse spots, separated by vertical light lines | Narrow in ♂** |
| *thomasi* | ♂ 92<br>♀ 78 | Enlarged | Abrupt | One median row | One | Paired | 17.2 (14–20), *N* = 18 | 28.4 (25–30), *N* = 17; usually ≥ 28 (75.5%) | Absent or broken into speckles | Distinct, fused with upper lateral light blotches | Absent | Split into transverse spots, separated by vertical light lines | Present |
| *undulatus* | ♂ 116<br>♀ 95 | Enlarged | Abrupt | One median row | One | One row†, posterior-most paired in one-half (52%) of specimens | 16.8 (13–20), *N* = 162 | 27.7 (25–30), *N* = 94 | Broken into speckles | Absent except in young | Absent | Split into transverse spots, separated by vertical light lines | Present |

*This study (not recorded in the cited references).
†'Single median row and on each side a smaller row' (Smith & Laufe, 1946).
‡On one side (both sexes).
§Dark lines between blotches considerably less than half as wide as blotches.
¶Dark bars between light blotches at least half as wide as blotches, usually wider.
**Averages and ranges are for numbers of lateral vertical light lines between the levels of the axilla and groin.

in the search of organismal, as opposed to gene, phylogenies (Moore, 1995, 1997). Because of the intrinsically stochastic nature of the coalescent process and the longer coalescence times of nuclear compared with mitochondrial genes (Moore, 1995; Wiens & Penkrot, 2002; Zink & Barrowclough, 2008), general corroboration between nuclear and mtDNA gene trees is not expected (especially for mtDNA trees with a shallow geographical structure), but when such corroboration is present (at least for some lineages in the phylogeny), it is evidence of probably long lineage isolation. We obtained sequences of an mtDNA fragment encompassing the genes encoding tRNAMet (in part), the second unit of the NADH dehydrogenase (ND2), tRNATrp and tRNAAla (in part), for approximately 1200 bp. In addition, we obtained sequence data from two nuclear markers: the nuclear intron RPS8 (427 bp) (Tod W. Reeder, pers. comm.), and the coding region amplified with the primers for the α-cardiac-actin gene (561 bp) of Waltari & Edwards (2002). The mitochondrial fragment was sequenced for all of the included samples. For the nuclear genes, an effort was made to sequence representative samples of each clade concordant with geography in the mitochondrial tree.

*Laboratory protocol*

We extracted DNA from liver or tail clips using the standard phenol-chloroform-isoamyl alcohol protocol (Hillis *et al*., 1996), or the extraction protocol for reptile shed skins of Fetzner (1999). All of the sequenced genes were amplified via PCR. The primers used to amplify and sequence these genes are detailed in Table S2. The PCR cycle parameters for the mitochondrial fragment were: an initial denaturation cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 45–53 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 4 min. The PCR cycle parameters for the nuclear fragments were: an initial denaturation cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 45–48 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 4 min. PCR products were cleaned with polyethylene glycol precipitation (Lis & Schleif, 1975). DNA templates were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The reaction products were cleaned using Sephadex columns and sequenced with an ABI 3100 automated Genetic Analyzer Sequencer.

## Phylogenetic analyses

We assembled, edited and aligned sequences using CLC Main Workbench 6.9.1 (CLC Bio). The alignment was adjusted visually with Mesquite v2.75 (Maddison & Maddison, 2011). Regions in the alignment that could not be unambiguously aligned were removed. All of the sequences were deposited in GenBank (Table S1).

We analysed the aligned DNA sequences using Bayesian and maximum-likelihood (ML) methods of phylogenetic inference. We performed partitioned analyses of the mitochondrial fragment and single model analyses of the individual nuclear markers. For the mitochondrial dataset, we assayed different combinations of partitions (partition strategies). To determine the best partition strategy, we used the Bayes factor as described by Brandley, Schmitz & Reeder (2005). To select the best substitution model for each partition, we used the nst = mixed option implemented in MrBayes v3.2 (Ronquist *et al*., 2012). This option uses rjMCMC to sample among all possible reversible substitution models (Ronquist *et al*., 2012). We estimated the marginal likelihood by calculating the harmonic mean of the likelihood values of the Markov Chain Monte Carlo (MCMC) samples. The harmonic mean was calculated by using the sump command in MrBayes (Newton & Raftery, 1994). The partition strategies compared and the selected models of evolution for each strategy are given in Table S3.

Bayesian settings included random starting trees and default priors except for the rate prior, which was set to variable. Analyses consisted of two runs (nruns = 2), each conducted with three heated and one cold Markov chains, sampling every 10000 generations for $10 \times 10^7$ generations. The parameters and tree output files from the two individual replicates were combined in LogCombiner 1.8.0 (Drummond, Rambaut & Suchard, 2013). We evaluated stationarity and convergence of likelihood scores between runs visualizing the output parameters with TRACER 1.5.0 (Rambaut & Drummond, 2009). We then discarded burn-in trees and estimated the maximum clade credibility (MCC) tree with nodal posterior probability (PP) support from the postburn-in trees using TreeAnnotator 1.8.0 (Rambaut & Drummond, 2013). We considered nodes with posterior probability values ≥ 95% as significantly supported (Felsenstein, 2004).

We performed ML analyses with RAxML 7.0.4 (Stamatakis, 2006a). We used the same model (GTRGAMMA) for each of four partitions for the mitochondrial fragment (one for the non-coding region and one for each codon position) and each nuclear gene. We used GTRCAT for the bootstrapping face with 1000 fast bootstrap replicates for the final tree inference (Stamatakis, 2006a, b). We considered nodes with a bootstrap value ≥ 70% as significantly supported (Hillis & Bull, 1993). All the phylogenetic analyses were performed in the Cyberinfrastructure for Phylogenetic Research (CIPRES; Miller, Pfeiffer & Schwartz, 2010).

In addition, we estimated a coalescent-based species tree using the program BEAST 1.8.0 (Drummond *et al*., 2012). We assigned the individuals to those sets

of populations supported by the molecular and/or morphological data as distinct, independent evolutionary lineages (see below). We unlinked the three genes (ND2, RPS8 and α-cardiac-actin) and selected the best substitution model for each gene with the use of the Bayesian information criterion implemented with jModelTest 2.1.4 (Darriba *et al*., 2012). We performed the BEAST analyses under a correlated lognormal relaxed molecular clock (mean clock rate fixed to the gene ND2) and the Yule process. We performed two replicates with 100 million generations each, and sampled every 10000 steps. We combined the Log and tree files from the two individual replicates in LogCombiner 1.8.0 (Rambaut & Drummond, 2013), and used the program Tracer 1.5 (Rambaut & Drummond, 2009) to evaluate the convergence of the trees and estimate the burn-in value. Finally, we estimated the MCC tree using TreeAnnotator 1.8.0 (Rambaut & Drummond, 2013).

*Testing alternative phylogenetic hypotheses*

To test whether alternative phylogenetic hypotheses not present in the preferred tree (i.e. the mitochondrial tree, see below) could be statistically rejected by the data (e.g. an alternative hypothesis holding as monophyletic a paraphyletic taxon in the Bayesian tree), we used a Bayesian approach for hypothesis testing (Huelsenbeck & Rannala, 2004; Brandley *et al*., 2005). If a phylogenetic hypothesis of interest was not included in the 95% set of credible trees for the mtDNA dataset, it was rejected. The hypotheses tested were the monophyly of *H. u. parvus* and *H. u. dexter*.

