# Conservation phylogenetics of helodermatid lizards using multiple molecular markers and a supertree approach

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## ABSTRACT

We analyzed both mitochondrial (MT-) and nuclear (N) DNAs in a conservation phylogenetic framework to examine deep and shallow histories of the Beaded Lizard (*Heloderma horridum*) and Gila Monster (*H. suspectum*) throughout their geographic ranges in North and Central America. Both MTDNA and intron markers clearly partitioned each species. One intron and MTDNA further subdivided *H. horridum* into its four recognized subspecies (*H. n. alvarezi, charlesbogerti, exasperatum,* and *horridum*). However, the two subspecies of *H. suspectum* (*H. s. suspectum* and *H. s. cinctum*) were undefined. A supertree approach sustained these relationships. Overall, the Helodermatidae is reaffirmed as an ancient and conserved group. Its most recent common ancestor (MRCA) was Lower Eocene [35.4 million years ago (mya)], with a ~25 my period of stasis before the MRCA of *H. horridum* diversified in Lower Miocene. Another ~5 my passed before *H. h. exasperatum* and *H. h. horridum* diverged, followed by ~1.5 my before *H. h. alvarezi* and *H. h. charlesbogerti* separated. *Heloderma suspectum* reflects an even longer period of stasis (~30 my) before diversifying from its MRCA. Both *H. suspectum* (México) and *H. h. alvarezi* also revealed evidence of historic range expansion following a recent bottleneck. Our conservation phylogenetic approach emphasizes the origin and diversification of this group, yields information on the manner by which past environmental variance may have impacted its populations and, in turn, allows us to disentangle historic impacts from contemporary impacts that might threaten its long-term persistence. The value of helodermatid conservation resides in natural services and medicinal products, particularly venom constituents, and these are only now being realized.

## 1. Introduction

Conservation genetics is the application of molecular methods to preserve species as dynamic entities capable of coping with environmental change (Frankham et al., 2003). Yet, to become the practical tool for biodiversity management many had envisioned (Vijenhoek et al., 1985), the discipline must move beyond a mere description of genetic diversity in single species (Milligan et al., 1994) and depict instead regional biodiversities in both space and time. The 'space' component can be readily grasped using phylogeography as a model (Avise, 2000, 2009), whereas the temporal component is less intuitive for it invokes historical (or phylogenetic) rather than contemporaneous time (Stockwell et al., 2003). A deep historical perspective is largely missing from molecular conservation genetics as generally practiced today. Instead, the field is often narrowly equated with relationships among extant populations mediated through measures of inbreeding depression, heterozygosity loss, and pedigree analysis (Avise, 1996; Hughes et al., 2008). The field must instead broaden and extend such that it no longer merely focuses on the numerous and diverse twigs in the tree of life, but also within its deeper and more substantive branches.

The logic of prioritizing conservation based upon a phylogenetic perspective was first articulated by Wilson (1992), who noted that a successful conservation strategy should place greater emphasis on sustaining as much evolutionary diversity as possible, especially with regard to substantive branches in the tree of life (reviewed by Crozier, 1997; Crozier et al., 2006). Others have been similarly evocative. Nee and May (1997) argued for the importance of visualizing historic biodiversity processes (e.g., extinctions and diversifications) through a phylogenetic lens, with the resulting perspectives often dictating modern conservation priorities. Willis and Birks (2006) emphasized the value of an extended temporal perspective to disentangle historic from recent variation so as to provide guidance for conservation priorities. These (and other)

viewpoints prompted Avise (2005) to suggest 'conservation phylogenetics' as a research focus, with the 'Tree of Life' as a potential metric of standardization (Avise et al., 2008). In this sense, a phylogenetic perspective for 'biodiversity genetics' (Avise, 2008), looms large as we move into a future clouded by global climate change and habitat fragmentation.

### 1.1. Biodiversity genetics as a geocentric science

Here, regions and the species therein must represent the research focus, rather than species within regions. This distinction, while subtle, directs biodiversity research as geo- rather than taxon-centric (Douglas et al., 2006, 2009). Our study spans the three deserts of western North America and the tropical dry forests along eastern and western versants of México/Guatemala, an area encompassing the entire distribution of *Heloderma*. Much of the conservation research in this region has focused on species inhabiting restricted drainages (Hedrick et al., 2001), isolated springs (Hurt, 2004), or elevated sky-islands (Masta, 2000; Knowles, 2001; Holycross and Douglas, 2007).These studies were often provoked by immediate conservation concerns, and consequently were conducted under a strong sense of urgency. Their focal species and restricted habitats were clearly pertinent for specific conservation purposes, yet did not offer the broad geographic perspective necessary for adaptive management at the regional level.

We suggest a regional perspective is an imperative to move biodiversity conservation from reactive to proactive. If we cannot achieve this in a timely fashion, then (as an example) biotic associations and regional linkages within the warm deserts of western North American will be damaged or lost due to mining, logging, and livestock activities coupled with groundwater pumping and land acquisition for urban growth. These anthropogenic impacts will force us yet again into post hoc crisis-mode if their impacts cannot be deflected with pre-emptive regional management. A clarification of faunal diversification within tropical dry forests and regional deserts is a necessary first step in a broader landscape perspective on the adaptive management of these fragile areas. In this sense, our evaluation of the Helodermatidae provides a robust and alternative perspective on the evolution of these regions (Riddle et al., 2000). Additionally, it contributes to a growing list of studies that address taxa within the region (Oakey et al., 2004; Jaeger et al., 2005; Douglas et al., 2006; Schulte et al., 2006; Mulcahy, 2008; Becerra et al., 2009), and may thus serve in tandem with the above as a benchmark for long-term management of biotic communities therein.

Our perspectives employ phylogeny not as a metaphor but instead as a barometer with which to gauge not only origins and histories of regions and species (as above), but their collective fates as well. Conservation phylogenetics is compelling in that it places biodiversity losses within an historical perspective, but serves more importantly as a tool with which to manage the lineages that survive (Davies et al., 2009). Yet, a broader application of this concept is seriously impeded by the lack of phylogenies for most lineages, much less a specific conservation perspective within which to imbed them. This situation is unlikely to soon change (Crozier et al., 2006) unless greater efforts are marshaled to generate phylogenies for rare and enigmatic taxa, and to place them within a conservation format (as herein).

### 1.2. Molecular markers and biodiversity studies

Molecular markers have made a profound and significant contribution to biodiversity studies in general and, in particular, to the enhancement of regional conservation perspectives. However, their benefits are somewhat constrained by practical limitations.

Monsen and Blouin (2003) noted that 83% (29/35) of studies that classified vertebrate populations into management units in the previous 8 years did so with but a single type of marker, and 76% of the latter (22/29) utilized MTDNA. Yet, this molecule has shortcomings that can limit its potential in recovering historic signal (reviewed by Douglas et al., 2002, 2006). Although MTDNA will continue to provide an invaluable window into the phylogeography of populations, clarifying the evolutionary histories of populations and grouping them into cohesive units for management purposes will be more precisely described and interpreted if both MTDNA and nuclear (N) DNAs are analyzed (Douglas et al., 2007).

Non-coding intron segments of nuclear eukaryotic genes represent such an alternative genetic marker (Friesen, 2000), but their function(s) within nuclear genomes remain uncertain (Johnson, 2004). Here, we applied MT- and NDNA markers to identify molecular diversification in Helodermatidae, [i.e., the Beaded Lizard (*Heloderma horridum*) and the Gila Monster (*H. suspectum*)]. We accomplished this by evaluating these species across their distributions, then imbedding this diversity within the Varanoidea, an ancient and monophyletic clade composed of the venomous New World helodermatids, the Old World Bornean Earless Monitor (*Lanthanotus borneennsis),* and the highly-diverse goannas or monitors (65 + *Varanus* species). We then discuss the Varanoidea as a component of the larger squamate clade Anguimorpha, which consists of Anguidae, Helodermatidae, Shinisauridae, Varanidae, Xenosauridae (Townsend et al., 2004, 2008; Vidal and Hedges, 2005, 2009), and occasionally Serpentes (Lee, 2009). Given these perspectives, we employed other major lizard and snake families in our analyses so as to gain a broader perspective and to establish, in a phylogenetic sense, the relative position of varanoid and anguimorph taxa.

## 2. Materials and methods

### 2.1. Study organisms

Within Varanoidea, *Heloderma* is composed of two broad-ranging species: Beaded Lizard (*H. horridum*) and Gila Monster (*H. suspectum*) (Bogert and Martin del Campo, 1956). The Beaded Lizard occurs along the Pacific foothills from southern Sonora to Chiapas (México), along Pacific drainages in southern Guatemala, and in two Atlantic drainages of Chiapas and eastern Guatemala (Campbell and Lamar 2004; Beck, 2005). Four subspecies are recognized: *H. h. alvarezi* (Rio Grijalva valley of central Chiapas to extreme western Guatemala), *H. h. charlesbogerti* (Rio Motagua Valley and adjacent foothills of eastern Guatemala), *H. h. exasperatum* (southern Sonora, northern Sinaloa), and *H. h. horridum* (western México) (Fig. 1).

The Gila Monster is distributed in the Mohave Desert of extreme southern Nevada, southwestern Utah, extreme southeastern California and northwestern Arizona, and throughout the Sonoran Desert region in Arizona and Sonora, México (exclusive of Baja California), and into the Chihuahuan Desert of southeastern Arizona and southwestern New Mexico (Campbell and Lamar, 2004; Beck, 2005). Two subspecies are recognized: *H. s. suspectum* (Reticulate Gila Monster) and *H. s. cinctum* (Banded Gila Monster) (Fig. 1).

