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Phylogenomics of Horned Lizards (Genus: *Phrynosoma*) Using Targeted Sequence Capture Data

Adam D. Leaché[1,2] and Charles W. Linkem[2]

New genome sequencing techniques are enabling phylogenetic studies to scale-up from using a handful of loci to hundreds or thousands of loci from throughout the genome. In this study, we use targeted sequence capture (TSC) data from 540 ultraconserved elements and 44 protein-coding genes to estimate the phylogenetic relationships among all 17 species of horned lizards in the genus *Phrynosoma*. Previous molecular phylogenetic analyses of *Phrynosoma* based on a few nuclear genes, restriction site associated DNA (RAD) sequencing, or mitochondrial DNA (mtDNA) have produced conflicting relationships. Some of these conflicts are likely the result of rapid speciation at the start of *Phrynosoma* diversification, whereas other examples of gene tree discordance appear to be caused by active and residual traces of hybridization. Concatenation and coalescent-based species tree phylogenetic analyses of these new TSC data support the same topology, and a divergence dating analysis suggests that the *Phrynosoma* crown group is up to 30 million years old. The new phylogenomic tree supports the recognition of four main clades within *Phrynosoma*, including Anota (*P. mcallii, P. solare,* and the *P. coronatum* complex), Doliosaurus (*P. modestum, P. goodei,* and *P. platyrhinos*), Tapaja (*P. ditmarsi, P. douglasii, P. hernandesi,* and *P. orbiculare*), and Brevicauda (*P. braconnieri, P. sherbrookei,* and *P. taurus*). The phylogeny provides strong support for the relationships among all species of *Phrynosoma* and provides a robust new framework for conducting comparative analyses.

HORNED lizards (*Phrynosoma*) are a peculiar group with respect to their morphology and behavior, and they have evolved a number of remarkable adaptations including anti-predator blood squirting, water harvesting, and a wide array of horn armaments (Sherbrooke, 2003). Not surprisingly, horned lizards have been the subjects of diverse research in ecology and evolutionary biology (Hodges, 2004; Meyers et al., 2006; Luxbacher and Knouft, 2009; Bergmann and Berk, 2012). The group currently includes 17 species with a collective distribution extending from Canada to Guatemala with most of the species diversity found in Mexico (Nieto-Montes de Oca et al., 2014). The phylogenetic relationships among *Phrynosoma* have been difficult to resolve for at least three reasons. First, rapid speciation events early in the diversification of *Phrynosoma* have made it difficult to resolve relationships with small amounts of data (Hodges and Zamudio, 2004). Second, hybridization and introgression between species has produced a mitochondrial DNA (mtDNA) gene tree that conflicts with nuclear gene trees (Leaché and McGuire, 2006). Third, incomplete lineage sorting has caused nuclear gene trees to conflict with one another, and as a result, phylogenetic analyses using concatenation and coalescent methods typically disagree (Nieto-Montes de Oca et al., 2014). These problems are not mutually exclusive, and the genealogical patterns produced by incomplete lineage sorting and horizontal gene transfer can be hard to tell apart (Leaché et al., 2014). Overcoming these challenges requires a large sample of loci from throughout the genome.

Phylogenetic studies of *Phrynosoma* using morphological data have identified several relationships that have since been corroborated by molecular phylogenetic studies. Phylogenetic relationships that were supported by some (but not all) of the morphological studies include a close relationship between the short-horned species *P. douglasii*, *P. orbiculare,* and *P. ditmarsi,* as well as a clade containing the short-tailed species *P. taurus* and *P. braconnieri* (Reeve, 1952; Presch, 1969; Montanucci, 1987; Reeder and Montanucci, 2001; Hodges and Zamudio, 2004). MtDNA gene trees have provided decisive support for a clade containing *P. mcallii* and *P. platyrhinos* (Reeder and Montanucci, 2001; Hodges and Zamudio, 2004; Leaché and McGuire, 2006; Nieto-Montes de Oca et al., 2014); however, analyses of nuclear genes do not support this clade (Leaché and McGuire, 2006). Hybrids between *P. mcallii* and *P. goodei* have been reported (Mulcahy et al., 2006), and a study of one hybrid using nuclear genes suggests that hybridization and mtDNA introgression have produced an mtDNA genealogy that does not match the species phylogeny (Leaché and McGuire, 2006). Introgressive hybridization may have transferred the *P. mcallii* mtDNA genome into the common ancestor of *P. goodei* and *P. platyrhinos,* and, as a result, this maternally inherited marker is tracking a genealogy that conflicts with the speciation history recorded in autosomal nuclear loci.