## Species concept

To re-evaluate the species limits in *H. undulatus*, we used the tree-based methods for delimiting species proposed by Wiens & Penkrot (2002) and Bond & Stockman (2008). These methods (hereafter WP and BS, respectively) use a lineage-based species concepts (*sensu* Wiley & Lieberman, 2011) but differ in the species properties or lines of evidence that they use for species delimitation. De Queiroz (2007) proposed that the primary and only necessary defining property of species is their existence as separately evolving lineages. Other properties acquired by lineages during the course of divergence (intrinsic reproductive isolation, genetic exchangeability, diagnosability, same niche or ecological interchangeability, monophyly, etc.) are secondary defining properties of species, many of which are more appropriately interpreted as different lines of evidence relevant to species delimitation only to the extent that they provide evidence of lineage separation.

Thus, a lineage might lack one or more of the secondary defining properties even if it is evolving separately from all other lineages, and does not have to have any of them to be considered a species. However, the presence of any one of those properties (if appropriately interpreted) is evidence for the existence of a species, although more properties and thus more lines of evidence are associated with a higher degree of corroboration (de Queiroz, 2007). This is consistent with the emerging general view that taxonomy needs to be pluralistic and integrate new approaches for species delimitation (e.g. Dayrat, 2005; Bond & Stockman, 2008; Padial *et al*., 2010; Schlick-Steiner *et al*., 2010).

## Species delimitation

The WP method is based on a haplotype phylogeny for a set of populations currently classified as a species (the focal species of the study) and as many species as possible that are closely related to this species. The method assumes a phylogeny of non-recombining mtDNA haplotypes of known locality and taxonomic designation. In addition, it assumes that the failure of haplotypes from a given locality to cluster together is potential evidence of gene flow with other populations, as is the general discordance between haplotype clades and the geographical areas from which the haplotypes are found. Species delimitation will depend on the relationship of the focal species to the other species [i.e. whether the focal species is exclusive *sensu* de Queiroz & Donoghue (1990) and subsequent authors] and on the general concordance between phylogeny and geography within the focal species. If the focal species is exclusive, the presence of significantly supported basal lineages (i.e. the oldest split or splits within a focal species) concordant with geography within the species is potential evidence of the absence of gene flow between these lineages (candidate species), and therefore suggests that the focal species may represent multiple species disguised by traditional taxonomy.

The WP method emphasizes the basal lineages that are concordant with geography as potentially distinct species because retained ancestral polymorphisms are most likely in populations that have split very recently (Neigel & Avise, 1986) and the problems of male-biased dispersal, female philopatry and coalescence of temporarily isolated populations are most likely to affect the more recent branches of the haplotype tree. Initially, the focal species of this study was *H. undulatus*. However, because the haplotypes of *H. undulatus* formed an exclusive group and were mainly distributed into lineages of identical subspecies designation, we subsequently added the subspecies of *H. undulatus* as focal species of the study.

The BS method is based on the cohesion species concept of Templeton (1989, 2001). In this concept, the boundaries of an evolutionary lineage are defined by the mechanisms that limit the action of gene flow, genetic drift and natural selection. These mechanisms fall into two major categories: genetic exchangeability and

ecological interchangeability. The first refers to the boundaries for gene flow (Templeton, Maskas & Cruzan, 2000). Individuals from different populations are genetically exchangeable if there is ample gene flow between populations (Crandall, 2000). The second refers to the ability of the descendants or genes of organisms to replace (through drift) or displace (through selection) the descendants or genes of other organisms in the lineage even if the lineage is not reproducing sexually (Templeton *et al*., 2000). Ecological interchangeability can be assessed by 'standard approaches' such as morphological differentiation or less conventional means such as niche-based distribution models. For a set of populations to qualify as a cohesion species they must be derived from a single evolutionary lineage and must be genetically exchangeable (GE) and/or ecologically interchangeable (EI) (Templeton, 2001).

Because several subspecies were previously recognized within *Holcosus undulatus* on the basis of their morphological differentiation, we evaluated the lineages within *H. undulatus* not only for the possibility of gene flow but also adaptive divergence potential (morphological differentiation) between them. For this, we relied on the traditional taxonomy of Hallowell (1860), Cope (1862) and Smith & Laufe (1946), and considered sister lineages to be morphologically differentiated if their populations belonged to different subspecies of *H. undulatus*. Evaluation of morphological differentiation between lineages also is important because recognizing mitochondrial introgression requires evaluating a mitochondrial gene tree against a nuclear background that identifies the participating taxa (nuclear loci or phenotypic differences that presumably have a nuclear basis; Smith, 1992).

Finally, we estimated genetic distances within and among lineages. Genetic distances among lineages have been used frequently to investigate species boundaries (e.g. Hebert *et al*., 2003, 2004; Lefébure *et al*., 2006; Zemlak *et al*., 2009). Although there is not a threshold to separate ranges of intra- and interspecific divergence, comparing genetic distances within and among clades may provide a clue on the divergence level of two clades and support species hypotheses.

## RESULTS

### Mitochondrial data

The mitochondrial dataset included 137 sequences of *H. undulatus* and 27 of the other taxa included in the analysis: *Ameiva ameiva* (4), *H. festivus* (3), *H. quadrilineatus* (1), West Indian *Ameiva* (13), *Aspidoscelis* (3) and *Leposoma parietale* (1). The dataset consisted of 1044 unambiguously aligned nucleotide positions corresponding to the ND2 gene and 137 corresponding to the adjacent tRNATrp and tRNAAla genes. There were only two indels in the tRNA regions, and no indels or stop codons in the ND2 region. Sequence nucleotide composition showed the typical negatively skewed proportion of guanine of mitochondrial genes (T = 24.0, C = 27.1, A = 39.2, G = 9.7).

Partitioning the dataset into four partitions (one for each ND2 codon position and one for the combined tRNAs) yielded the greatest improvement of mean $-\ln L$ (Table S3). All Bayesian analyses reached stationarity and convergence before $1 \times 10^7$ generations.

The MCC tree for the Middle American species of *Holcosus* and representatives of other teiid genera is shown in Figure S1. The haplotypes of *H. undulatus*, *H. festivus*, *A. ameiva*, West Indian *Ameiva* and *Aspidoscelis* all formed significantly supported clades, and *H. quadrilineatus* was significantly supported as sister taxon to the *H. undulatus* clade. Our results suggest that *Ameiva* is paraphyletic with respect to *Aspidoscelis* and do not support the monophyly of *Holcosus*. However, the relationships among the *H. festivus*, (*H. quadrilineatus* + *H. undulatus*) and (*Ameiva* + *Aspidoscelis*) clades were not significantly supported. The above main clades and the significantly supported relationships among them also were significantly supported in the ML tree (data not shown).