We collected 135 tissue samples to represent our ingroup, the two extant species of Helodermatidae, which consisted of 48 *H. horridum* (13 *H. h. alvarezi*, 11 *H. h. charlesbogerti*, 15 *H. h. exasperatum*, 9 *H. h. horridum*) and 87 *H. suspectum* (*H. s cinctum* and *H. s. suspectum*) sampled from populations throughout their range. Our "lizard" outgroups consisted of 8 species of Varanidae [*Varanus beccari* (n = 1), *V. dumerilii* (n = 1), *V. exanthematicus* (n = 1), *V. griseus* (n = 1), *V. indicus* (n = 1), *V. komodoensis* (n = 1), *V. niloticus* (n = 1), and *V. tristis* (n = 2)],
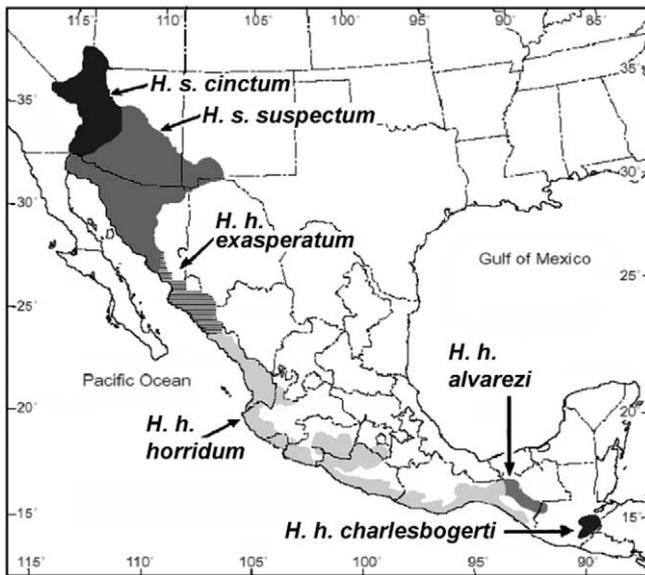
**Fig. 1.** Map depicting distribution of *Heloderma horridum* and *H. suspectum* with shaded areas depicting putative subspecific ranges (following Campbell and Lamar, 2004).

one specimen each of the monotypic Shinisauridae (*Shinisaurus croc-odilurus*) and Lanthanotidae (*Lanthanotus borneensis*), 3 species of Anguidae [*Abronia graminea, Elgaria kingii* (*n* = 2), *Ophisaurus ventralis*], one Xenosauridae (*Xenosaurus rectocollaris*), one Cordylidae (*Cordylus warreni*), 7 Scincidae (*Corucia zebrata, Plestiodon egregius, P. laticeps, Mabuya bistriata, Paracontias holomelas, Scincella lateralis, Tiliqua scincoides*), 5 Iguania (Crotaphytidae: *Gambelia wislizenii;* Iguanidae: *Iguana iguana, Sauromalus ater;* Phrynosomatidae: *Phrynosoma platyrhinos, Sceloporus occidentalis*), 4 Teiidae (*Aspidoscelis inornata, Cnemidophorus ocellifer, Tupinambus quadrilineatus, T. rufrescens*), one Chamaeleonidae (*Furcifer oustaleti*), 3 Agamidae (*Calotes versicolor, Chlamydosaurus kingii, Pogona vitticeps*), 4 Geckkota (Gekkonidae: *Gehyra mutilata, Hemidactylus turcicus, Teratoscincus keyserlingii*; Eublepharidae: *Eublepharis macularis*), 2 Amphisbaenia (Amphisbaenidae: *Amphisbaena schmidti*; Rhineuridae: *Rhineura floridana*), 4 Lacertidae (*Lacerta viridis, Mesalina guttulata, Podarcis muralis, Takydromus tachydromoides*), and one Sphenodontidae (*Sphenodon punctatus*).

In Serpentes, we examined one Acrochordidae (*Acrochordus granulatus*), 2 species of Boidae [*Boa constrictor* (*n* = 3), *Lichanura trivirgata*], 7 species of Pythonidae [(*Liasis mackloti* (*n* = 1), *Morelia boeleni* (*n* = 2), *M. viridis* (*n* = 2), *Python anchietae* (*n* = 2), *P. molurus, P. regius* (*n* = 2), *P. reticulatus* (*n* = 2)], one Cylindrophiidae (*Cylindrophis rufus*), and one Xenopeltidae (*Xenopeltis unicolor*). Our outgroup sample totaled 67 individuals representing 25 families and 59 species. However, sequence data across all of the molecular markers could not be derived for every individual and/or species.

## 2.2. DNA sampling, extraction amplification and alignment

For both ingroup and outgroup taxa, approximately 0.1 ml whole blood was removed from the caudal vein of living animals and stored in 1 ml of 95% ethanol. Samples of liver and tail muscle were recovered from recent road-killed specimens and similarly stored. Genomic DNA was extracted using the Puregene DNA Isolation Kit (Gentra Systems). Mitochondrial ATPase 8 and ATPase 6 genes were amplified across 153 individuals (135 helodermatids + 18 outgroup taxa) using primers specified in Douglas et al. (2006b). Primers for α-enolase (ENOL, Friesen et al., 1997) amplified intron 8 and small parts of exon 8 and 9 over 145 individuals

(135 helodermatids + 10 outgroup taxa), whereas primers for ornithine decarboxylase (OD: Friesen et al., 1999) annealed in exons 6 and 8 and amplified introns 6 and 7 over 147 individuals (135 helodermatids + 12 outgroup taxa). Additional sequence data were obtained from GenBank, either as entire mitochondrial genomes from which target genes were excised, or as specific introns (see Acknowledgments).

Double-stranded sequencing reactions were conducted with fluorescently-labeled dideoxy terminators according to manufacturer recommendations [Applied Biosystems Inc. (ABI), Forest City, CA]. Labeled extension products were analyzed with an automated DNA sequencer (ABI Prism 3100). Sequences were edited using Sequencher (Gene Codes, Ann Arbor MI) and aligned with Clustal-X (Aladdin Systems, Heidelberg, Germany) using default gap costs, with alignments checked visually. MacClade (v. 4.01, W.P. Maddison and D.R. Maddison, 2001; http://macclade.org/) was used to convert individuals to their respective haplotypes or genotypes. As a mechanism to combine sequences for our analyses, we applied the partition homogeneity test (=incongruence length difference test; Farris et al., 1994), as implemented in Paup* (ver. 4.04b10, Swofford, 2001; http://paup.csit.fsu.edu/).

Gaps have not been widely accepted as phylogenetic markers (Kawakita et al., 2003) and consequently are most likely ignored as missing data, considered a fifth base, or simply excluded from analyses. Furthermore, gaps are not part of most explicit models of sequence evolution, and their evaluation has traditionally been restricted to parsimony methods (but see below). However, gaps clearly contain useful phylogenetic signal concordant with that of base substitutions (Graham et al., 2000; Simmons et al., 2001). Simple indel coding (SIC: Simmons and Ochoterena, 2000), a method that successfully converted indels into analyzable data, was broadened and extended by Müller (2005, 2006) into multiple complex indel coding (MCIC), and subsequently programmed (along with older algorithms) into a readily available software (SeqState: http://www.nees.uni-bonn.de/downloads/SeqState). Gaps are coded and subjected to a stepmatrix analysis that determines number of changes needed to transform one sequence into another. Parsimony reconstruction methods then determine the ancestral states that minimize the number of character change steps, given the tree and the observed character distribution, with the stepmatrix explicitly specifying the number of steps from state to state. Optimal number of transitions in the stepmatrix is derived from pair-wise alignment, with selection being that transition with the greatest similarity and the least number of steps. The result is that indels (and peripheral bases) are successfully converted into coded data that can then serve as input into parsimony (and other) programs.

## 2.3. Sequence neutrality, divergence and mismatch distributions

The HKA test (Hudson et al., 1987) was applied to our MTDNA data so as to ensure selective neutrality for both ATPase genes, using observed and expected differences within two sister species, and the differences between these for the DNA regions being compared (using DNAsp 4.5; Rozas et al., 2003). We also tested neutrality using the McDonald–Kreitman (MK) test (McDonald and Kreitman, 1991), again with DNAsp.

To examine regional biodiversity, we derived molecular diversity indices and sequence divergence values for our MTDNA data across the 4 *H. horridum* subspecies, and 4 regional groupings of *H. suspectum* (i.e., Arizona, México, New Mexico, and Utah) that corresponded to molecular clades (Section 2.5). Haplotype (*h*) and nucleotide (*π*) diversities were calculated in DNAsp using the coalescent with 1000 replications. Pair-wise sequence divergence (*p*) values corrected for within-group variance and based on 1000 bootstrapped sequences were generated using Mega3 (Kumar

et al., 2004). To ascertain clock-like behavior in our ᴍᴛDNA sequences, we applied Tajima's (1993) test to compare representative sequences from Helodermatidae vs outgroups.

We calculated Tajima's D-statistic and, if neutrality was sustained, applied this statistic to infer demographic history (Tajima, 1989; see Douglas et al., 2006, 2009 for interpretation). However, causation is difficult to ascertain when Tajima's D deviates significantly from zero. To clarify, we tested for neutrality and computed Fu's Fs (Fu, 1997) which is particularly useful in detecting population expansion. We contrasted these with values for Fu and Li's F* and D*, which provide additional information regarding expansion vs background selection when calculated in tandem with Fu's Fs. We derived Tajima's D, Fu's Fs, and Fu and Li's F* and D* in DNAꜱᴘ using the coalescent with 1,000 replications.