A phylogenetic taxonomy was proposed for *Phrynosoma* by Leaché and McGuire (2006) based on a combined analysis of nuclear and mtDNA data that specifically excluded the introgressed mtDNA data for *P. platyrhinos* and *P. goodei*. The four clades proposed include Anota (*P. mcallii, P. solare,* and the *P. coronatum* complex), Doliosaurus (*P. modestum, P. goodei,* and *P. platyrhinos*), Tapaja (*P. ditmarsi, P. douglasii, P. hernandesi,* and *P. orbiculare*), and Brevicauda (*P. braconnieri* and *P. taurus*). The relationships among these groups were unclear, and two species, *P. asio* and *P. cornutum*, were assigned to the crown clade *Phrynosoma* (Leaché and McGuire, 2006). The support for these groups by subsequent phylogenetic studies has been mixed for a variety of reasons. First, several studies concatenate mtDNA with nuclear data without accounting for mtDNA introgression in *P. platyrhinos* and *P. goodei*, and therefore have supported a *P. mcallii* and *P. platyrhinos* clade that renders Anota and Doliosaurus non-monophyletic (Wiens et al., 2010, 2013; Pyron et al., 2013). Second, a recent concatenation analysis of six nuclear genes

[1] Burke Museum of Natural History and Culture, University of Washington, Seattle, Washington 98195; E-mail: leache@uw.edu. Send reprint requests to this address.
[2] Department of Biology, University of Washington, Seattle, Washington 98195.
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corroborated the four species groups and added a new species to the Brevicauda clade (*P. sherbrookei*); however, a coalescent based analysis of the same data only provided support for two of the groups (Tapaja and Brevicauda; Nieto-Montes de Oca et al., 2014). The differing performance of concatenation and coalescent-based phylogenetic inference is an indication that more loci are required to overcome problems associated with incomplete lineage sorting (Leaché and Rannala, 2011). Third, a phylogenetic study using thousands of restriction site associated DNA (RAD) loci also failed to recover the monophyly of Anota and Doliosaurus (Leaché et al., 2015a). These RAD loci, despite their large numbers, provided ambiguous support for the initial divergences in the genus, and only supported the monophyly of Tapaja and Brevicauda.

Targeted sequence capture (TSC) methods (also referred to as hybrid enrichment) have become a viable option for obtaining large comparative genomic data sets (Faircloth et al., 2012; Lemmon et al., 2012). Sequence capture methods use short probes (60–120 base pairs) to hybridize to specific genomic regions, which are subsequently isolated and sequenced using next-generation sequencing (Gnirke et al., 2009; McCormack et al., 2012; Lemmon and Lemmon, 2013). This method was recently used to estimate the phylogenetic relationships among genera in the family Phrynosomatidae (Leaché et al., 2015b). The TSC data supported a fully resolved and strongly supported phylogeny for phrynosomatid lizards; however, the study only included one species of *Phrynosoma* (*P. sherbrookei*). TSC data show great potential for resolving the difficult relationships that result from rapid radiations (Crawford et al., 2012; Faircloth et al., 2013; McCormack et al., 2013; Linkem et al., unpubl.), and in this study we conduct phylogenetic analyses of TSC data for all species of *Phrynosoma*.

MATERIALS AND METHODS

Sampling.—We included one sample to represent each species of *Phrynosoma* (Table 1). For outgroups, we included one sample for each genus of sand lizard (*Callisaurus draconoides*, *Cophosaurus texanus*, *Holbrookia maculata,* and *Uma notata*), and *Sceloporus bicanthalis* was included to root trees when necessary. The TSC data for *P. sherbrookei* and the sand lizards were published in a previous study of phrynosomatid lizard phylogeny (Leaché et al., 2015b), and the data for the remaining species are new to this study.

Targeted sequence capture data.—The sequence capture experiments that we conducted utilize a set of RNA probes specific for iguanian lizards (Leaché et al., 2015b). We synthesized 1,170 custom probes to target 585 loci (two 120 bp probes per locus) using the MYbaits target enrichment kit (MYcroarray Inc., Ann Arbor, MI). The probes target 541 ultraconserved elements (UCEs) that are a subset of the Tetrapods-UCE-5Kv1 probes (ultraconserved.org) and 44 nuclear loci used for the Squamate Tree of Life project (Squamate ToL loci; Wiens et al., 2012).

Whole genomic DNA was extracted from tissues using a NaCl extraction method (MacManes, 2013). Genomic DNA (400 ng) was sonicated to a target peak of 400 bp using a Bioruptor Pico (Diagenode Inc.). Genomic libraries were prepared using an Illumina TruSeq Nano library preparation kit. The samples were hybridized to the RNA-probes in the presence of a blocking mixture composed of forward and reverse compliments of the Illumina TruSeq Nano Adapters, with inosines in place of the indices, as well as chicken blocking mix (Chicken Hybloc, Applied Genetics Lab Inc.) and salmon blocking mix to reduce repetitive DNA binding to beads. Libraries were incubated with the RNA probes for 24 hours at 65°C. Post-hybridized libraries were enriched using TruSeq adapter primers with Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) for 20 cycles. Enriched libraries were cleaned with AMPure XP beads. We quantified enriched libraries using qPCR (Applied Biosystems Inc.) with primers targeting five loci mapping to different chromosomes in the genome of *Anolis*. Library quality was verified using an Agilent Tape-station 2200 (Agilent Tech.). These samples were pooled in equimolar ratios and sequenced (100 bp paired-end reads; combined with 60 samples for other projects) using an Illumina HiSeq2000 at the QB3 facility at UC Berkeley. Detailed methods for the collection of TSC data are provided in Linkem et al. (unpubl.).