The MCC tree for the *Holcosus undulatus* clade showed a pronounced population structure that was strongly concordant with geography at both deep and shallow levels. The clade was composed of six significantly supported clades in both the Bayesian and the ML analyses (Fig. 2). These clades were concordant with geography, allopatric or parapatric, and intermediately to moderately highly divergent from each other (p = 7.4–11.4). Their geographical distributions are shown in Figure 3. Of the six clades, two were geographically distributed on the Pacific versant of Mexico west of the Isthmus of Tehuantepec. One clade was composed of two significantly supported subclades: one with all of the haplotypes of *H. u. dexter* from the Pacific versant of north-western Guerrero (hereafter the north-western *H. u. dexter* lineage), and the other one with all of the haplotypes of *H. u. sinister*. The latter haplotypes were grouped into three significantly supported subclades concordant with geography; however, the relationships among these subclades were not significantly supported. The other clade was composed of all of the haplotypes of *H. u. dexter* from the Pacific versant of south-eastern Guerrero and south-western Oaxaca (hereafter the south-eastern *H. u. dexter* lineage) and a nested, significantly supported clade with all of the haplotypes of *H. u. undulatus*.

Two clades were geographically distributed on the Atlantic versant of Mexico. One was composed of two large, but not significantly supported subclades: one with all of the haplotypes of *H. u. amphigrammus* and the other one with all of the haplotypes of *H. u.*

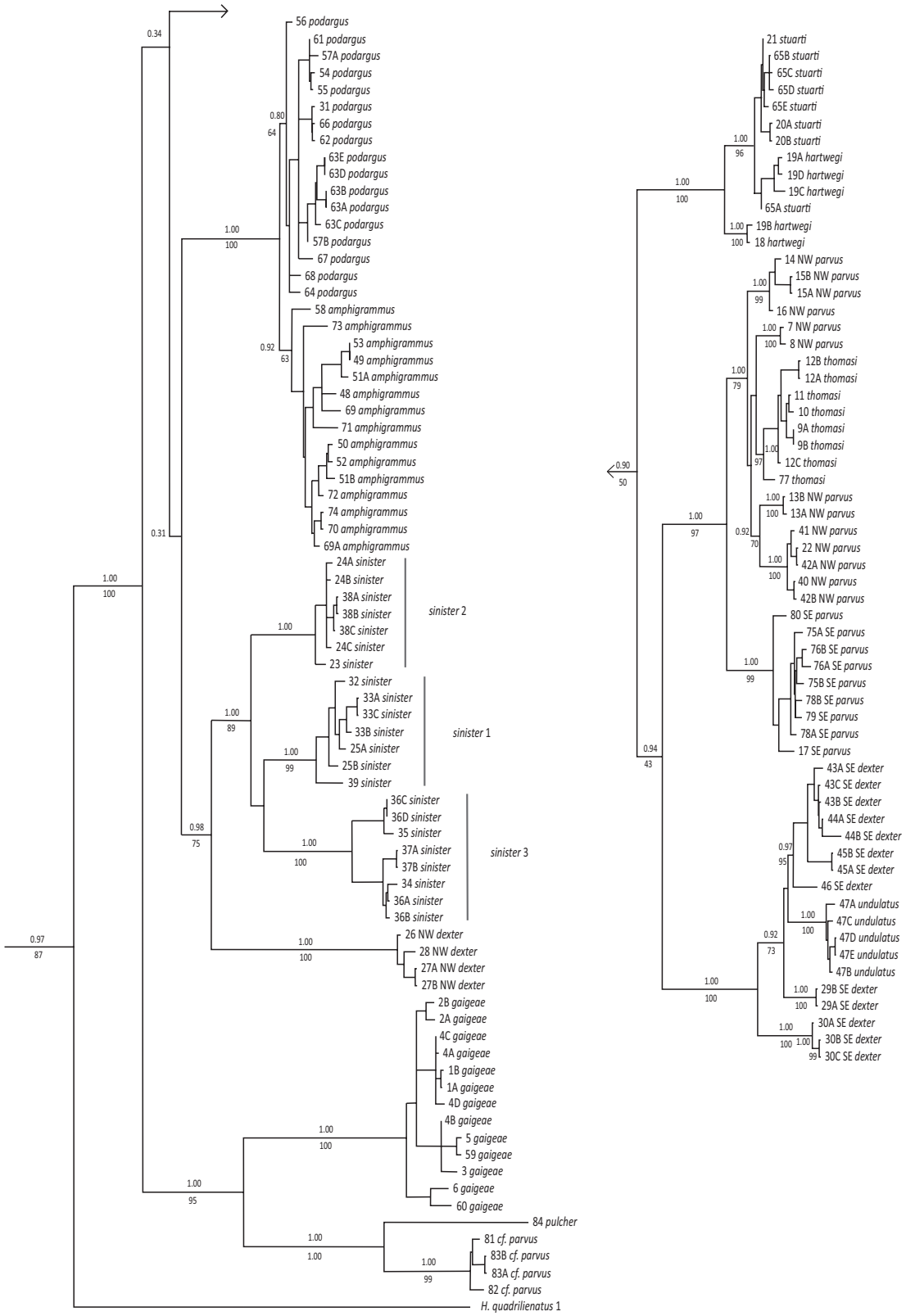



**Figure 2.** Bayesian MCC tree of *H. undulatus* based on a partitioned analysis of the mitochondrial dataset. Outgroups are not shown. Numbers next to branches indicate posterior probability/bootstrap support values.