Mismatch distributions reflect the frequency of pair-wise differences among individuals, and can provide information regarding demographic history (Rodgers and Harpending, 1992). Here, a recent bottleneck followed by rapid, population expansion yields a unimodal mismatch distribution that approximates a Poisson distribution. We tested for this scenario using DNAꜱᴘ.

### 2.4. Phylogenetic analyses

We utilized MrBayes (ver. 3.1.2; Huelsenbeck and Rohnquist, 2001) to partition our ᴍᴛDNA and OD data into two sections each: sequence vs standard discrete data (i.e., MCIC-coded indels), with each being analyzed under separate models. The General Time Reversible (nst = 6) model with gamma-shaped rate variation and with a proportion of invariant sites (rates = invgamma) was used for our molecular data, whereas our coded indel data were evaluated using a model analogous to Jukes-Cantor but developed instead for discrete morphological data (Lewis, 2001). However, the limited number of available character states in this model ($n = 9$) forced us to derive a phylogenetic tree for our ENOL data using maximum parsimony (MP), in that ambiguous regions within these data required 12 character states for representation.

Minimum length trees for our MP analyses were obtained using Paup*. Shortest trees were sought using heuristic searches that employed accelerated character transformation (ACCTRAN) optimization, tree bisection-reconnection (TBR) branch swapping, retention of minimal trees (MULPARS), and collapse of zero-length branches to yield polytomies. Five replications were performed and most parsimonious trees derived using majority-rule (MR) consensus. We also compiled an MR consensus for trees generated using Bayesian analysis of ᴍᴛDNA and OD data.

### 2.5. Supertree approaches

Analyses employing multiple data sets are becoming increasingly prevalent, not only as more and varied molecular markers are employed (as herein) but also as whole genomes become sequenced. These data are most commonly analyzed using one of two approaches (Philippe et al., 2005): 'supermatrix,' where all relevant data are *a priori* combined before analysis (sometimes referred to as 'total evidence;' Matthee et al., 2004), and 'supertree,' in which each of several source topologies with partially overlapping taxa are compiled first into a data matrix, then into a single comprehensive tree. We summarized our diverse trees in this study using the supertree approach.

Supertree methods do have limitations (Goloboff and Pol, 2002), to include 'signal enhancement' (Bininda-Emonds, 2004; Bininda-Emonds et al., 2007), a situation where clade-relationships in a supertree contradict those found in one (or several) source trees. Furthermore, the assessment of uncertainty and confidence in supertree relationships is also a difficulty (Moore et al., 2007), as is interpretation of supertrees as phylogenetic hypotheses which,

in turn, can provoke unjustified conclusions. Yet, the most serious argument is that supertrees are open to interpretation, in that they are derived from trees rather than directly from primary data.

Our sequences were amenable for a supertree approach in that identical numbers of ingroup but varying numbers of outgroup individuals were amplified for each marker. Our resulting MR consensus trees served as input to program TɴT (Goloboff, P., Farris, J., Nixon, K. 1999. TɴT: Tree Analysis Using New Technology; www.cladistics.org). There, we derived a matrix using MRP (matrix representation using parsimony; Baum, 1992; Ragan, 1992), but with compatibility (per Goloboff and Pol, 2002), rather than parsimony. We then employed tree fusing, sectorial searches, and tree drifting in TɴT so as to produce 22 equal-length ($L = 101$) trees that reduced to a single majority-rule consensus supertree.

We also analyzed our tree data using two additional approaches: a semi-strict supertree in TɴT (Goloboff and Pol, 2002), and a Majority-Rule (+) supertree (per Cotton and Wilkinson, 2007; Dong and Fernández-Baca, 2009; Dong et al., 2009). Strict and semi-strict consensuses are a preferred means of summarizing results, in that each consensus group has an unambiguous interpretation. It is present in all (or some) input trees but absent (or contradicted) in none (Goloboff and Pol, 2002). Although, both fail to provide any confidence and/or level of uncertainty in the overall supertree relationships they derive, each can identify monophyletic clades that, in turn, provide a coarse level of certitude.

### 2.6. Molecular estimation of divergence times

Divergence times estimated from DNA sequence data are becoming increasingly common in evolutionary and systematic studies (Douglas et al., 2006, 2009), and are being driven by an accelerated development of optimal estimation procedures (Renner, 2005; Welch and Bromham, 2005; Rutschmann, 2006). We inferred divergence times from our ᴍᴛDNA data by applying a semi-parametric penalized likelihood (PL) approach using a truncated Newton (TN) optimization algorithm as implemented in the software ʀ8ꜱ (Sanderson, 2003) and described in Douglas et al. (2006, 2009). Time estimates are derived using a parametric model having a different substitution rate on every branch, coupled with a non-parametric 'roughness' penalty that costs the model if rates change too quickly from branch-to-branch. The contribution of each is determined by a smoothing parameter. When large, roughness dominates and the model is reasonably clock-like, yet when small, roughness contributes little and considerable rate variation is allowed. Optimal smoothing is determined by a data-driven and iteratively run cross-validation procedure. Each terminal branch is removed in turn with model parameters re-estimated and the expected number of substitutions predicted on that branch. Performance is evaluated as a normalized 'chi-square-like' score. Once we determined the lowest cross-validation smoothing value, we then optimized the PL approach using this value and the gamma shape parameter and executed 10 searches (with multiple re-starts within searches).

One stipulation with estimating divergence times from DNA sequence data is the need to fix or constrain (either minimally or maximally) fossil calibrations for nodes. We used the following seven dates in our estimation procedure: Squamata (*Sphenodon*; Evans, 2003) = 250 mya fixed constraint; Anguimorpha (*Parviraptor estesi*; Evans, 1994) = 166 mya maximum constraint; Teiidae (*Ptilodtodon*; Nydam and Cifelli, 2002) = 112 mya minimum constraint; Helodermatidae (*Primaderma nessovi*; Nydam, 2000) = 106 mya minimum constraint; Cordylidae (*Konkasaurus*; Krause et al., 2003) = 68 mya minimum constraint; Shinisauridae (*Bahndiwivici*; Conrad, 2006) = 50 mya minimum constraint; and African *Varanus* (Smith et al., 2008) = 31 mya minimum constraint.

# 3. Results

## 3.1. Sequence amplification and alignment of ambiguous indels

PCR amplifications and automated sequencing of ATPase 8 and ATPase 6 resulted in 836 base pairs (bp) of sequence converted into 46 haplotypes (30 ingroup + 16 outgroup), plus an additional 21 outgroup haplotypes obtained from GenBank ($n = 67$ total). For ENOL, 216 bp were sequenced, resulting in 32 genotypes (2 ingroup + 30 outgroup), plus an additional 8 outgroup genotypes from GenBank ($n = 40$ total). Lastly, OD yielded 647 bp, resulting in 31 genotypes (7 ingroup + 24 outgroup) ($n = 31$ total).

After our MTDNA data were aligned, SeqState converted 30 ambiguous regions into new characters. For these data, 172 (20%) were constant, 47 (5%) non-informative, and 644 (75%) parsimony-informative. With regard to the indel-converted characters, one (3%) was constant, 16 (53%) uninformative, and 13 (44%) parsimony-informative.

For ENOL, 216 bases were sequenced and, following sequence alignment, 11 ambiguously aligned regions were delimited and re-coded. Within the aligned sequences, 91 bp (34%) were constant, 32 (12%) non-informative, and 144 (55%) parsimony-informative. Of the 11 recoded indel regions, 4 (36%) non-informative, and 7 (64%) parsimony-informative. Similarly, 44 ambiguous regions were discovered once the 619 bp of OD sequence were aligned. For these data, 134 (22%) were constant, 107 (17%) non-informative and 301 (49%) parsimony-informative, whereas for the indel-converted data, one (2%) was constant, 15 (34%) were non-informative, while 28 (64%) were parsimony-informative. While parsimony-informative characters varied percentage-wise among sequenced regions as well as among recoded indels, MTDNA reflected highest percentage of parsimony-informative sequence characters but also lowest percentage of parsimony-informative recoded characters. In sequence vs recoded indels, parsimony-informative characters were 75% vs 44% for ATPase 8/6, 55% vs 64% for ENOL, and 44% vs 64% for OD.

## 3.2. Sequence neutrality, divergence and demographic histories

Combining MTDNA sequences was supported by a non-significant partition homogeneity test (Paup*: $P > 0.42$). All sequences were evolving neutrally [HKA: $(0.90 < P < 0.99)$, MK: $(0.83 < P < 0.99)$] and in a rate-uniform manner (Tajima's test, $P > 0.20$).