Bioinformatics.—The raw DNA sequence reads were demultiplexed based on unique sequence tags using Casava (Illumina). We removed low-quality reads, trimmed low-quality ends, and removed adapter sequences using Trimmomatic (Bolger et al., 2014). The clean reads were assembled for each species using the *de novo* assembler IDBA (Peng et al., 2010). We ran IDBA iteratively over k-mer values from 50 to 90 with a step length of 10. We used phyluce (Faircloth et al., 2012) to assemble loci across species. We performed multiple sequence alignments for each locus using MAFFT (Katoh and Standley, 2013), and we trimmed long ragged-ends to reduce missing or incomplete data.

Phylogenetic analysis.—We estimated phylogenetic trees using concatenation and coalescent-based species tree inference. For the concatenation analyses, we conducted maximum likelihood (ML) analyses with RAxML v8.0.2 (Stamatakis, 2014) and Bayesian divergence dating analyses with BEAST v1.8.1 (Drummond et al., 2012). All analyses were unpartitioned, and although allowing each locus to have a separate clock rate and nucleotide substitution model has advantages, the large number of parameters that this introduces into the analysis makes the computation times prohibitive. The ML analyses used the GTRGAMMA model, and branch support was estimated using 1,000 bootstrap replicates. For the BEAST analyses, we tested clock models (strict clock versus the uncorrelated lognormal relaxed clock) using marginal likelihood estimation (Baele et al., 2013). Marginal likelihoods were estimated using path sampling and stepping-stone analyses (Baele et al., 2012), both with 100 sampling steps with 100,000 generations for each step. The strict clock was rejected (2 x $\log_e$ Bayes Factor = 222.9), and an uncorrelated lognormal relaxed clock was used for the final analyses. We assumed a Yule tree prior, and an HKY+Γ model of nucleotide substitution. These models are relatively simple and were used to help the Bayesian analysis reach apparent stationarity, which was difficult to achieve with more complex models. We time-calibrated the tree using a secondary calibration point obtained from a previous study of phrynosomatid lizards that included four fossils (Wiens et al., 2013). We assumed that the crown group age for phrynosomatid lizards was on average 55 million years old (normal distribution, mean = 55, standard deviation = 4), resulting in a 95% highest probability density (HPD) ranging from 48.4 to

Table 1. List of specimens included in the analysis, voucher numbers, and a summary of the TSC data. Standard museum abbreviations follow Sabaj Pérez (2014), and RRM refers to the personal field series of Richard R. Montanucci.

Species	Voucher	Raw reads	Clean reads	Nuclear loci captured	Nuclear loci k-mer depth
Phrynosoma asio	UWBM 7281	8,326,520	6,695,036	565	12,469
Phrynosoma blainvillii	CAS 200652	2,747,116	2,470,118	565	4,433
Phrynosoma braconnieri	UWBM 7282	7,207,490	6,453,604	561	11,491
Phrynosoma cerroense	MVZ 161206	4,217,154	3,859,106	579	7,941
Phrynosoma cornutum	MVZ 238582	3,395,606	2,836,972	575	2,788
Phrynosoma coronatum	UABC 1007	1,827,658	1,441,742	364	1,559
Phrynosoma ditmarsi	RRM 2459	2,286,766	1,948,510	576	2,119
Phrynosoma douglasii	UWBM 7227	8,858,212	8,134,806	515	17,838
Phrynosoma goodei	CAS 229922	7,122,242	6,483,980	574	14,156
Phrynosoma hernandesi	MVZ 245875	1,683,090	1,236,108	573	1,489
Phrynosoma mcallii	CAS 229923	943,436	768,346	538	869
Phrynosoma modestum	MVZ 238583	1,011,108	783,804	545	911
Phrynosoma orbiculare	UWBM 7285	831,042	609,540	508	614
Phrynosoma platyrhinos	MVZ 161495	23,645,034	19,061,802	563	10,870
Phrynosoma sherbrookei	UWBM 7286	7,634,142	6,971,920	579	14,107
Phrynosoma solare	MVZ 241510	460,944	344,526	410	458
Phrynosoma taurus	UWBM 7296	8,318,130	5,988,802	559	4,243
Outgroups					
Callisaurus draconoides	MVZ 265543	9,622,116	9,035,068	575	23,280
Cophosaurus texanus	UWBM 7347	9,176,180	8,625,204	573	24,401
Holbrookia maculata	UWBM 7362	12,314,136	11,604,340	573	31,000
Sceloporus bicanthalis	UWBM 7307	3,227,968	2,926,904	583	6,437
Uma notata	SDSNH 76166	2,332,400	2,099,068	577	4,232

61.6 Ma. Two replicate analyses of 10 million generations each were run, sampling every 1,000 steps, and discarding the first 25% prior to combining the results using LogCombiner v1.8. We calculated a maximum clade credibility (MCC) tree using TreeAnnotator v1.8.