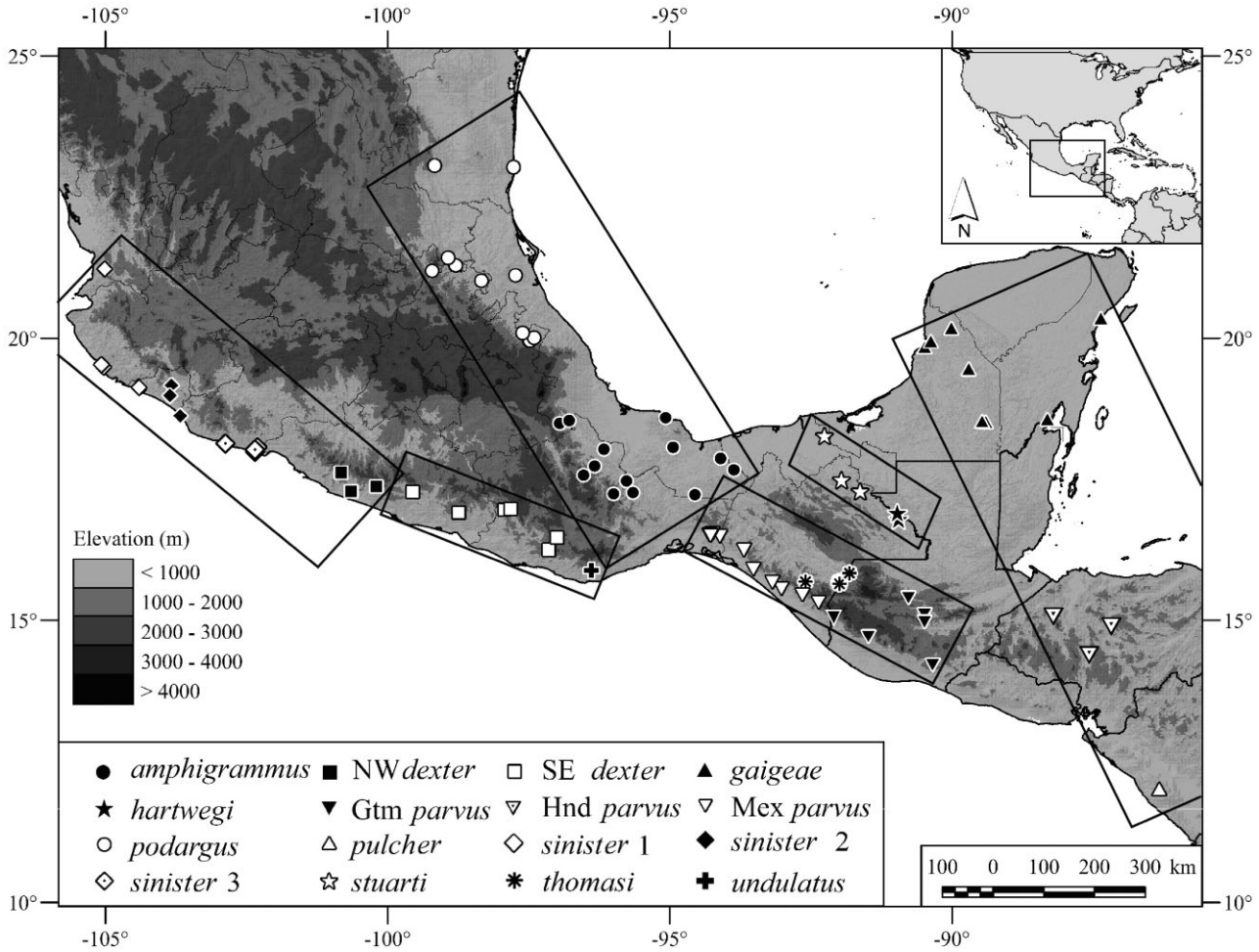



**Figure 3.** Geographical distribution of the six major, significantly supported haplotype clades identified in the Bayesian and ML phylogenetic analyses of the mitochondrial genes.

*podargus*. The other clade was composed of all of the haplotypes of *H. u. hartwegi* and *H. u. stuarti*. However, these subspecies were not exclusive with respect to each other.

One clade was geographically distributed mostly on the Pacific versant of Mexico east of the Isthmus of Tehuantepec and Guatemala, but it also included some haplotypes from central Guatemala. This clade was composed of all the haplotypes of *H. u. parvus* from Mexico and Guatemala and a nested, significantly supported clade with all the haplotypes of *H. u. thomasi*.

The last clade was geographically distributed on the Atlantic versant of Mexico (the Peninsula of Yucatán) and Honduras, and the Pacific versant of Nicaragua. This clade was composed of two significantly supported subclades: one with all the haplotypes of *H. u. gaigeae*, and another one with the only haplotype of *H. u. pulcher* as sister to a significantly supported clade with all the haplotypes of *H. u. parvus* from Honduras (hereafter the Honduran *H. u. parvus* lineage).

The relationships among the above six major clades were not significantly supported, although the (Mexican *H. u. parvus* + *H. u. thomasi*) clade was only marginally not significantly supported as sister to the (southeastern *H. u. dexter* + *H. u. undulatus*) clade in the Bayesian analysis (PP = 0.94).

Thus, *H. u. gaigeae*, *H. u. sinister*, *H. u. thomasi* and *H. u. undulatus* were all significantly supported as exclusive lineages concordant with geography, whereas *H. u. amphigrammus* and *H. u. podargus* also were exclusive and concordant with geography, but were not significantly supported. In contrast, the haplotypes of *H. u. dexter* were not exclusive but formed two separate, significantly supported lineages concordant with geography, and one of them was non-exclusive with respect to *H. u. undulatus*. Similarly, the haplotypes of *H. u. parvus* were not exclusive but formed two separate, significantly supported lineages concordant with geography, and one of them was non-exclusive with respect to *H. u. thomasi*. Finally, the combined

**Table 3.** Genetic divergences (A) among the six significantly supported, major mitochondrial clades of *Holcosus undulatus*, and (B) between lineages within the clades

| Clade | | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| A | | | | | | | |
| 1 | Gtm *H. u. parvus* + (Mex *H. u. parvus* + *H. u. thomasi*) | | | | | | |
| 2 | SE *H. u. dexter* + *H. u. undulatus* | 8.2 | | | | | |
| 3 | *H. u. stuarti* + *H. u. hartwegi* | 7.4 | 8.1 | | | | |
| 4 | *H. u. amphigrammus* + *H. u. podargus* | 7.9 | 8.8 | 7.8 | | | |
| 5 | NW *H. u. dexter* + *H. u. sinister* | 9.1 | 9.8 | 8.9 | 8.2 | | |
| 6 | *H. u. gaigeae* + (Hnd *H. u. parvus* + *H. u. pulcher*) | 10.7 | 11.1 | 11.2 | 10.2 | 11.4 | |
| 7 | *H. quadrilineatus* | 16.1 | 17.0 | 16.2 | 15.6 | 15.9 | 16.8 |
| B | | | | | | | |
| | Gtm *H. u. parvus* – (Mex *H. u. parvus* + *H. u. thomasi*) | 4 | | | | | |
| | · Mex *H. u. parvus* – *H. u. thomasi* | 2.2 | | | | | |
| | SE *H. u. dexter* – *H. u. undulatus* | 3.2 | | | | | |
| | *H. u. amphigrammus* – *H. u. podargus* | 1.7 | | | | | |
| | NW *H. u. dexter* – *H. u. sinister* | 9 | | | | | |
| | · *H. u. sinister* 1 – *H. u. sinister* 2 – *H. u. sinister* 3 (average) | 5.9 | | | | | |
| | *H. u. gaigeae* – (Hnd *H. u. parvus* + *H. u. pulcher*) | 12 | | | | | |
| | · Hnd *H. u. parvus* – *H. u. pulcher* | 8.4 | | | | | |

haplotypes of *H. u. hartwegi* and *H. u. stuarti* comprised a significantly supported clade concordant with geography, but were not mutually exclusive. Because only one sample of *H. u. pulcher* was available, the exclusivity of this subspecies could not be evaluated.

The ML tree (not shown) was similar to the Bayesian tree, except that the *H. u. podargus* clade and all of the relationships among the six significantly supported major clades were not significantly supported.

Genetic divergences (uncorrected p-distances, expressed as percentages; Table 3) within the significantly supported, major clades were moderately high between north-western *H. u. dexter* and *H. u. sinister* (9.0), *H. u. pulcher* and Honduran *H. u. parvus* (8.4), and (*H. u. pulcher* + Honduran *H. u. parvus*) and *H. u. gaigeae* (12.0); intermediate among the *H. u. sinister* subclades (5.9); moderate between south-eastern *H. u. dexter* and *H. u. undulatus* (3.0) and Mexican and Guatemalan *H. u. parvus* (4.0); and low between *H. u. amphigrammus* and *H. u. podargus* (1.7).