Percent MTDNA sequence divergence (sd) was calculated among the four subspecies of *H. horridum*, and ranged from a high of 9.8% (±0.1) between *H. h. exasperatum* vs *H. h. charlesbogerti*, to a low of 1% (±0.3) between *H. h. alvarezi* vs *H. h. charlesbogerti* (Table 1). *Heloderma h. horridum* differed from the other subspecies at 5.4–7.1% (±0.6–0.8). The four regional groupings of *H. suspectum* showed little divergence from one another, with the greatest value
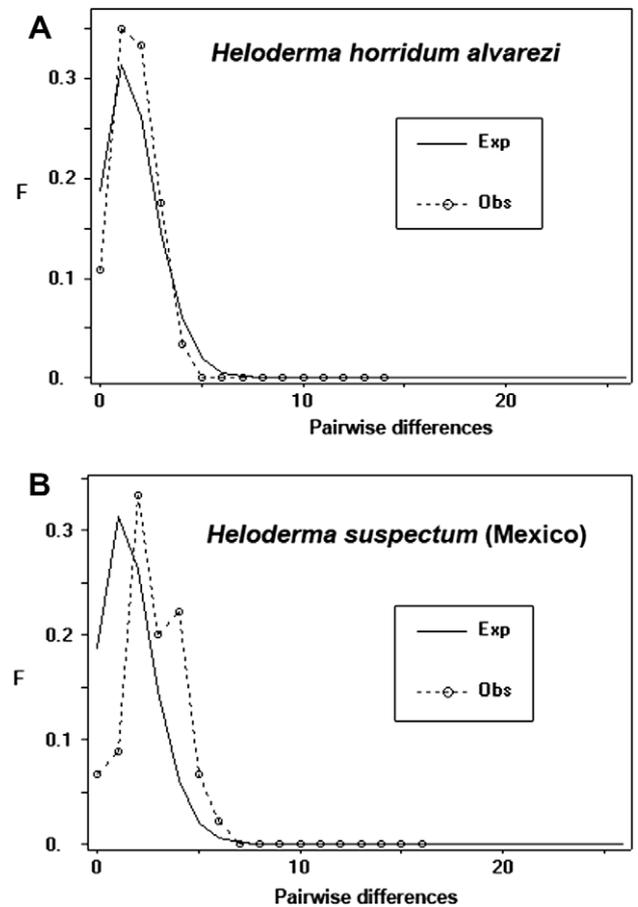


**Fig. 2.** Results of mismatch distribution analyses (MDA) conducted for (A) *Heloderma horridum alvarezi* and (B) *H. suspectum* (México) depicting pair-wise differences among individuals (dashed line) plotted against expected occurrence (solid line) under a model of population expansion.

between México vs Utah/New Mexico [1.4% (±0.4) for each], while the least was 0.3% (±0.1) between Utah/New Mexico vs the remainder of the range (Table 1).

In comparisons across species, the four *H. horridum* subspecies were most similar to *H. suspectum* from México (range = 18.3–19.9% ± 1.4), and they differed from *Varanus* on average at 25.9% (Table 1). Both *H. h. alvarezi* and *H. suspectum* showed evidence for historic range expansion by demonstrating significant values for Fu's Fs coupled with non-significant values for Fu and Li's F* and D*. Both also reflected negative values for Tajima's D (also indicative of expansion) but the latter were not significant. Mismatch distributions for *H. h. alvarezi* and *H. suspectum* (México) yielded a distinct curve consistent with the hypothesis that each

**Table 1**
Pair-wise sequence divergence between molecular clades as identified in Figs. 3B and 5. Values in lower triangle are percent sequence divergence (*p*-distances corrected for within-group variability) while those in upper triangle are standard errors. Clade abbreviations are: HHA = *Heloderma horridum alvarezi*; HHH = *H. h. horridum*; HHC = *H. h. charlesbogerti*; HHE = *H. h. exasperatum*; HSUT = *H. suspectum* (Utah); HS = *H. suspectum* (Arizona); HSNM = *H. suspectum* (New Mexico); HSMX = *H. suspectum* (Mexico); VAR = *Varanus*.

|       | HHA   | HHH   | HHC   | HHE   | HSUT  | HS    | HSNM  | HSMX  | VAR   |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| HHA   | ×     | 0.006 | 0.003 | 0.01  | 0.014 | 0.014 | 0.014 | 0.014 | 0.012 |
| HHH   | 0.054 | ×     | 0.006 | 0.008 | 0.014 | 0.014 | 0.014 | 0.014 | 0.011 |
| HHC   | 0.01  | 0.062 | ×     | 0.01  | 0.014 | 0.014 | 0.014 | 0.014 | 0.012 |
| HHE   | 0.093 | 0.071 | 0.098 | ×     | 0.015 | 0.014 | 0.015 | 0.014 | 0.012 |
| HSUT  | 0.203 | 0.191 | 0.209 | 0.208 | ×     | 0.001 | 0     | 0.004 | 0.012 |
| HS    | 0.195 | 0.184 | 0.201 | 0.199 | 0.003 | ×     | 0.001 | 0.001 | 0.012 |
| HSNM  | 0.203 | 0.191 | 0.209 | 0.208 | 0     | 0.003 | ×     | 0.004 | 0.012 |
| HSMX  | 0.193 | 0.183 | 0.199 | 0.197 | 0.014 | 0.004 | 0.014 | ×     | 0.012 |
| VAR   | 0.262 | 0.249 | 0.264 | 0.263 | 0.257 | 0.254 | 0.257 | 0.258 | ×     |

**Table 2**
Molecular diversity in subspecies of *Heloderma horridum* and in regional clades of *H. suspectum*. Subspecies are: *H. h. alvarezi* = HHA; *H. h. charlesbogerti* = HHC; *H. h. exasperatum* = HHE; *H. h. horridum* = HHH. Regional clades are: *H. suspectum* Utah = HSUT; *H. suspectum* = HS; *H. suspectum* = HS; *H. suspectum* New Mexico = HSNM; *H. suspectum* Mexico = HSMX. Provided are N = sample size; H = number of haplotypes; h = haplotype diversity (standard deviation in parentheses); Pi = nucleotide diversity (standard deviation in parentheses); T-D = Tajima's D; Fu's Fs = Fu-Fs; Fu and Li's D = F-L-D; Fu and Li's F = F-L-F ([*]significance at P < 0.05).

| Region | N | H | h | Pi | T-D | Fu-Fs | F-L-D | F-L-F |
|---|---|---|---|---|---|---|---|---|
| HHA | 16 | 10 | 0.892 (0.063) | 0.002 (0.0003) | −1.405 | **−6.724 (P < 0.0000)** | −1.49 | −1.69 |
| HHC | 11 | 2 | 0.182 (0.144) | 0.0002 (0.0002) | −1.129 | −0.41 (P < 0.37) | −1.29 | −1.39 |
| HHE | 16 | 7 | 0.742 (0.105) | 0.002 (0.001) | **−1.879**[*] | −1.702 (P < 0.12) | **−2.41**[*] | **−2.61**[*] |
| HHH | 9 | 4 | 0.694 (0.147) | 0.0025 (0.0006) | 0.497 | 0.45 (P < 0.57) | 0.69 | 0.71 |
| HSUT | 4 | 1 | n/a | n/a | n/a | n/a | n/a | n/a |
| HS | 52 | 15 | 0.778 (0.045) | 0.008 (0.0003) | 1.029 | 0.247 (P < 0.60) | −0.27 | −0.22 |
| HSNM | 4 | 1 | n/a | n/a | n/a | n/a | n/a | n/a |
| HSMX | 10 | 8 | 0.933 (0.077) | 0.0032 (0.0006) | −1.356 | **−3.911 (P < 0.006)** | −1.60 | −1.73 |

had undergone a recent bottleneck and subsequent expansion (Fig. 2 and Table 2).

### 3.3. Phylogenetic analyses of individual markers

For MTDNA data, Bayesian analyses ran for 400k generations with a sampling frequency every 100 generations and a burn-in of 30k generations to yield 2922 trees. For OD, 300k generations (with parameters as above) yielded 400 trees. The number of generations implemented for each marker was determined when the standard deviation of split frequencies reached 0.01. For ENOL, MP analyses yielded 120 most parsimonious trees each with Length = 646. Majority-rule trees are depicted in Figs. 3–5, respectively, for each of these markers.

Our MTDNA tree (Fig. 3A) is rooted at *Sphenodon punctatus* and depicts a basal clade, containing Gekkonidae, Agamidae and Chamaeleonidae, as sister taxon to the remaining lineages of the tree. Gekkonidae is identified as sister taxon to the other two. Moreover, the one chameleonid species (a Madagascan taxon, *Furcifer oustaleti*), is sister to *Calotes versicolor*, thus rendering Agamidae as polyphletic. Next is a clade consisting of three lizard families + Serpentes. Here, Lacertidae is identified, albeit with low support, as sister taxon to Amphisbaenia (Amphisbaenidae + Rhineuridae) + Serpentes.

The next clade is Cordylidae + Scincidae, followed by a clade composed of Crotaphytidae + Phrynosomatidae as sister taxon to Iguanidae. This clade is sister taxon to all remaining lineages, which represent Anguimorpha, here recovered as monophyletic
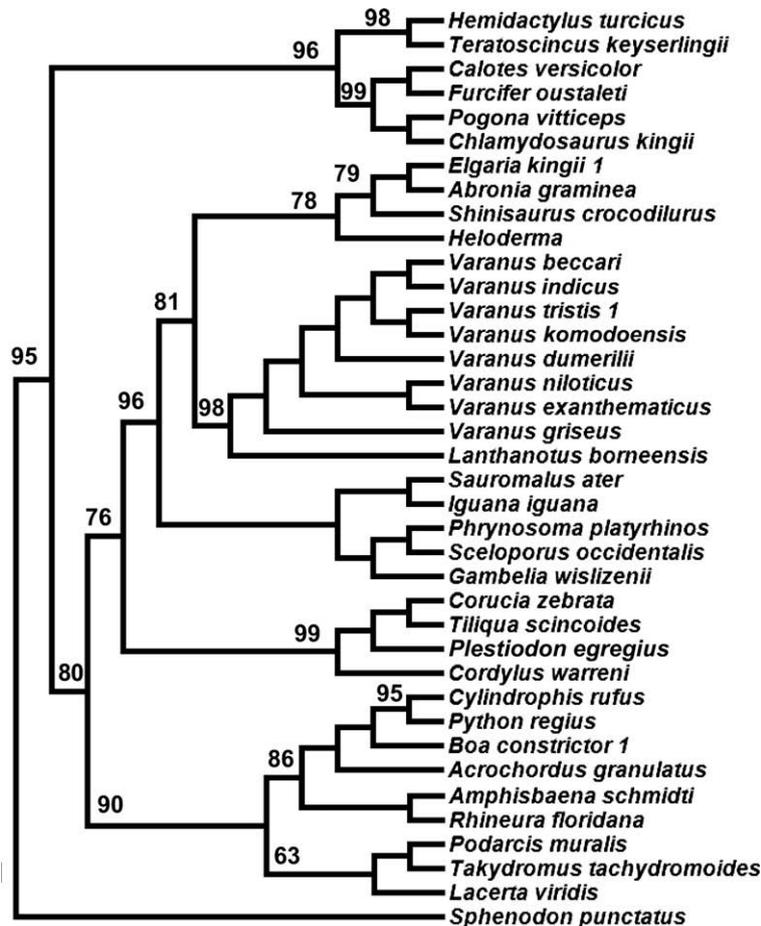