A common practice in species tree inference using phylogenomic data is to bypass the sequence data and instead use summary statistics to estimate the species tree. Reducing the sequence data to summary statistics (gene trees) drastically reduces computation time, but this comes at the cost of ignoring an important source of variability in the data. We estimated a species tree using SVDquartets (Chifman and Kubatko, 2014), a new coalescent-based species tree inference method that uses the full sequence data. This method infers the topology among randomly sampled quartets of species using a coalescent model, and then a quartet method is used to assemble the randomly sampled quartets into a species tree. Reducing the species tree inference problem into quartets makes the analysis of large numbers of loci feasible. We randomly sampled 20,000 quartets from the data matrix, and used the program Quartet MaxCut v.2.1.0 (Snir and Rao, 2012) to infer a species tree from the sampled quartets. We measured uncertainty in relationships using nonparametric bootstrapping with 100 replicates. The bootstrap values were mapped to the species tree estimated from the original data matrix using SumTrees v.3.3.1 (Sukumaran and Holder, 2010).

RESULTS

Targeted sequence capture data.—A summary of the TSC data is provided in Table 1. The maximum number of loci captured (out of 585 total) across the 22 species included in the study was 583 loci for *Sceloporus bicanthalis*. The minimum number of loci captured was 364 for *Phrynosoma coronatum*. We assembled two data sets for phylogenetic analysis: an incomplete data matrix that included all loci that were sequenced for at least three species, and a complete data set that only contained loci that were sequenced for all 22 species. The incomplete data set introduces more missing data, but it adds 2.5X the number of informative sites. The incomplete data matrix contained 584 loci with some loci missing for some species. The incomplete data matrix contained 338,332 positions and 10,799 parsimony-informative characters. The total amount of missing data in the incomplete data matrix was 12.9%. The complete data matrix included 223 loci that were sequenced for all 22 species. The complete data matrix contained 138,087 aligned nucleotide positions and 4,404 parsimony-informative characters. The total amount of missing data in the complete data matrix was 8.3%.

A summary of the variation in the TSC loci is provided in Table 2. On average, the UCE loci were less variable compared to the Squamate ToL loci. We measured variability in terms of percentage of variable sites and as the number of parsimony-informative characters. On average, the UCE loci were approximately 575 bp, with 9% variation and 18 parsimony informative characters. In comparison, the Squamate ToL loci were longer (mean = 638 bp), more variable (mean = 19% variability), and contained more parsimony informative characters (mean = 26). The variability within the loci decreased by half or more when only species of *Phrynosoma* were considered.

Phylogenetic analysis.—Phylogenetic analyses of the TSC data using concatenation and the coalescent-based species tree inference supported the same topology (Fig. 1). This