### Nuclear data

The RPS8 and α-cardiac actin datasets consisted of approximately 511 and 561 unambiguously aligned nucleotide positions, respectively. Individual nuclear gene trees showed less resolution and much weaker support than the trees from the mitochondrial dataset (Figs S2, S3). Of the significantly supported mitochondrial lineages included and represented by more than one sample, only the *H. u. gaigeae* and *H. u. podargus* lineages were recovered in both the Bayesian and the ML analyses of RPS8; and of these, only the *H. u. gaigeae* lineage was significantly supported in both analyses. Similarly, only the Honduran *H. u. parvus* and *H. u. sinister* lineages were recovered in the Bayesian and ML analyses of α-cardiac actin; and of these, only the Honduran *H. u. parvus* lineage was significantly supported in the ML (but not the Bayesian) analysis. In addition, the *H. u. stuarti* samples were significantly supported as exclusive and a clade composed of one of the Guatemalan *H. u. parvus*, the two Honduran *H. u. parvus*, and the *H. u. hartwegi* samples was significantly supported in both the Bayesian and the ML analyses of RPS8. None of the six major clades in the mitochondrial tree was recovered. Most importantly, however, the nuclear markers did not contradict the relationships recovered by the mitochondrial marker.

### Species tree

The coalescent-based species tree is shown in Figure 4. The tree was fully resolved but differed from the mitochondrial tree mainly in that: (1) the (*H. u. podargus* + *H. u. amphigrammus*) and (*H. u. stuarti* + *H. u. hartwegi*) clades were significantly supported and marginally not significantly supported (PP = 0.91), respectively; and (2) it recovered all of the major, significantly supported mitochondrial clades except for the (*H. u. gaigeae* + Honduran *H. u. parvus*) clade. In the species tree, the Honduran *H. u. parvus* and *H. u. gaigeae* were the most and second-most closely related species, respectively, to the (Mexican–Guatemalan *H. u. parvus* + *H. u. thomasi*) clade, although these relationships were not significantly

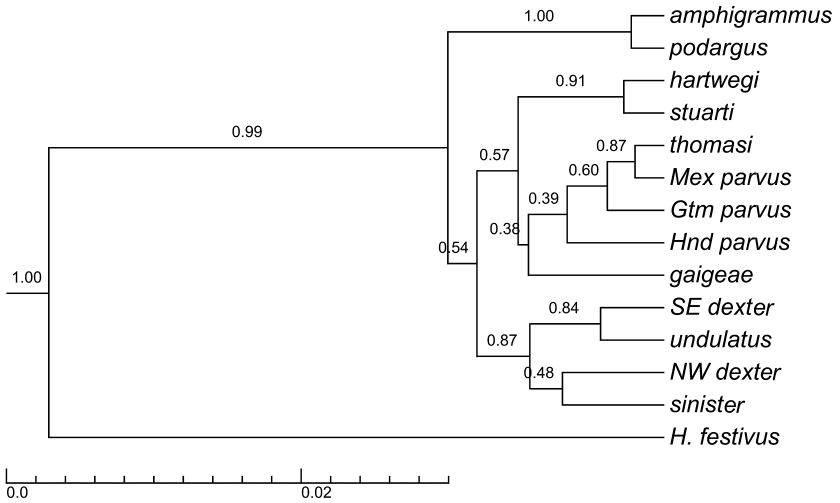



**Figure 4.** Bayesian MCC species tree of *H. undulatus* inferred by simultaneous gene tree and species tree analysis of the mitochondrial and nuclear genes with *BEAST. Numbers above the branches are posterior probabilities.

supported, and these five species were intermediately supported as the sister group to the (*H. u. hartwegi* + *H. u. stuarti*) clade. All of these species comprise a Central American group that is distributed from the Isthmus of Tehuantepec, Mexico, south and east (including the Yucatán Peninsula) to Nicaragua. This group was intermediately supported as sister to a relatively well-supported group composed of the (south-eastern *H. u. dexter* + *H. u. undulatus*) and (north-western *H. u. dexter* + *H. u. sinister*) clades, which is distributed on the Pacific versant of Mexico from Nayarit to the Isthmus of Tehuantepec in Oaxaca. Finally, the (*H. u. amphigrammus* + *H. u. podargus*) clade, from the Atlantic versant of Mexico west of the Isthmus of Tehuantepec, was significantly supported as sister to all of the other species.

### Testing alternative phylogenetic hypotheses

The Bayesian approach to hypothesis testing showed that the alternative hypotheses of the exclusivity of *H. u. dexter* and *H. u. parvus* could be statistically rejected because the alternative sets of relationships were not present in the 95% set of credible trees. In contrast, the south-eastern *H. u. dexter* lineage was recovered as exclusive in 1317 out of 7121 trees in the 95% set of credible trees. Thus, the alternative hypothesis of the exclusivity of this group could not be statistically rejected.

## DISCUSSION

*Holcosus undulatus* was significantly supported as an exclusive lineage distinct and highly divergent from all the other Middle American *Holcosus* in all the mtDNA analyses (see above). However, note that these analyses did not include *H. leptophrys* or *H. chaitzami*. Also, the haplotypes of *H. undulatus* were distributed into six significantly supported, major lineages (see above). All these lineages were concordant with geography, allopatric or parapatric, and highly divergent genetically from each other. The deep genetic structure of the *H. undulatus* populations in the mitochondrial tree and the distribution of the haplotypes predominantly into lineages of identical subspecific designation and concordant with geography suggest strongly that *H. undulatus* is a species complex diversified in Mexico and Central America, and that significant species diversity has been concealed in the genus. Below, we apply the WP and BS methods to the focal species of this study (*H. undulatus* and its subspecies), discuss phylogenetic relationships among the *H. undulatus* lineages and propose pertinent taxonomic changes.

In the WP method, the basal lineages of an exclusive focal species are considered as potentially distinct species if there is no gene flow between them, regardless of their geographical distribution. In the BS method, the daughter lineages of an exclusive focal species also are considered as potentially distinct species if they are allopatric and therefore no gene flow between them is possible (i.e. they are non-GE), whether they are EI or not. However, if there is no evidence of gene flow between the daughter lineages but they are parapatric (i.e. genealogical exchangeability cannot be ruled out), whether they are considered as potentially distinct species depends on the nature of the parapatry and the evidence of adaptive divergence: when the sister lineages are separated by a historical barrier to gene flow (i.e. 'significant' geographical or habitat breaks), they may be interpreted as distinct species

with niche conservation if they are EI, and as potentially distinct species if they are not. When the sister lineages are not separated by a historical barrier to gene flow, they are considered as a single cohesion species if they are EI, and as potentially distinct species if they are not.

### Species limits within basal clades

*(North-western* H. u. dexter + H. u. sinister*) and (*H. u. gaigeae + *(Honduran* H. u. parvus + H. u. pulcher*)) clades*

In each of these clades, the daughter lineages corresponded to distinct subspecies and were significantly supported and concordant with geography. In the WP method, this suggests that the daughter lineages of each clade may represent distinct species. In addition, these lineages were allopatric (Figs 2, 3) and highly divergent genetically (p = 10.0–11.1 and 12.0%, respectively), which supports that suggestion. Furthermore, the subspecies *H. u. sinister* and *H. u. gaigeae* were exclusive. Similarly, in the BS method, the above evidence suggests that the daughter lineages of each basal clade are non-GE. Also, because of their adaptive divergence, they are non-EI (Table 2). Thus, the daughter lineages of each basal clade must be reset as emergent focal taxa.