**Fig. 3A.** Majority-rule (MR) consensus tree (2922 trees) derived from a partitioned Bayesian analysis of haplotypes spanning 836 base pair (bp) of ATPase 8 and ATPase 6 MTDNA across 153 individuals, coupled with 30 ambiguous sequence regions containing insertions-deletions that were recoded using program SeqState. Nodes with <100% support are designated, while nodes = 100% are not.

group with moderate support. Anguimorpha, falls into two clades, one placing Helodermatidae (represented as *H. suspectum*) as sister taxon to Shinisauridae + Anguidae, whereas the other places Lanthanotidae as sister taxon to Varanidae.

Helodermatidae is expanded (Fig. 3B) to depict its constituent 28 haplotypes (107 individuals), with *H. horridum* and *H. suspectum* as sister taxa. Within *H. horridum*, each of the 4 subspecies forms a distinct clade. One places *Heloderma h. charlesbogerti* as sister taxon to a somewhat undifferentiated *H. h. alvarezi*, whereas the other shows *H. h. exasperatum* and *H. h. horridum* as clearly differentiated sister taxa.

Within *H. suspectum*, we detected no molecular evidence for its two recognized subspecies, *H. s. suspectum* (Reticulate Gila monster) and *H. s. cinctum* (Banded Gila monster). In fact, putative *H. s. cinctum* from Utah and Nevada [(UT) 1–5; (NV) 1] share a haplotype with *H. s. suspectum* from New Mexico [(NM) 1–5].

The ENOL-tree (Fig. 4) depicts Gekkota (Eublepharidae + Gekkonidae) as sister taxon to a clade containing all other lineages, although the latter is only weakly supported. Serpentes is recovered as monophyletic group and placed as sister taxon to the remainder of the tree. Here, a clade formed by Lacertidae (represented by *Mesalina guttulata*) + Teiidae is shown as sister taxon to Scincidae + Anguimorpha. Congruent with the mtDNA topology, Anguimorpha is recovered as a monophyletic group, although relationships within differ. Helodermatidae (*H. suspectum* and *H. horridum*) is now depicted as sister taxon to the other Anguimorpha. These fall into two clades, one formed by Anguidae as sister taxon to Shinisauridae + Xenosauridae, and another representing Varanidae. All *H. suspectum* and *H. horridum* revealed their respective species-specific genotypes without subspecific division.

The OD-tree (Fig. 5), rooted at Gekkonidae, depicts a polyphyletic Scincidae, where one species (*Tiliqua scincoides*) is placed as the basal taxon to a clade that contains another Scincidae (*Corucia zebrata*) as sister taxon to the remaining lineages. Serpentes and Anguimorpha each form a monophyletic group and are recovered

as sister taxa. Anguimorpha is split into two clades, one representing Varanidae, whereas the other is composed of Anguidae as sister taxon to Shinisauridae + Helodermatidae. Within Helodermatidae, *H. suspectum* and *H. horridum* are clearly identified as sister taxa, but subspecies of the latter form a polytomy (trichotomy), with one group corresponding to *H. h. exasperatum* and another to *H. h. horridum*, respectively, whereas a third consists of a clade containing *H. h. charlesbogerti* and *H. h. alvarezi* as sister taxa.

### 3.4. Phylogenetic analyses using supertree approaches

The supertree composite of our MR trees is depicted in Fig. 6, and rooted at Sphenodontidae. A basal clade consisting of Chamaeleonidae + Agamidae as sister taxon to Gekkonidae is followed by a composite clade that includes Amphisbaenia (Amphisbaenidae + Rhineuridae), Eublepharidae and Lacertidae, although node support for these groups is low. The supertree topology recovers a well-defined Serpentes as sister taxon to the remaining lineages, with a composite clade combining Iguania + Teiidae as sister taxon to Scincidae + Anguimorpha, both well-supported as monophyletic groups. Within Anguimorpha, Helodermatidae is placed basal to a clade containing two groups, one including Shinisauridae + Xenosauridae as sister taxon to Anguidae, while the other places Lathanotidae as sister taxon to Varanidae, Aberrant placement of some species (Gekkonidae: *Gehyra mutilata*; Lacertidae: *Mesalina guttulata*) and low node support for other groupings are likely a result of having but a single marker (ENOL) representing these taxa.

In Helodermatidae, *Heloderma suspectum* is placed as sister taxon to *H. horridium*, which exhibits a polytomy consisting of *H. h. horridum*, *H. h. exasperatum* and a clade with *H. h. alvarezi* + *H. h. charlesbogerti*, a topography that effectively recovers the 4 subspecies as distinct taxa. By employing compatibility (Estabrook, 2008) to analyze the matrix representing our three input trees (i.e., by finding those trees determined by the largest clique of compatible characters), we determined how frequently a group is recovered,
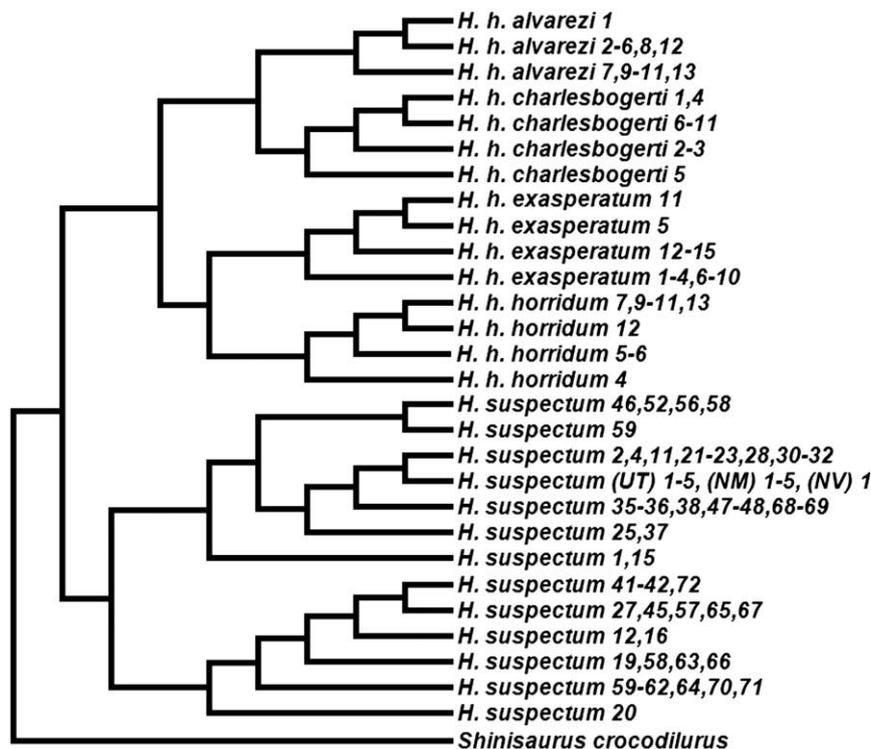


**Fig. 3B.** Continuation of the partitioned Bayesian analysis of haplotypes shown in Fig. 3A. *Heloderma* now expanded into constituent haplotypes. All nodes at 100% (values not displayed). Numbers following leaf names refer to individuals.
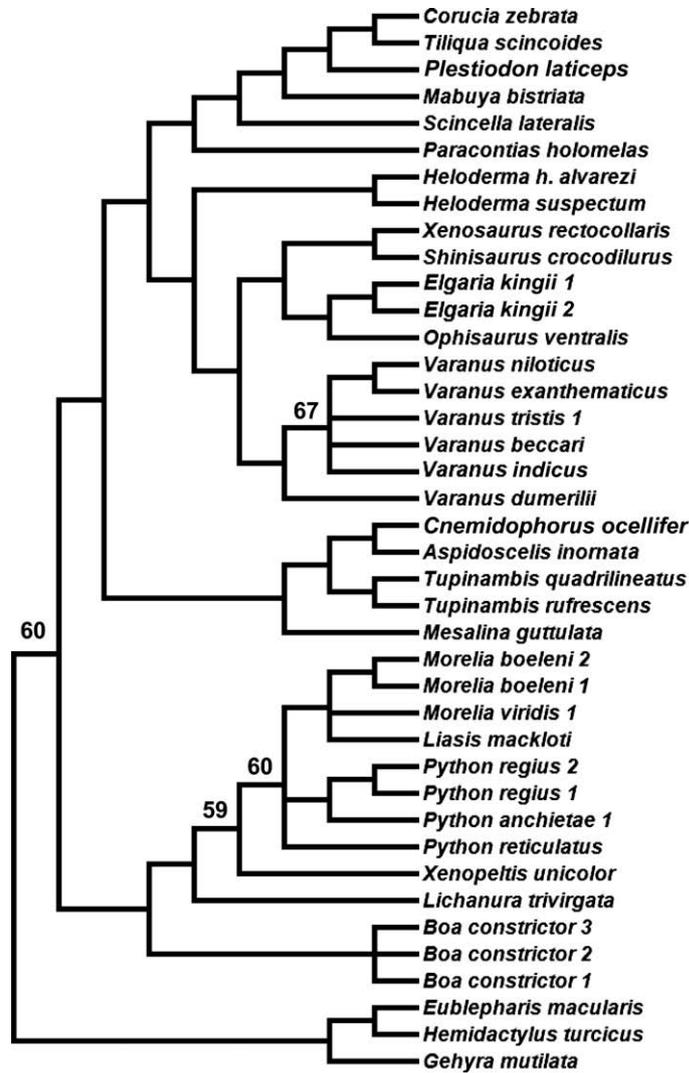
**Fig. 4.** Maximum parsimony analysis of α-enolase (ENOL) genotypes from 216 bp of intron 8 and small parts of exon 8 and 9 over 145 individuals coupled with 11 ambiguous sequence regions containing insertions-deletions that were recoded using program SeqState. Nodes with <100% support are designated, while nodes = 100% are not.

and (by so doing) produced results more reasonable than those from MRP (Goloboff and Pol, 2002). We also employed semi-strict and majority-rule (+) supertree approaches to analyze our MRP data and each yielded a monophyletic Anguidae, Varanidae, and Helodermatidae (trees not shown).