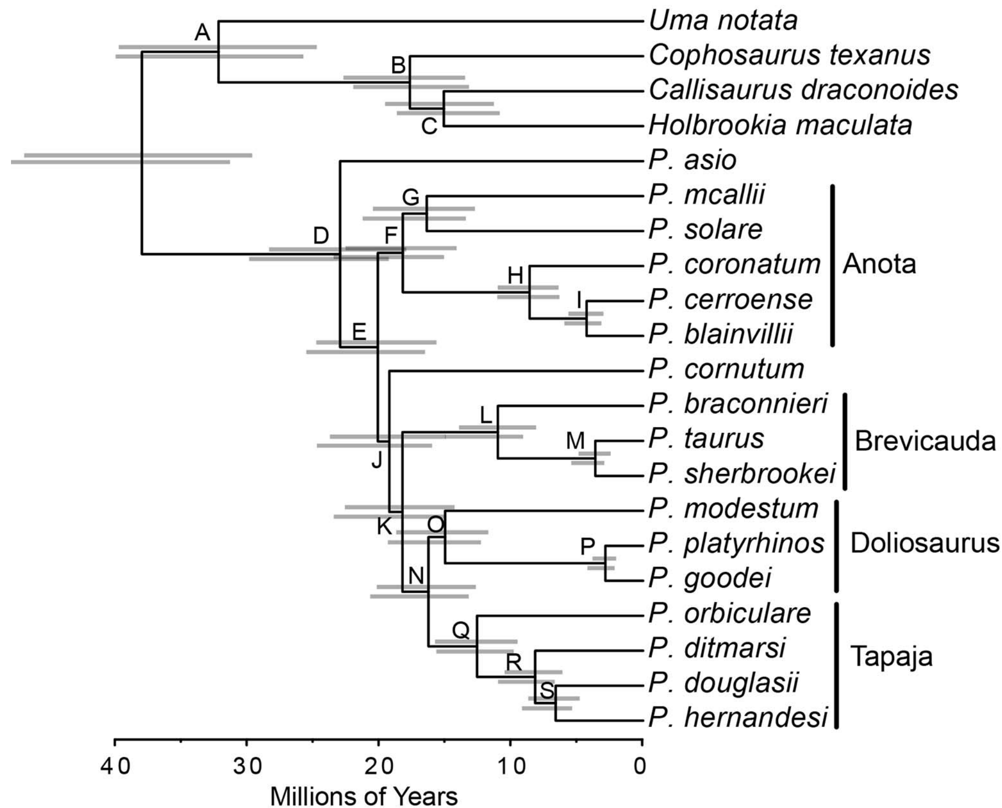

Fig. 1. Time-calibrated phylogeny for *Phrynosoma* and sand lizards based on a Bayesian relaxed-clock analysis of 584 nuclear loci. The dual error bars on each node show the 95% highest posterior density (HPD) for divergence times from the incomplete (top; 584 loci) and complete (bottom; 223 loci) data sets. The support values that correspond to each lettered branch are provided in Table 3.

topology was supported by analyses of the incomplete data (584 loci) and the complete data (223 loci). The time-calibrated phylogeny estimated using 584 loci provides an estimated divergence time for the *Phrynosoma* crown group of up to 30 million years old (mean = 24.4 Ma). *Phrynosoma asio* is supported as the sister taxon to the rest of the species, which are divided into two clades. One clade contains all of the members of Anota. Within Anota, *P. mcallii* and *P. solare* form a clade that is sister to the three species belonging to the *P. coronatum* complex (*P. blainvillii, P. cerroense,* and *P. coronatum*). The other clade contains *P. cornutum,* which is the sister taxon to a clade containing Brevicauda and a Doliosaurus + Tapaja clade. Within Brevicauda, *P. sherbrookei* and *P. taurus* are sister taxa. Within Doliosaurus, *P. platyrhinos* and *P. goodei* form a clade. Within Tapaja, *P. orbiculare* is sister to the remaining species, and *P. douglasii* and *P. hernandesi* form a clade.

The phylogenetic relationships among the sand lizards (i.e., *Callisaurus, Cophosaurus, Holbrookia,* and *Uma*) match a previous study that used the same data (Leaché et al., 2015b). Specifically, *Uma* is sister to the remaining sand lizards, and *Holbrookia* and *Callisaurus* form a clade. The monophyly of the earless lizards (*Holbrookia* and *Cophosaurus*) is not supported by the TSC data.

The support for relationships from the concatenation and coalescent analyses of the incomplete and complete data sets is summarized in Table 3. Concatenation analyses of the incomplete data set (584 loci) provided strong support for every branch in the tree using BEAST (posterior probability = 1.0) and RAxML (bootstrap ≥ 98%). The coalescent-based approach provided weaker support (bootstrap values <70%) for the shortest internal branches in the phylogeny. Specifically, weaker support was provided for the monophyly of Anota and Doliosaurus, the sister group relationship between *P. solare* and *P. mcallii,* and the monophyly of the clade containing Brevicauda, Doliosaurus, and Tapaja. Phylogenetic analysis of 223 loci (the complete data set) provides weaker support for these same branches.

Table 2. Variation in the phrynosomatid lizard TSC data. Only loci that were captured for ≥ 3 species are included in the calculations. The mean and (minimum–maximum) values are provided for locus length (in base pairs), variation (% variable characters), and number of parsimony informative characters (PIC).

	All taxa			*Phrynosoma*		
Loci	Length	Variation	PIC	Length	Variation	PIC
UCEs	575 (306–1,114)	9 (1–20)	18 (0–61)	575 (306–1,114)	5 (0–13)	9 (0–32)
Squamate ToL	638 (327–805)	19 (7–41)	26 (1–61)	638 (327–805)	5 (2–7)	12 (0–33)

Table 3. Support for phylogenetic relationships from concatenation and coalescent analyses of 584 nuclear loci. If the support for a branch was different based on analyses of the complete data (i.e., 223 loci shared among all species), then the alternative support value is shown in parentheses.