In the (Honduran *H. u. parvus* + *H. u. pulcher*) emergent focal taxon, the daughter lineages also corresponded to distinct subspecies (if one of them was represented by a single haplotype), and the Honduran *H. u. parvus* lineage was significantly supported and concordant with geography. In addition, the Honduran *H. u. parvus* and *H. u. pulcher* haplotypes originated from distant areas on the Atlantic and Pacific versants of Honduras and Nicaragua, respectively, and were highly divergent from each other (p = 8.4). Thus, gene flow between these subspecies seems unlikely. In the WP method, this suggests that the Honduran *H. u. parvus* lineage and *H. u. pulcher* may represent distinct species; in the BS method, it suggests that they are non-GE and non-EI, and therefore the hypothesis of a single cohesion species for this clade must be rejected.

*(South-eastern* H. u. dexter + H. u. undulatus*) clade*

In this clade, the basal lineages were significantly supported, concordant with geography, and geographically disjunct. In the WP method, this suggests that the lineages may represent distinct species. However, there were large unsampled areas between their geographical distributions, and the basal-most clades were composed of few individuals from single localities. This suggests instead that the apparent absence of gene flow between the basal lineages is probably an artefact of insufficient sampling, and the basal lineages actually represent a single species.

By contrast, the haplotypes of *H. u. dexter* were not exclusive with respect to those of *H. u. undulatus*, which comprised a significantly supported, exclusive lineage concordant with geography. In the WP method, if there is gene flow between the basal lineages of a focal species, and if the haplotypes of the focal species are not exclusive with respect to the haplotypes of a second species that is distinct and exclusive, then the focal species may represent a single non-exclusive species. Therefore, the south-eastern *H. u. dexter* and *H. u. undulatus* lineages may represent distinct species, even though they were only slightly divergent from each other (p = 3.2). However, our sampling of both *H. u. undulatus* and the area of intergradation between *H. u. dexter* and *H. u. undulatus* in south-central Oaxaca (Smith & Laufe, 1946; Fig. 1) was considerably limited; in fact, our sample of *H. u. undulatus* came from a single locality in the latter area. Thus, additional sampling is needed to conclusively corroborate the existence of two distinct species in this basal clade. For the time being, we conservatively treat this basal clade as a single species (*H. undulatus*, because of the law of priority).

In the BS method, the daughter lineages of this basal clade were concordant with geography and appeared to be geographically disjunct, which suggests they are non-GE. However, one of the lineages was not significantly supported in the Bayesian analysis, and the other was composed of samples from a single locality. Also, the area between their geographical distributions was not sampled. Thus, we consider the daughter lineages to be actually GE. In addition, the populations of *H. u. dexter* in both daughter lineages lacked adaptive divergence. Thus, these populations are EI, and should be treated as a single cohesion species. The low genetic divergence between the daughter lineages supports this suggestion. In addition, the above evidence suggests that the *H. u. undulatus* lineage is both non-GE and non-EI with the *H. u. dexter* lineage, and it may represent a separate cohesion species even though is only slightly divergent genetically from it (but see our reservations above).

*(Mexican–Guatemalan* H. u. parvus + H. u. thomasi*) clade*

In this clade, the basal lineages were significantly supported and concordant with geography, even though they were parapatric in extreme south-eastern Chiapas and represented by relatively numerous samples (Figs 2, 3). In the WP method, this suggests the absence of gene flow between the daughter lineages and therefore that they may represent two distinct, independent evolutionary lineages, although they were only slightly divergent from each other (p = 4.0): one composed of the populations of *H. u. parvus* from Guatemala and the Tacaná volcano in extreme south-eastern Chiapas

(hereafter the Guatemalan *H. u. parvus* lineage), and the other one composed of the remaining populations of *H. u. parvus* from Mexico (hereafter the Mexican *H. u. parvus* lineage) and the populations of *H. u. thomasi*.

In the (Mexican *H. u. parvus* + *H. u. thomasi*) subclade, the basal lineages were neither consistently significantly supported nor concordant with geography, which suggests they represent the same evolutionary species. However, the haplotypes of *H. u. parvus* were not exclusive with respect to those of *H. u. thomasi*, which comprised a significantly supported, exclusive lineage concordant with geography. In the WP method, this suggests that the Mexican *H. u. parvus* and *H. u. thomasi* lineages may represent distinct species (see above), even though they were only slightly divergent genetically from each other (p = 2.1–2.2). This suggestion is supported by the relatively extensive sampling of both lineages and their geographical isolation by the highlands of the Sierra Madre de Chiapas and western portion of the Central Depression of Chiapas (Figs 1, 3).

In the BS method, the above evidence suggests the absence of gene flow between the daughter lineages of this basal clade. However, because the daughter lineages are parapatric in the absence of major geographical or habitat breaks, they are potentially GE, and considerable weight is placed on evidence of adaptive divergence. Because the populations of *H. u. parvus* in both daughter lineages lacked adaptive divergence, they are EI, and should be treated as a single cohesion species. Genetic divergence between the daughter lineages was low (≤ 4.0). In addition, the above evidence suggests that the *H. u. thomasi* lineage is both non-GE and non-EI with the *H. u. parvus* lineage, and it may represent a separate cohesion species even though it is only slightly divergent genetically from it (see above).

*(*Holcosus u. amphigrammus + H. u. podargus*) clade*

In this clade, the daughter lineages corresponded to distinct subspecies, both of which were concordant with geography and were exclusive. In the WP method, this suggests the absence of gene flow between the *H. u. amphigrammus* and *H. u. podargus* lineages, and therefore that they may represent distinct species. However, these lineages were not significantly supported, and were only slightly divergent genetically from each other (p = 1.7).

Although Smith & Laufe (1946) stated that *H. u. amphigrammus* and *H. u. podargus* intergrade in northern Veracruz, our field work showed that their geographical distributions actually meet at the eastern end of the Mexican Transvolcanic Belt in central Veracruz. A division of the Gulf Coastal Plain into northern and southern portions at central Veracruz has been documented in other groups such as toads (Mulcahy & Mendelson, 2000), frogs (Zaldívar-Riverón, León-Regagnon & Nieto-Montes de Oca, 2004), and reptiles and mammals (Pérez-Higareda & Navarro, 1980). This phylogeographical break has been explained by the repeated inundation of the coastal floodplain resulting from rising and lowering sea levels throughout much of the Pleistocene (Beard, Sangree & Smith, 1982). In the BS method, because there was no evidence of gene flow between the daughter lineages of the basal clade and because the lineages appeared to be parapatric but separated by a phylogeographical break, they are considered to be non-GE. Also, because of their adaptive divergence, the daughter lineages are non-EI, and thus the hypothesis of a single species should be rejected, and these subspecies reset as emergent focal taxa.

It has been argued that poorly supported clades are unreliable because they may have been recovered by chance if their sample size is small (Erixon *et al*., 2003). However, sample size for both *H. u. amphigrammus* and *H. u. podargus* was rather large (15 and 17 haplotypes, respectively). This suggests that the concordance with geography of the *H. u. amphigrammus* and *H. u. podargus* lineages actually reflects reduced or absent gene flow between them, and that their weak support and low divergence might be explained by causes such as a recent origin or introgressive hybridization from *H. u. amphigrammus* into *H. u. podargus* followed by a complete replacement sweep across its entire distribution (Funk & Omland, 2003; Rheindt & Edwards, 2011). However, until further data corroborate that *H. u. amphigrammus* and *H. u. podargus* are distinct species, we prefer to treat this basal clade as a single species.