### 3.5. Molecular estimation of divergence times

Five iterations of the TN method were required to establish an optimal smoothing parameter of 3.27 (log 10 = 0.51455). Inserting this and the gamma value into method TN yielded divergence times depicted in Table 3. The MRCA of Anguimorpha (maximally constrained at 166 mya) resulted instead in an estimated divergence time of 127 mya (=Early Cretaceous), while the MRCA node for Lanthanotidae was estimated to be Mid Cretaceous (108 mya). Shinisauridae (minimally constrained to 50 mya), was estimated at 59.7 mya (=Early Paleocene) (Table 3). The MRCA for Varanidae was placed in Early Eocene while the MRCA for Serpentes was Mid Eocene (42.6 mya; Table 3).

The MRCA for Helodermatidae (minimally configured at 106 mya) was estimated at 35.4 mya (Late Eocene, at the Eocene–Oligocene juncture). The MRCA of *Heloderma horridum* was dated to 9.71 mya (Lower Miocene), with considerable stasis

(5+ mya) before it subsequently diversified again. The first was restricted to *H. h. exasperatum* and *H. h. horridum* (4.42 mya), and the second to *H. h. alvarezi* and *H. h. charlesbogerti* (3.02 mya). However, *H. suspectum* appears not to have diversified until the Pleistocene (2 mya), suggesting a period of stasis that persisted for >7 mya since its divergence from *H. horridum*.

## 4. Discussion

Our study used a molecular approach to investigate both deeper and more recent histories of the Beaded Lizard (*H. horridum*) and Gila Monster (*H. suspectum*) throughout their geographic ranges in North and Central America. We employed MT- and NDNAs to examine the phylogenetic diversity of this group in the context of the larger squamate clades Varanoidea and Anguimorpha, as well as other major Squamate lineages, including Serpentes. Furthermore, we summarized our data using a supertree approach, as a means of providing consensus among disparate phylogenetic signals. We discuss these results at several levels by: (a) dissecting the deep historical relationships among these Squamate clades; (b) evaluating diversification within the Helodermatidae and its biogeographic significance; (c) juxtaposing congruence among molecular divergence times and clade diversification; and (d)
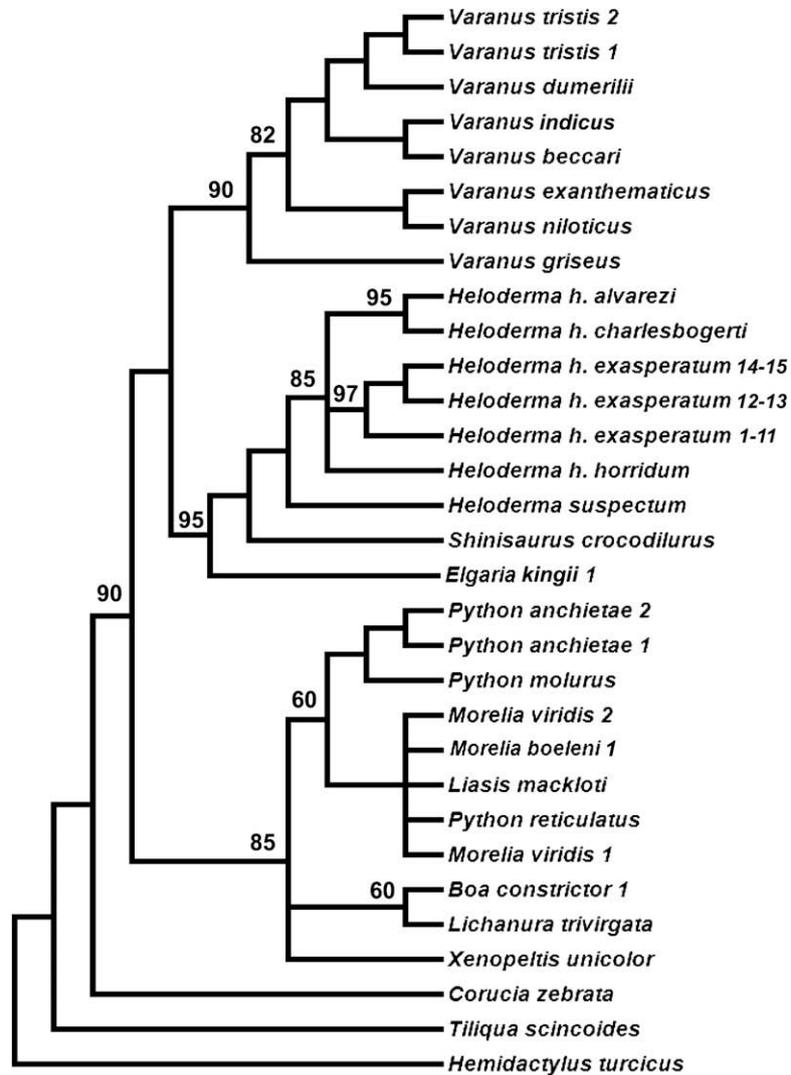
**Fig. 5.** Partitioned Bayesian analysis of genotypes stemming from 647 bp of ornithine decarboxylase (OD) exons 6 and 8 over 147 individuals coupled with 44 ambiguous sequence regions containing insertions-deletions that were recoded using program SeqState. Nodes with <100% support are designated, while nodes = 100% are not.

interpreting the conservation of helodermatid lizards with this phylogeny. Finally, we discuss (e) the considerable and to date unrecognized economic value of conserving helodermatid lizards.

### 4.1. Phylogenetic relationships within Varanoidea, Anguimorpha, and Squamata

Our phylogenetic hypotheses (Figs. 3–6) support both the monophyly of the Helodermatidae and Anguimorpha (though sister relationships vary), as well as earlier studies evaluating them (and most major Squamate lineages; Townsend et al., 2004; Vidal and Hedges, 2004, 2005, 2009; Dong and Kumazawa, 2005; Kumazawa, 2007; Douglas and Arnason, 2009; Rui et al., 2009). However, we failed to support the monophyly of Varanoidea, in that Helodermatidae instead has closer relations to Anguidae + Shinisauridae (Fig. 3A) or Shinisauridae (Fig. 5). Moreover, in some of our analyses, Anguidae, Shinisauridae, and/or Xenosauridae were closer to Varanidae than to Helodermatidae (Figs. 4 and 6; corroborated by Townsend et al., 2004; Dong and Kumazawa, 2005; Zhou et al., 2006; Douglas and Arnason, 2009; Rui et al., 2009; Vidal and Hedges, 2009). Our analyses often depicted Varanidae as sister to Lanthanotidae (Fig. 3A), a result supported by other molecular (Ast, 2001; Townsend et al., 2004; Vidal and

Hedges, 2009), and morphological studies (McDowell and Bogert, 1954; Pregill et al., 1986).

Inclusion of Serpentes within Anguimorpha depends upon which DNA markers are applied and how they are analyzed, with diagnoses or definitions varying among studies. Lee (2009) combined morphology and DNA-based markers to demonstrate that Serpentes is sister to extant anguimorphs (not recovered in most recent molecular analyses, see above). In contrast, Anguimorpha (less Serpentes) has instead shown a sister relationship with Iguanidae (or Iguania), Scincidae + Lacertidae, or Acrodontia. A robust positioning of Serpentes within Squamata has in fact been problematic. Townsend et al. (2004) suggested that long-branch attraction between Serpentes and Agamidae and Chamaeleonidae (acrodonts) may be one reason why the former often links with Lacertidae and Amphisbaenia (for example). Our mtDNA analysis (Fig. 3A) reflects just such a topology, with Serpentes as sister taxon to Amphisbaenidae + Rhineuridae, and this clade in turn as sister taxon to Lacertidae.