Branch	Concatenation BEAST	Concatenation RAxML	Coalescent SVDquartets
A	1.0	100%	100%
B	1.0	100%	100%
C	1.0	100% (96%)	100% (92%)
D	1.0	100%	100%
E	1.0	100%	99% (98%)
F	1.0	100%	83% (70%)
G	1.0	100%	77% (61%)
H	1.0	100%	100%
I	1.0	100%	100%
J	1.0	98% (62%)	99% (96%)
K	1.0	98% (84%)	97% (47%)
L	1.0	100%	100%
M	1.0	100%	100%
N	1.0	100%	100%
O	1.0	99% (94%)	90% (72%)
P	1.0	100%	100%
Q	1.0	100%	100%
R	1.0	100%	100%
S	1.0	100%	99% (93%)

DISCUSSION

Phrynosoma *phylogeny*.—The new TSC data presented here provide strong support for the phylogenetic relationships among *Phrynosoma* (Fig. 1) and support the phylogenetic taxonomy recommended for *Phrynosoma* by Leaché and McGuire (2006). Specifically, the monophyly of Anota, Brevicauda, Doliosaurus, and Tapaja are corroborated by the TSC data. In addition, the TSC phylogeny resolves the placements of *P. asio* and *P. cornutum,* two species that have traditionally been difficult to place in the phylogeny. The same topology was supported by concatenation and coalescent-based species tree analyses, although the latter provided relatively weak support for several of the shortest branches in the phylogeny (Table 3).

Concatenation and coalescent phylogenetic analyses support the same topology, which helps put to rest any concerns that incomplete lineage sorting could be misleading the phylogeny. Incomplete lineage sorting can generate gene tree discordance, and phylogenetic analyses that use coalescent models as opposed to concatenation have a higher probability of overcoming this challenge (reviewed by Edwards, 2009). A recent phylogenetic analysis of *Phrynosoma* using six nuclear genes produced conflicting results with coalescent and concatenation analyses (Nieto-Montes de Oca et al., 2014), but the use of hundreds of nuclear loci seems to have ameliorated the discrepancies that can arise from using different models for phylogenetic analysis with smaller data sets.

Previous phylogenetic studies of *Phrynosoma* utilizing either one or a few loci have supported conflicting phylogenetic trees, especially when comparing mtDNA gene trees to nuclear DNA trees (Leaché and McGuire, 2006; Nieto-Montes de Oca et al., 2014). We did not include any mtDNA data in this study, because the history of that one particular gene has been studied by numerous previous publications (Reeder and Montanucci, 2001; Hodges and Zamudio, 2004; Leaché and McGuire, 2006; Wiens et al., 2010; Nieto-Montes de Oca et al., 2014). Different regions of the mtDNA genome have consistently supported a sister group relationship between *P. mcallii* and *P. platyrhinos* (and *P. goodei*), which is strongly discordant with nuclear gene trees. Introgression of mtDNA haplotypes from *P. mcallii* into the common ancestor of *P. platyrhinos* and *P. goodei* is a likely mechanism for this discordance. Current phylogenetic models are not able to accommodate reticulate genealogical histories into phylogenetic inference without including multiple samples for each species (Gerard et al., 2011). Analyzing nuclear and mtDNA jointly could provide new insights into the speciation history of *Phrynosoma*, but this will require sampling multiple individuals per species and the use of new phylogenetic models that consider reticulate evolutionary histories.

The divergence times that we estimated for *Phrynosoma* are limited by the use of one calibration on the age of the phrynosomatid crown group. The prior probability distribution that we used is a secondary calibration obtained from a study that included four fossil calibrations (Wiens et al., 2013). Our assumptions about the mean age of the clade (55 Ma), and the standard deviation around that estimate, have a heavy influence on the estimated ages for the rest of the phylogeny. Incorporating fossil information directly into the analysis can provide more accurate divergence time estimates and is more easily justifiable (reviewed by Parham et al., 2011), and this creates a real need for new morphological phylogenetic studies of *Phrynosoma* that incorporate fossil taxa. However, the majority of *Phrynosoma* fossils are ascribed to extant species or extinct species (*P. anzaense* and *P. josecitensis*) that are too young to date speciation events (reviewed by Presch, 1969; Cassiliano, 1999; Mead et al., 1999; Parmley and Bahn, 2012). Molecular dating using mutation rate estimates for specific genes may be necessary for obtaining more refined divergence time estimates in *Phrynosoma*.

Systematics, phylogeography, and species delimitation.—The number of species recognized within *Phrynosoma* has grown in recent years as a result of phylogeographic studies exposing previously unrecognized genetic diversity within species. Currently, *Phrynosoma* contains 17 species divided into four natural groups: (1) Tapaja, the viviparous short-horned lizards: *P. ditmarsi*, *P. douglasii*, *P. hernandesi*, and *P. orbiculare*; (2) Anota, containing species with prominent cranial horns: *P. mcallii*, *P. solare*, and the *P. coronatum* group (*P. blainvillii*, *P. cerroense*, and *P. coronatum*); (3) Doliosaurus, containing three species lacking antipredator blood-squirting: *P. modestum, P. platyrhinos,* and *P. goodei*; and (4) Brevicauda, containing three viviparous species with extremely short tails that generally lack anti-predator blood squirting: *P. braconnieri*, *P. sherbrookei,* and *P. taurus*. The position of *P. cornutum* and *P. asio* relative to these groups is estimated with certainty by the TSC data presented here (Fig. 1).