*(*Holcosus u. hartwegi + H. u. stuarti*) clade*

In this clade, although both daughter lineages were significantly supported, they were not concordant with geography (i.e. both the haplotypes of *H. u. hartwegi* and *H. u. stuarti* from a given locality failed to cluster together; Figs 2, 3), which is potential evidence for gene flow with other populations. Thus, although the clade was composed of haplotypes of two distinct subspecies, they were not exclusive with respect to each other. In the WP method, this suggests that these taxa represent a single species. Similarly, in the BS method, because the daughter lineages of this basal clade were not concordant with geography or taxonomic designation, they may be considered to be both GE and EI. This suggests that the hypothesis of a single cohesion species cannot be rejected.

However, *H. u. hartwegi* and *H. u. stuarti* differ in body length, size and arrangement of gular scales, numbers of femoral pores and lamellae under the fourth toe, and dorsolateral colour pattern (Smith & Laufe, 1946; Table 2). In fact, Smith & Laufe (1946: 22)

regarded *H. u. stuarti* to be so widely different from *H. u. hartwegi* (and *H. u. gaigeae*) that 'it might well be considered a member of a different species'. Smith & Laufe (1946) also stated that *H. u. hartwegi* and *H. u. stuarti* occur in close geographical proximity, but that no incontrovertible intergrades between the two were known. Furthermore, there appears to be a sharp difference in ecological preference: *H. u. hartwegi* occurs in dense, high inland forest, whereas *H. u. stuarti* inhabits mixed scrub-savanna coastal areas (Smith & Laufe, 1946). Thus, the morphological and ecological evidence suggests strongly that *H. u. hartwegi* and *H. u. stuarti* are distinct species.

However, *H. u. hartwegi* and *H. u. stuarti* were not mutually exclusive in their haplotype phylogeny. This pattern might be explained by incomplete lineage sorting if *H. u. hartwegi* and *H. u. stuarti* actually were sister species that have diverged recently. However, this seems unlikely given the morphological divergence between them. Current gene flow also seems unlikely, as there is no evidence of morphological intermediates (Smith & Laufe, 1946). Another possible explanation for the non-exclusivity of *H. u. stuarti* and *H. u. hartwegi* is introgressive hybridization, as they share similar mtDNA haplotypes but are otherwise divergent species (Funk & Omland, 2003).

### *An alternative scenario for* Holcosus undulatus dexter

The above discussion assumed that the non-monophyly of *H. u. dexter* is explained by imperfect taxonomy (Funk & Omland, 2003), i.e. that the north-western *H. u. dexter* lineage actually represents a cryptic species morphologically similar to, yet distinct from, true *H. u. dexter*. However, we did not find evident morphological differences between the two *H. u. dexter* lineages despite the high genetic divergence between them. Also, it seems odd that such apparently distantly related lineages should be morphologically identical and parapatric.

An alternative explanation is that one of the lineages of *H. u. dexter* does not actually correspond to the native mitochondrial genome of *H. u. dexter* but represents the captured mitochondrial genome of some other lineage. The haplotypes of the south-eastern *H. u. dexter* and *H. u. undulatus* lineages are only slightly divergent from each other, and these two subspecies were reported to intergrade in south-central Oaxaca (Smith & Laufe, 1946). This suggests that the south-eastern *H. u. dexter* lineage might actually represent the captured mitochondrial genome of *H. u. undulatus* (if the latter subspecies actually is distinct from *H. u. dexter*, see above). The north-western *H. u. dexter* lineage is highly divergent from *H. u. sinister* and geographically isolated from it by the Balsas River Basin (Smith & Laufe, 1946); thus, introgressive hybridization from *H. u. sinister* into the north-western *H. u. dexter* lineage seems less likely. Under this scenario, the two mitochondrial lineages of *H. u. dexter* would represent one and the same geographically continuous, morphologically homogeneous lineage that has been introgressed by *H. u. undulatus* over most of its geographical distribution.

A similar scenario could be conceived as an alternative explanation for the non-monophyly of *H. u. parvus*. However, the high genetic distances between the Honduran *H. u. parvus* lineage and its most closely related subspecies in the mitochondrial tree, *H. u. gaigeae* and *H. u. pulcher* (p = 10.7–13.3), do not support this hypothesis.

## Species limits at lower levels

The WP method emphasizes the basal lineages of a focal species as potentially distinct species. However, because it is possible that each of these lineages might contain multiple species, it uses the same reasoning to detect such cases. Similarly, in the BS method, the emergent focal taxa are evaluated in the same manner as the basal taxa, and evaluation progresses through the tree towards the tips, until the daughters are determined to be a single cohesion species. This is important because emphasizing only the basal lineages within the focal species might overlook those taxa that represent the most recent adaptations to a changing environment and may be important sources of future evolutionary potential (Wang *et al*., 2008). However, at these progressively lower levels, small sample sizes may limit our ability to confidently rule out gene flow with other lineages.

In this study, sampling of most of the lineages identified as potentially distinct species within the basal clades, as well as the potential areas of contact between the geographical distributions of these lineages, was generally not extensive. Thus, within most of those lineages, the haplotypes generally did not form significantly supported subclades concordant with geography, or, if they did, there were relatively large unsampled areas among their distributions. Also, the populations of each of the potential species were morphologically homogeneous, and their haplotypes only slightly divergent from each other. Thus, the available evidence suggests that all of these lineages each represent a single species. The only clear exception is the *H. u. sinister* lineage.

The three *Holcosus undulatus sinister* lineages were significantly supported, concordant with geography, allopatric or parapatric, and intermediately divergent from each other (p = 4.8–6.4%). Furthermore, in males and females of the *H. u. sinister* 1 and *H. u. sinister* 3 lineages the throat was consistently orange, whereas in males and females of the geographically

intermediate *H. u. sinister* 2 lineage the throat was consistently yellow. This suggests that each lineage may represent a separate species. This is supported by the moderately high genetic divergences and apparently fixed throat colour differences among them. However, throat colour polymorphism is well known to occur in several populations of *H. undulatus* (Echternacht, 1971).

### Comparison of used methods

The WP and BS methods differ in the species properties that they use for species delimitation. In the WP method, the basal lineages of a focal species are considered as potentially distinct species if there is no evidence of gene flow between them, regardless of their geographical distribution. In contrast, in the BS method non-GE sister lineages that are parapatric but not separated by a 'significant' geographical or habitat break are not considered as potentially distinct species unless they show evidence of adaptive divergence potential (i.e. they are non-EI). This is because genealogies are influenced by chance in the form of genetic drift. Therefore, phylogeographical breaks in a continuously distributed species might develop in the absence of geographical barriers as a result of stochastic causes, if the average individual dispersal distances and/or population size of the species are low (Irwin, 2002).