Our ENOL-tree (Fig. 4) depicts Serpentes basal to a clade consisting of Teiidae + Lacertidae as sister to Scincidae + anguimorphs (Helodermatidae + Varanidae (Anguidae (Shinisauridae + Xenosauridae))). In contrast, our OD-tree (Fig. 5) depicts Serpentes as sister to Varanidae + (Anguidae (Shinisauridae + Helodermatidae)), thus
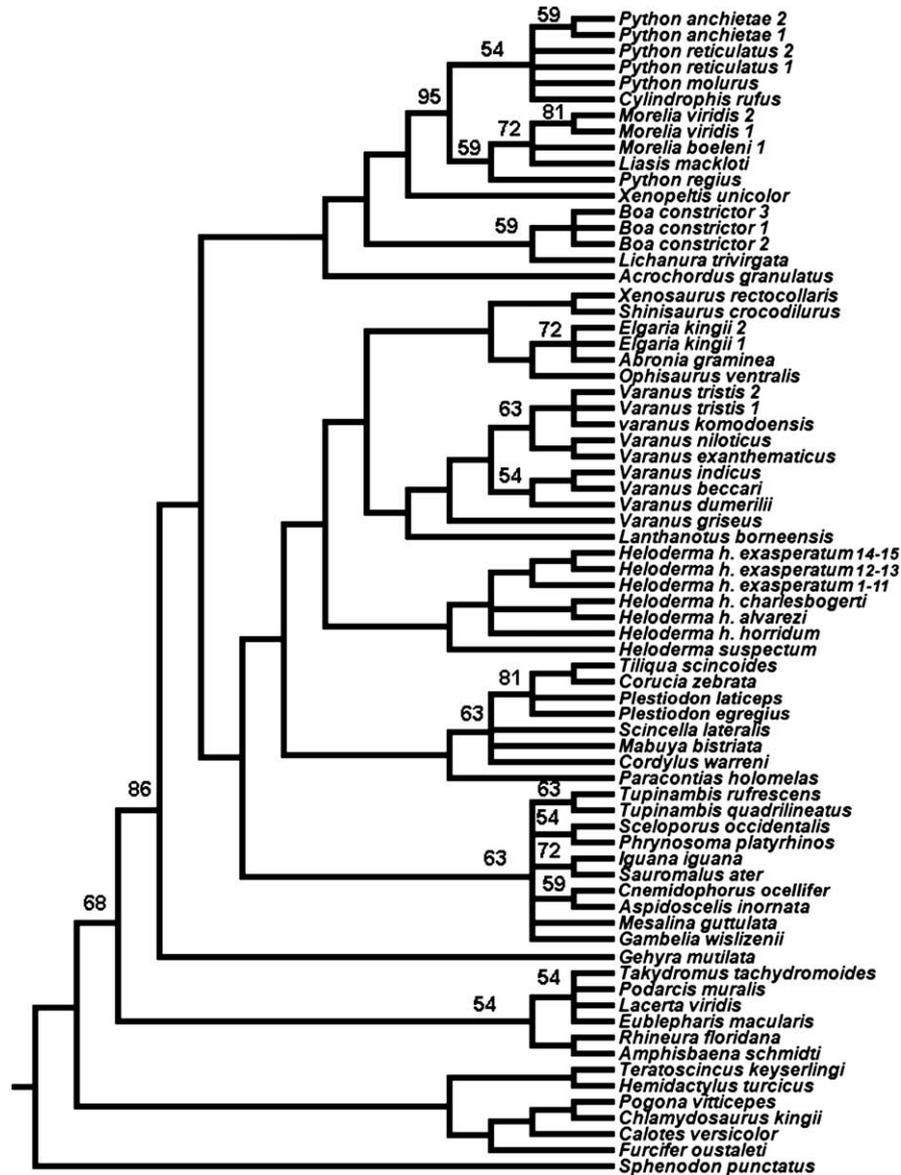
**Fig. 6.** Supertree for all taxa produced by matrix representation a compatibility approach to matrix representation using parsimony (MRP). Input consisted of separate Bayesian trees [mtDNA atpase 8 and ATPase 6 (Figs. 3A and B) and ornithine decarboxylase (OD) intron (Fig. 5)] as well as a maximum parsimony (MP) tree based on analysis of the α-enolase (ENOL) intron (Fig. 4). Trees were compiled using TnT (Tree technology using New Technology: Goloboff, 1999). Nodes with <80% support are designated, while nodes >80% are not.

rendering a monophyletic Anguimorpha (Lee, 2009). Finally, our supertree topology places Serpentes as basal to a mixed clade containing Teiidae + Iguania (Crotaphytidae, Iguanidae, Phyrnosomatidae) as sister taxon to Scincidae + Cordylidae and anguimorphs (Helodermatidae, Varanidae, Anguidae, Shinisauridae + Xenosauridae). Clearly, additional perspectives and analyses are needed before Serpentes can be confidently imbedded within the larger Squamata clade (Albert et al., 2009; Schulte and Cartwright, 2009).

In our mtDNA majority-rule consensus tree (Fig. 3A), we show *Heloderma* (*H. suspectum*) as sister taxon to Anguidae + Shinisauridae. Similar relationships [i.e., (Helodermatidae (Anguidae + Xenosauridae)) and Helodermatidae + Anguidae] have been recovered in other studies (Townsend et al., 2004; Vidal and Hedges, 2004, 2005; Kumazawa, 2007; Douglas and Arnason, 2009). Likewise, our partitioned Bayesian analysis of OD recovered *Heloderma* as sister taxon to Shinisauridae (Fig. 5). Finally, our MP analysis of ENOL has *Heloderma* as sister taxon to all other anguimorphs

(Fig. 4). None recovered Helodermatidae as sister taxon to Varanidae, or to Varanidae + Lanthanotidae (McDowell and Bogert, 1954; Bogert and Martin del Campo, 1956; Pregill et al., 1986; Estes et al., 1988; Townsend et al., 2004).

We note some mixed post-outgroup configurations within our supertree (Fig. 6). For example, Lacertidae, Teiidae, Cordylidae, and Scincidae fall within more derived clades. Yet, many other relationships (i.e., Serpentes and Anguimorpha) adequately recover marker tree topologies, as well as phylogenetic perspectives overall. We suggest the supertree may reflect a reasonable compilation of the diversity found within constituent trees simply because composite OTUs (operational taxonomic units) are relatively similar within each tree (mtDNA = 38; ENOL = 40; OD = 31). Albeit, the different topologies place Helodermatidae in varying relationships to Anguidae and Varanidae, the supertree recovers distinct *H. suspectum* and *H. horridum*, as well as clear divergences within the latter that correspond to (and exceed) subspecific designations (per Douglas et al., 2007).

**Table 3**
Divergence times for selected clades in Figs. 3A and B, determined using a semi-parametric penalized likelihood (PL) approach implemented in the software R8S. Node refers to taxonomic clades. Mean age of the node is presented in millions of years (mya), with numbers in parentheses representing confidence intervals (CI values, in mya). Configuration (of node) is: Max = maximum; Min = minimum; E = estimated. HHC/HHA = *Heloderma h. charlesbogerti/H. h. alvarezi*; HHE/HHH = *H. h. exasperatum/H. h. horridum*.

| Node | Mean age (CI values) | Configuration |
|---|---|---|
| Iguanidae | 146.1 (117.9–174.3) | E |
| Anguimorpha | 127.1 (105.5–148.7) | Max (166) |
| Lanthanotidae | 108.3 (83.3–133.2) | E |
| Shinisauridae | 59.7 (17.6–100.3) | Min (50) |
| Varanidae | 48.7 (30.7–73.6) | Min (31) |
| Serpentes | 42.6 (26.5–64.6) | E |
| Anguidae | 40.5 (23.1–65.4) | E |
| Helodermatidae | 35.4 (29.4–41.4) | Min (106) |
| *H. horridum* | 9.71 (7.86–11.6) | E |
| HHE/HHH | 4.42 (2.35–6.89) | E |
| HHC/HHA | 3.02 (1.65–4.99) | E |
| *H. suspectum* | 2.0 (1.2–2.9) | E |

## 4.2. Helodermatid evolution within the warm deserts of North America

Embedding the evolution of *Heloderma* within a larger phylogenetic context allows us to derive a timeline for its diversification, and consequently, to place these events within a scenario of long-term environmental change in the desert ecosystems in North America (Douglas et al., 2006). The Sonoran Desert, in particular (MacMahon, 1997), was well established by Late Miocene (8–5 mya), thus making it one of the youngest biotic communities of North America (Axelrod, 1979). As the climate in North America became drier and more variable during Miocene, *Heloderma* evolved into a subtropical and desert specialist (Pregill et al., 1986), and by so doing, diverged in lifestyle from highly predaceous (Nydam, 2000) to increasingly sedentary (Beck, 2005). Extant *Heloderma* now reflect low metabolic rates and activity levels, ingest large meals, and have a considerable physiological capacity for fat storage (Beck, 2005 and references therein). These (and other) traits reflect adaptations to harsh arid environments, and enable helodermatids to subsist for long periods without feeding.

During much of the Quaternary, the typical environment of southwestern North America was woodlands and forests (as evidenced in *Neotoma* middens), with desert scrub found only at low elevations (<300 m) along the Colorado River (Van Devender and Spaulding, 1979). In early Holocene (ca. 8 kybp), a rapid and widespread warming trend (Pielou, 1991) eliminated these woodlands. Desert-adapted plants increased in abundance and dispersed into new areas (Weng and Jackson, 1999), concomitant with drastic effects on regional faunas (Douglas et al., 2003; Hull and Girman, 2005).

Given the conservation concerns regarding *H. suspectum* (see below), its historical demography is thus of considerable interest. We were particularly interested in its overall genetic diversity, whether it is parceled into geographic regions, and how the latter are related one to another in an evolutionary context. Our conservation phylogenetic perspective was thus germane to these issues, yet we were surprised to discover very low levels of genetic diversity and a lack of geographic substructure in this species.

