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**Abstract**

Biodiversity reduction and loss continues to progress at an alarming rate, and thus there is widespread interest in utilizing rapid and efficient methods for quantifying and delimiting taxonomic diversity. Single-locus species-delimitation methods have become popular, in part due to the adoption of the DNA barcoding paradigm. These techniques can be broadly classified into tree-based and distance-based methods depending on whether species are delimited based on a constructed genealogy. Although the relative performance of these methods has been tested repeatedly with simulations, additional studies are needed to assess congruence with empirical data. We compiled a large data set of mitochondrial ND4 sequences from horned lizards (*Phrynosoma*) to elucidate congruence using four tree-based (single-threshold GMYC, multiple-threshold GMYC, bPTP, mPTP) and one distance-based (ABGD) species delimitation models. We were particularly interested in cases with highly uneven sampling and/or large differences in intraspecific diversity. Results showed a high degree of discordance among methods, with multiple-threshold GMYC and bPTP suggesting an unrealistically high number of species (29 and 26 species within the *P. douglasii* complex alone). The single-threshold GMYC model was the most conservative, likely a result of difficulty in locating the inflection point in the genealogies. mPTP and ABGD appeared to be the most stable across sampling regimes and suggested the presence of additional cryptic species that warrant further investigation. These results suggest that the mPTP model may be preferable in empirical data sets with highly uneven sampling or large differences in effective population sizes of species.

*Key Words:ABGD, GMYC, ND4,* Phrynosoma*, PTP, speciation*

## Introduction

Proper identification and delimitation of species is of utmost importance for most fields of biology (de Queiroz 2007). Many research programs address fundamental questions through a comparative framework, necessitating the use of both a robust phylogeny and accurate species assignments for hypothesis testing. Molecular-based species-delimitation methods can generally be classified into single- or multilocus, and discovery and validation-based techniques (Carstens *et al.* 2013). DNA barcoding threshold methods (Hebert *et al.* 2003, 2004; Hebert & Gregory 2005; Edgar 2010; Puillandre *et al.* 2012a) comprise one common example of a single-locus technique, where threshold or cut-off values are used to differentiate inter- from intraspecific divergences. The Refined Single Linkage (RESL) method, for example, is a popular clustering algorithm implemented within the Barcode of Life Data Systems (Ratnasingham & Hebert 2013) to delineate operational taxonomic units (OTUs) based on animal COI barcode data. Although these threshold-type methods continue to be a quick and effective way to document and describe diversity, they do not take into account tree structure and often rely on arbitrarily defined thresholds (e.g., 2–3% pairwise sequence divergence) to delimit species (Blaxter 2004; Hebert & Gregory 2005; Hamilton *et al.* 2011).

More recently, coalescent-based methods of species delimitation have become common, in part due to the continual ease in which researchers can generate vast quantities of molecular data (Leaché & Fujita 2010; Fujita *et al.* 2012). Bayesian multilocus coalescent methods, for example, can explicitly account for gene tree/species tree incongruence when delimiting species and estimating a species tree (Yang & Rannala 2014; Jones 2014). Although an attractive alternative to threshold-type methods, the utility of many Bayesian multilocus coalescent

68 methods for large data sets remains uncertain due to the relatively large computational demand
69 of the algorithms (Yang & Rannala 2010, 2014; Satler *et al.* 2013; Leaché *et al.* 2014).
70 Single-locus, coalescent-based methods like the General Mixed Yule Coalescent model
71 (GMYC; Pons *et al.* 2006; Fujisawa & Barraclough 2013) have become a popular tree-based
72 species-delimitation technique often applied to barcoding data (e.g., animal mitochondrial DNA).
73 The GMYC model uses maximum likelihood and an ultrametric gene tree to model the transition
74 between inter- and intraspecific branching patterns. Branching patterns older than the inferred
75 threshold represent speciation events (Yule process), whereas younger branching indicates
76 neutral coalescence within species. GMYC has been used in numerous empirical studies (e.g.,
77 Monaghan *et al.* 2009; e.g. Barraclough *et al.* 2009; Hamilton *et al.* 2011; Gebiola *et al.* 2012;
78 Esselstyn *et al.* 2012; Blair *et al.* 2015), and recent simulations and empirical data suggest that
79 the method is fairly robust to different assumptions (Reid & Carstens 2012; Esselstyn *et al.* 2012;
80 Fujisawa & Barraclough 2013; Talavera *et al.* 2013; Tang *et al.* 2014). The Poisson Tree
81 Processes (PTP/bPTP) model is similar in that it seeks to model the transition in branch lengths
82 between versus within species (Zhang *et al.* 2013). However, PTP estimates branching processes
83 using the expected number of substitutions (versus time in GMYC), and thus utilizes a non-
84 ultrametric phylogenetic tree as input. One limitation of the original PTP model is that it assumes
85 only two independent distributions to model branch lengths (one exponential distribution for
86 speciation and one exponential distribution for coalescence). This generally ignores the
87 stochastic variation among species due to different population sizes and demographic histories.
88 Conversely, the recently-developed multi-rate Poisson Tree Processes model (mPTP) fits
89 multiple independent exponential distributions to each delimited species to explicitly account for
90 differences in sampling intensity and/or population history (Kapli *et al.* 2016). Although the