Bond & Stockman (2008) used the spiders of the *Aptostichus atomarius* complex as their model system. These spiders are fossorial, sedentary, sit-and-wait predators prone to extreme population structuring (see Bond *et al*., 2001, 2006; Arnedo & Ferrández, 2007; Starrett & Hedin, 2007). Unlike Bond & Stockman's (2008) model system, most teiids (including *Holcosus*) are good runners and active wide-ranging foragers. These characteristics may hinder the formation of deep phylogeographical breaks as a result of stochastic causes. Additionally, genetic structure in *H. undulatus* did not extend to the tips of the mtDNA tree. Except for the *H. u. sinister* lineage, the basal lineages were moderately to intermediately genetically structured. If small female dispersal distances were the general cause for genetic structure in *H. undulatus*, we would expect that those lineages were structured too, especially if they have wide geographical distributions.

We argue that, rather than being the result of stochastic causes, deep genetic structure in the absence of extrinsic barriers to gene flow in *H. undulatus* probably indicates that some intrinsic reproductive isolation is operating. Therefore, as long as they are well sampled, significantly supported sister lineages concordant with geography and highly divergent in the mtDNA tree probably represent reproductively isolated, evolutionary independent lineages, even in the absence of geographical barriers and adaptive divergence.

### Phylogeny

The six major mitochondrial clades were significantly supported in both the Bayesian and the ML analyses, and were also recovered (although most not significantly supported) in the species tree, except for the (*H. u. gaigeae* + Honduran *H. u. parvus*) clade. However, the phylogenetic relationships among the major clades within *H. undulatus* remain poorly resolved.

By contrast, the sister taxa relationship between the daughter lineages of each of the major clades seems highly probable on the basis of their morphology and geographical distribution, except for *H. u. stuarti* and *H. u. hartwegi*, whose actual position in the tree is uncertain, as their relationships are probably obscured by ancestral gene flow. In the species tree, the ((south-eastern *H. u. dexter* + *H. u. undulatus*) + (north-western *H. u. dexter* + *H. u. sinister*)) clade, which is distributed on the Pacific versant of Mexico west of the Isthmus of Tehuantepec, is not only geographically congruent but also contains only those species having one row of preanals (Smith & Laufe, 1946). Similarly, the (((Honduran *H. u. parvus* + (Guatemalan *H. u. parvus* + (Mexican *H. u. parvus* + *H. u. thomasi*))) clade is distributed east of the Isthmus of Tehuantepec, and contains those taxa sharing two rows of preanals and abruptly enlarged median gulars arranged in a single longitudinal row (Smith & Laufe, 1946). Therefore, it can be expected that further data will support these relationships.

### Taxonomic conclusions

Our results suggest that, at least, the following lineages should be recognized as distinct evolutionary species:

(*H. u. amphigrammus* + *H. u. podargus*) = *H. amphigrammus*
*H. u. gaigeae* = *H. gaigeae*
*H. u. hartwegi* = *H. hartwegi*
(Guatemalan–Mexican *H. u. parvus*) = *H. parvus*
*H. u. sinister* = *H. sinister*
*H. u. stuarti* = *H. stuarti*
*H. u. thomasi* = *H. thomasi*
(South-eastern *H. u. dexter* + *H. u. undulatus*) = *H. undulatus*

However, note that (1) the (*H. u. amphigrammus* + *H. u. podargus*), Guatemalan–Mexican *H. parvus*, and (south-eastern *H. u. dexter* + *H. u. undulatus*) lineages could each be composed of two distinct species; and (2) *Holcosus hartwegi* and *H. stuarti* are recognized on morphological and ecological grounds, and the apparent gene flow between them is attributed to introgressive hybridization.

In addition, the north-western *H. u. dexter* and Honduran *H. u. parvus* lineages may represent cryptic, yet distinct, independent species, provided that the non-monophly of *H. u. dexter* and *H. u. parvus* in the mitochondrial tree is explained by imperfect taxonomy. Further research on the systematics of these two taxa is needed to identify the cause of their non-monophyly and, if appropriate, formally describe the potentially undescribed species. Further research may also uncover additional species within the *H. u. sinister* lineage. Several approaches are available for delimiting species using coalescent techniques (Fujita *et al*., 2012), but the most robust methods rely on the availability of data for several independent loci. Future research with multilocus data, analysed under a coalescent approach, will be decisive for elucidating the species boundaries within *H. dexter*, *H. sinister* and *H. parvus*.

The above conclusions have implications for the status of *H. u. miadis* and *H. u. pulcher*. Evidently, it is realistically not possible that these two subspecies are conspecific with *H. undulatus*. Thus, their status should depend on whether they are distinct from the species identified herein and from each other. We could not include *H. u. miadis* in our analysis. However, this subspecies, known only from Corn Islands, Nicaragua, is allopatric with respect to the other subspecies of *H. undulatus*, and can be distinguished from these subspecies on the basis of its unique colour pattern and several other morphological characters (Echternacht, 1970, 1971). Thus, we also consider *H. u. miadis* to represent a distinct species endemic to Corn Islands. Similarly, both the WP and the BS methods suggest that *H. u. pulcher* may represent a distinct species, although this needs corroboration through additional sampling.

This work has significant implications for the conservation of Mexican and Central American *Holcosus*. Currently, *H. undulatus* is perceived as a common, widely distributed, single species with no conservation problems. Nonetheless, our study indicates that several of its once recognized subspecies actually represent distinct, independent evolutionary species, and therefore the number of species of Mexican and Central American *Holcosus* has been severely underestimated. Evidently, these species have more restricted geographical distributions and their own biological and ecological properties, and their conservation status and extinction risk should be assessed separately. Thus, the basic knowledge of their existence is essential for their conservation.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** The inter-relationships of selected lineages of Teiidae, including the phylogenetic placement of *H. undulatus*, inferred by a partitioned Bayesian analysis of the mitochondrial genes. The tree is an MCC tree. Numbers next to branches indicate posterior probability/bootstrap support values.

**Figure S2.** Bayesian MCC tree of *H. undulatus* based on an analysis of the nuclear marker RPS8. Numbers next to branches indicate posterior probability/bootstrap support values.

**Figure S3.** Bayesian MCC tree of *H. undulatus* based on an analysis of the nuclear marker α-cardiac-actin. Outgroups are not shown. Numbers next to branches indicate posterior probability/bootstrap support values.

**Table S1.** Taxonomic designation, ID, voucher number, locality and GenBank accession numbers for the DNA sequences used in this study. Acronyms for specimen numbers are either scientific collection acronyms (CAS, MVZ, MZFC, UTA) listed in Sabaj-Pérez (2014), field numbers for specimens to be catalogued in the MZFC (AMH, ANMO, ART, DHL, IDF, ISZ, JAC, JCBH, JLAL, JRM, LCM, LNG, LMOO, NLMM, UOGV) or the UTA (MSM) collections, or field numbers assigned to sequences downloaded from Genbank (ALS, BWMC). CR, Costa Rica; Dom Rep, Dominican Republic; Gtm, Guatemala; Hnd, Honduras; Mex, Mexico; Nic, Nicaragua; Sur, Surinam; USA, United States of America.

**Table S2.** Primers used in study.

**Table S3.** Partition strategies, harmonic mean of the likelihood values of the MCMC samples, and GTR submodels with the highest posterior probability (the submodels represented by six numbers specify different substitution rates in the order $r_{AC}$; $r_{AG}$; $r_{AT}$; $r_{CG}$; $r_{CT}$; $r_{GT}$).