If low genetic diversity, like that reported for *H. suspectum*, were uncovered in an endangered species, possible explanations would include small population sizes, demographic instability, inbreeding, bottleneck effects, and the loss of intervening gene flow due to severe habitat reduction and fragmentation (per a molecular conservation genetics approach discussed above). Consequently, our approach (phylogenetic conservation using geographically

widespread species) suggests that biodiversity perspectives within the warm deserts of North America should be reconsidered.

Patterns of low genetic diversity, similar to that found in *H. suspectum*, were also uncovered in two rattlesnake species with ranges restricted primarily to the Sonoran Desert (*Crotalus ruber* and *C. tigris*; Douglas et al., 2006). This offers an hypothesis that numerous (and diverse) species were similarly impacted in this region by a biome-wide phenomenon. The fluctuating climatic regimes of the Pleistocene, driven by numerous glacial advances and retreats, lend anecdotal support for this hypothesis, in that they have been implicated as drivers for low genetic variability in other (more diverse) organisms inhabiting the western North American deserts (Hunter et al., 2001; Smith and Farrell, 2005a,b; Douglas et al., 2006).

### 4.3. Clade diversification and its congruence with molecular divergence times

The evolution of *H. suspectum* also reflects a long period of stasis (~30 mya) following an initial split from a common ancestor with *H. horridum* in the Early Eocene. Subsequent evolution did not seemingly occur until Mid and Late Pliocene, and Early Pleistocene (Table 3). Fossil data for helodermatids are fairly complete, and *H. suspectum* is well established in southwestern North America into Late Pleistocene (Pianka and King, 2004; Beck, 2005; Bhullar and Smith, 2008). Van Devender (2002) suggested *H. horridum* and *H. suspectum* each evolved in isolation, based on obvious morphological differences and the fact that their distributions overlap in southern Sonora, México. The Mid Miocene uplift of the Sierra Madre Occidental separated the MRCA of *H. horridum* and the extinct *H. texana*, with the latter evolving in the Chihuahuan Desert east of the Continental Divide (Van Devender, 2002). It apparently dispersed northward into Texas (Yatkola, 1976) and northwest across the Continental Divide into a region termed Mohavia (Morafka, 1977). Continued uplift and colder conditions presumably led to the extinction of eastern *H. texana*, whereas western populations survived and evolved as *H. s. cinctum*. *Heloderma suspectum* subsequently dispersed south and came into contact with *H. horridum* after both were independently derived species, with the Mohave Desert *H. s. cinctum* presently representing the sister subspecies of *H. s. suspectum* (Van Devender 2002). However, newer perspectives conflict somewhat with this scenario. For example, the application of banded vs reticulate color patterns as traditional delimiters of geographic differentiation in *H. suspectum* has proven invalid (Beck, 2005, Plates 15–17). Also, our molecular data lack a phylogeographic signal for this species and, in combination with the above, serve to refute subspecific categories in *H. suspectum*.

The inherent difficulties with diagnosing component clades in the Helodermatidae, coupled with the broader issue of recognizing a monophyletic Varanoidea, serve to underscore the validity of our conservation phylogenetic approach. Previous morphological (and other molecular) methodologies have been unable to unravel these connections, due either to sample size issues, perspectives, or both. We discuss below the methods and protocols by which these data can be used to potentially conserve and manage helodermatid lizards.

### 4.4. Phylogenetics and the conservation of helodermatid lizards

The most apparent threats to *Heloderma* occur at northern and southern boundaries of its distribution, particularly the latter (Fig. 1). For example, *H. horridum* occurs almost exclusively within or adjacent to the Tropical Dry Forest (i.e., TDF) biome, the most threatened of the major forest types (Stoner and Sanchez-Azofeifa, 2009). When Spaniards entered the New World, TDF ranged from Panama northward to western México (~550,000 km$^2$). Today only

0.09% of that region (480 km$^2$) has official conservation status, and less than 2% of TDF is sufficiently intact to attract attention of conservationists (Janzen, 1988). Although tropical rainforests contain greater total species diversity, dry forests are much more diverse (Sanchez-Azofeifa et al., 2005), particularly with regard to species 'activities' (i.e., adaptations of organisms to seasonal drought, diversifications of growth forms in plants, strategies of insect parasitoids, etc.; Janzen, 1988; Becerra et al., 2009). The importance of these aspects is amplified below with regard to speciation.

In western México, TDF is geographically and ecologically isolated from other such remnants (Ceballos, 1995), yet it harbors an elevated number of endemic vertebrates. For example, 173 reptile and amphibian species (43% of the Méxican fauna) are restricted to western TDF (García-Aguayo and Ceballos, 1994; Ceballos, 1995). Dry forest habitat is fast disappearing due to human activities (Williams-Linera and Lorea, 2009). Although several TDF sites were identified as candidates for protection by CONABIO (Comisión Nacional para el Conocimiento y Uso de la Biodiversidad), those containing the majority of locality records for both species of *Heloderma* were not included. This is unfortunate because the southern *H. horridum* populations contain the greatest levels of genetic diversity. Furthermore, unlike *H. suspectum*, our analyses robustly support the current subspecific designations within *H. horridum*. However, these particular lineages almost certainly circumscribe more than a single species (Figs. 3A, 5, 6). Thus, one benefit of a conservation phylogenetic perspective is that it can properly identify biodiversity to its correct (and thus manageable) taxonomic level. A phylogenetic evaluation of the Helodermatidae, using independent markers evolving at different rates, allowed us to track clade divergences at various temporal scales, which in turn underscored the variability among the two Helodermatid species with regard to their evolutionary time lines. This, in turn, impacts our perspectives regarding their conservation status.

While this study can clearly visualize cryptic (as well as over-extended) biodiversity, can it also estimate the potential for phylogenetic biodiversity loss as well? Indeed it can, particularly if other vertebrate groups are employed as a relevant benchmark. Davies et al. (2009) argued that mammalian species are more likely to be 'at risk' when they have few close relatives, or are habitat specialists with narrow tolerances and reduced geographic ranges. Other mitigating factors are species-distributions within temperate regions that juxtapose with those of other threatened species, and which manifest elevated human densities. These can be easily reflected to the Helodermatidae. *Heloderma horridum* is a habitat-specialist with few close relatives in a reduced geographic region of elevated conservation concern and with a burgeoning human population as well.

Similarly, phylogenetic analyses can also identify recently diversified clades and the regions within which they are found, with the premise that such clades or regions might represent engines of speciation. The fact that *H. horridum* (sensu lato) exhibits the most derived characters of the Helodermatidae (Fig. 3A, 5, 6) suggests that TDF habitat is indeed such an engine, and thus every effort should be made to allocate reserves and to designate national parks within this region.

We can also apply the phylogenetic findings in this study as a baseline for conservation decisions regarding *H. suspectum*. Unlike *H. horridum*, the lack of significant genetic variability in *H. suspectum* stands in contrast to its demonstrated antiquity. This provokes apprehension in that species of conservation concern (such as *H. suspectum*) are impacted first by genetic factors long before other aspects drive them to extinction (Spielman et al., 2004). As with *H. horridum*, this clade has few close relatives yet is limited to a restricted geographic area that contains a burgeoning human population (Sullivan et al., 2004; Beck, 2005; Kwiatkowski et al., 2008). Most importantly, any conservation investment in *H.*

*suspectum* will likely be ineffective if economic and/or political factors push in the opposite direction. Conservation perspectives should instead juxtapose the phylogenetic history of this species within a socio-economic matrix (McNeely, 2006) so as to attract as broad a target audience as possible, including politicians and the lay public (Avise et al., 2008).

### 4.5. The economic value of helodermatid conservation

Venom constituents are a cornucopia for pharmaceutical companies, and this aspect is a widely recognized attribute of *Heloderma* (Beck, 2005). Helodermatid venom is unique among reptiles in showing the highest number of bioactive peptides (Bertanccini, 1976). Most important in this regard is exendin-4, found only in *H. suspectum*, the sister to all remaining Helodermatidae. Exenatide (synthesized from exendin-4 as Byetta, Amylin/Eli Lilly, San Diego, CA) enhances insulin release in response to elevated blood glucose and improves glycemic control by replicating the pharmacological attributes of mammalian glucagon-like peptide-1 (GLP-1) (Lam and See, 2006). As such, it is a new treatment for adult-onset type-II *diabetes mellitus*, a serious health issue that accounts for the majority (>90%) of the 18 million cases in the United States alone, and 150 million worldwide (Pollack, 2002; Lam and See, 2006). This is an important aspect, and for a variety of reasons. Biomedical applications that flow from the practical application of biodiversity conservation (such as drug development from natural products) have been sluggish in emerging (Timmerman et al., 1999; but see Li and Vederas, 2009). This, in turn, has tilted politicians and the pubic away from biodiversity conservation and towards immediate economic gains produced by altering natural habitats and ecosystem services (Palmer et al., 2004). Thus, the development by a large pharmaceutical company of a potential remediation for type-II diabetes based upon bio-molecules from a non-charismatic and venomous lizard unequivocally illustrates the economic benefits that biodiversity can provide. Conservation phylogenetics not only places saliva/venom constituents of Helodermatidae within the context of deep history, but simultaneously provides a strong economic incentive for why this clade should be conserved.

## 5. Conclusion

Phylogenetic data can point to the origins of biodiversity. Their careful assembly and scrutiny often yield information on the manner by which particular clades have responded to past environmental challenges and, in turn, how they may respond to ongoing human-mediated alterations of the environment. In this sense, phylogenetic analyses can be profitably juxtaposed with those detailing habitat fragmentation and global change, to not only understand impacts of historic climatic shifts but to potentially disentangle them from contemporary effects (Douglas et al., 2003; Willis and Birks, 2006). This is imperative when species-loss must be evaluated or predicted in a conservation context.

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