Most *Phrynosoma* exhibit considerable geographic variation in coloration and cranial horn morphology, and some species are still delimited based solely on these and other morphological sources of variation. Maintaining an integrative approach to systematics that considers diverse types of data from morphology, molecular genetics, and

ecology should provide the most accurate species delimitations possible (Padial et al., 2010); however, the imperiled populations that represent unique phylogeographic groups, whether recognized as species or not, are of great conservation importance and should be managed accordingly. Below, we provide a summary of the phylogeographic and species delimitation studies that have been conducted on *Phrynosoma,* and highlight areas in need of additional research.

Phylogeographic investigations have been conducted on most of the short-horned species within Tapaja. The first phylogeographic study within *Phrynosoma* focused on the relationships among populations of *P. douglasii,* and the clear geographic structuring of mtDNA lineages resulted in the recognition of *P. hernandesi* as a distinct species (Zamudio et al., 1997). The study identified two divergent mtDNA clades within *P. hernandesi*, and a species delimitation study is needed to test whether these mtDNA clades represent unique evolutionary lineages. The genetic structure of *P. hernandesi* has been investigated in detail in Canada using mtDNA and nuclear data (Leung et al., 2014), and similar studies across the entire range of this species from Canada to Mexico are needed (Burt, 2006). The geographic range of *P. ditmarsi* is limited to oak woodlands within the Sonoran realm of the Madrean Archipelago and a few nearby locations (Enderson et al., 2014), and the genetic differentiation among populations remains unstudied. A detailed phylogeographic study of *P. orbiculare* identified 11 geographically structured mtDNA lineages that began diverging in the Miocene (Bryson et al., 2012). These findings suggest that there are multiple evolutionary lineages within *P. orbiculare*, and future studies should incorporate multilocus genetic data to aid in the delimitation of *P. orbiculare* lineages.

The short-tailed species within Brevicauda have relatively limited distributions in southern Mexico, and two species, *Phrynosoma braconnieri* and *P. taurus,* show large levels of geographic variation in coloration and cranial horn morphology across their ranges. These species are rare and difficult to find, and acquiring the genetic materials necessary for thorough phylogeographic investigations will be challenging. The recent discovery of *P. sherbrookei* added a new species to the Brevicauda clade, and a preliminary investigation of the genetic diversity within this species revealed low genetic variation compared to *P. braconnieri* and *P. taurus* (Nieto-Montes de Oca et al., 2014). These Mexican endemic species are considered threatened and under special protection, and additional information on the genetic diversity among populations could have important management consequences.

The evolutionary history between species belonging to Anota and Doliosaurus is intertwined. Early molecular phylogenetic studies of *Phrynosoma* using mtDNA found strong support for a clade containing *P. mcallii* and *P. platyrhinos* (Reeder and Montanucci, 2001; Hodges and Zamudio, 2004). The first detailed phylogeographic investigation of *Phrynosoma platyrhinos* and *P. mcallii* conducted by Mulcahy et al. (2006) identified two important evolutionary patterns. First, the study found genetic structure among populations of *P. platyrhinos* geographically coincident with the Gila and Colorado rivers, and as a result recognized *P. goodei* as a distinct evolutionary species (Mulcahy et al., 2006). Second, by focusing on an area of sympatry between *P. goodei* and *P. mcallii*, a number of putative hybrid individuals were identified that carried mtDNA haplotypes belonging to either species. This study, along with a subsequent study that confirmed the hybrid using nuclear DNA (Leaché and McGuire, 2006), helped establish the important role that hybridization and mtDNA introgression have played in generating discordance between nuclear and mtDNA gene trees in *Phrynosoma*. The hybrid zone between *P. mcallii* and *P. goodei* provides evidence that mtDNA genomes can move across species boundaries. It is possible that an authentic *P. platyrhinos* or *P. goodei* mtDNA haplotype could still be discovered with more extensive geographic sampling, and the expectation is that a non-introgressed mtDNA haplotype would be more closely related to *P. modestum* than to *P. mcallii*. A recent fine-scale phylogeographic study of *P. platyrhinos* and *P. goodei* using mtDNA discovered high levels of population structure (Jezkova et al., 2015), but it did not discover any new mtDNA haplotypes that support the introgression hypothesis.

Species delimitation within the *Phrynosoma coronatum* complex has been studied in great detail using morphological data, and a morphometric statistical analysis conducted by Montanucci (2004) resulted in the description of a new species, *P. wigginsi*. This species was not supported as a distinct evolutionary lineage by phylogenetic analyses of mtDNA and nuclear data (Leaché et al., 2009), and we consider *P. wigginsi* as a synonym with *P. cerroense*. The *coronatum* complex currently contains three species, *P. coronatum, P. blainvillii,* and *P. cerroense,* but this complex contains a considerable amount of genetic variation among populations that is not reflected by this current taxonomy.

Four additional species with broad distributions have yet to be examined at a population level. These include *Phrynosoma asio, P. cornutum, P. modestum,* and *P. solare*. Limited sampling for each of these species (two to four specimens) was included in a recent phylogenetic analysis of *Phrynosoma* using RAD loci (Leaché et al., 2015a), but more extensive sampling will be necessary in order to describe the phylogeographic structure of these widespread species.

Targeted sequence capture data.—The application of TSC data for phylogenomic analysis offers the potential for obtaining hundreds or thousands of loci, but the data obtained for any given locus can be incomplete or missing for some species in the study. For example, we targeted 585 nuclear loci, but the maximum number of loci captured in our study was 583 loci for *Sceloporus bicanthalis* (Table 1). Deciding how many loci to include in the final alignment is a critical aspect of TSC data analysis, because missing data can cause problems for phylogenetic analysis (Lemmon et al., 2009; Wiens and Morrill, 2011; Roure et al., 2013). We addressed this issue by contrasting phylogenetic trees estimated from a data set that contained missing loci for some species (the incomplete matrix = 584 loci) to a data set that contained no missing loci (complete matrix = 223 loci). With respect to our study, the levels of missing data present in the TSC data produced different branch support values (Table 3), but not different topologies. We do not think that TSC data will become a panacea for missing data problems. Instead, it is more likely that the number of loci obtained for this particular study system was sufficient for overcoming any consequence that would otherwise manifest from missing data.

The TSC data have a number of advantages and disadvantages compared to other phylogenomic data collection

approaches. The resulting sequence data are similar to traditional Sanger sequencing data and are therefore easy to analyze with many existing phylogenetic methods. However, the volume of data produced by TSC can prevent the use of highly parameterized methods, such as partitioned Bayesian phylogenetics. One solution is to simplify the models (the approach taken here), or to use summary statistic methods (Liu et al., 2010). The use of RNA-probes covering conservative gene regions allows for the collection of homologous DNA across diverse taxa, but targeting conservative gene regions limits the potential information content of each locus making accurate estimation of individual gene trees difficult. Gene homology can be readily verified through comparison to the conservative probes, making bioinformatics of the raw sequence data simple and accurate. However, new tools are needed for assessing the quality of the final locus assemblies, since it is difficult to adequately evaluate the alignment of each locus by eye.

We were able to produce high-quality genomic libraries from starting DNA quantities of 100 ng. By skipping the shearing step, degraded DNA (or ancient DNA) can be used without a loss of sample homology or coverage (Carpenter et al., 2013), allowing for the inclusion of museum specimens (Bi et al., 2013; Guschanski et al., 2013). The number of capture probes (and targeted loci) can be tailored to specific project goals to increase multiplexing efficiency and gene homology. We selected a subset of the Tetrapods-UCE-5Kv1 UCE probes (Faircloth et al., 2012; ultraconserved.org) that most closely matched genomes of taxa of interest, and then augmented this with probes for protein-coding genes commonly used in reptile phylogenetics (Wiens et al., 2012). This unique set of probes allows us to capture homologous loci with high coverage while multiplexing as many as 80 samples. Since our probes are homologous with other UCE probes and other reptile coding genes, the sequences obtained for this study can be easily incorporated into future studies and combined with existing data.

The rapid availability of genomic data is providing strong support for many relationships that were previously unresolved (Crawford et al., 2012, 2015; Faircloth et al., 2013). Most of these studies sub-sample the genome by targeting a specific type of genomic feature (e.g., exons, introns, UCEs, microRNAs, RNA, restriction site associated DNA). The recently published phylogenies from bird genomes (Jarvis et al., 2014) suggest that different genomic elements (i.e., exons, UCEs, or introns) can produce unique and strongly conflicting topologies. Heterogeneity of evolutionary rates across different genomic elements may result in a variable response to species diversification events. Genomic elements under different types of evolutionary constraint (exons vs. introns) may support unique genealogies that are inconsistent with phylogenies estimated from the whole genome. The extent of differential evolution across targeted genomic elements, and the effect that targeting a single type of element will have on phylogenetic accuracy, remains unclear. It is possible that future phylogenetic studies focusing on alternative genomic elements may provide strong support for an alternative phylogeny of *Phrynosoma*. The sequencing of whole genomes will help unlock a wealth of new phylogenomic data and help us begin to identify the genomic basis of the specialized morphological adaptations that make *Phrynosoma* such a unique component of North America's lizard fauna.

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