mPTP model may potentially lead to more accurate delimitations versus other single-locus methods, testing and comparison using empirical data characterized by highly heterogeneous sampling intensity and/or large differences in genetic diversity among species is lacking.

Horned lizards (*Phrynosoma*) are a genus of phrynosomatid lizards consisting of 17–21 species distributed from Canada to Guatemala (Leaché & Linkem 2015; Montanucci 2015). The unique morphology and behavior of these lizards, including ocular blood squirting (Sherbrooke 2003), has made them the target of numerous systematic investigations. Early studies based on mitochondrial DNA (mtDNA) sequences yielded conflicting phylogenetic relationships with both nuclear and morphological data (e.g. Hodges and Zamudio, 2004; Leaché and McGuire, 2006; Reeder and Montanucci, 2001), presumably because of mtDNA introgression (Leaché & McGuire 2006; Mulcahy *et al.* 2006). More recently, next-generation sequencing has been used to estimate a robust phylogeny for the genus, with results suggesting that cladogenesis initiated in the Miocene around 22 million years ago (Leaché & Linkem 2015). Phylogeographic studies have also been conducted on several species including *P. douglasii* (Zamudio *et al.* 1997), *P. mcallii* (Mulcahy *et al.* 2006), *P. platyrhinos* (Jezkova *et al.* 2016), *P. coronatum* (Leaché *et al.* 2009), and *P. orbiculare* (Bryson *et al.* 2012). Many of these studies have indicated the presence of undocumented cryptic diversity, but whether any of this diversity may warrant species status has not been evaluated in detail for most groups (but see Leaché *et al.* 2009). These studies have also revealed substantially different levels of intraspecific diversity, ranging from deep lineages within *P. orbiculare* to low levels of genetic diversity in *P. mcallii*. More recent taxonomic work on the genus has suggested that levels of diversity may be underestimated at a clade-wide level (Nieto-Montes de Oca *et al*. 2014; Montanucci 2015).

Given the need for additional empirical studies to compare and contrast single-locus "discovery-based" species delimitation methods, particularly in cases with large differences in sampling intensity and levels of intraspecific diversity, in this study we assess congruence among four tree-based and one distance-based methods of delimiting horned lizard species. We particularly focus on the utility of one method (mPTP) with the ability to accommodate highly heterogeneous data sets comprised of species with dramatically different levels of molecular diversity. Based on our results, we provide further quantitative evidence for undescribed species in the genus.

## Materials and Methods

### *Data collection*

We obtained from GenBank a total of 368 orthologs of the mitochondrial ND4 gene from multiple representatives of *Phrynosoma*. Although many molecular phylogenetic studies have been performed on the genus, there have been relatively few phylogeographic investigations of species using the same marker. We extracted from GenBank multiple sequences from *P. mcallii* (Mulcahy *et al.* 2006), *P. platyrhinos* (Jezkova *et al.* 2016), *P. orbiculare* (Bryson *et al.* 2012), and the *P.douglasii* complex (Zamudio *et al.* 1997). We included singletons of several other species, including *P. cornutum*, *P. coronatum*, *P. solare*, *P. asio*, and *P. taurus*. Although the COI gene is the generally accepted standard for most animal barcoding studies (Hebert *et al.* 2003; Ratnasingham & Hebert 2013), ND4 likely serves as a good proxy due to the linked inheritance of mtDNA. Full sampling information can be found in Supplementary Table S1. All gene trees and alignments used in this study can be found on Dryad (doi:10.5061/dryad.r7989).

*Phylogenetic analysis*

The primary goal of our study was to test multiple methods of single-locus species delimitation of horned lizards based on ND4 GenBank data. We were particularly interested in testing for similarities and differences in those methods requiring an ultrametric tree (GMYC-type methods) versus those that do not rely on temporal calibration (PTP-type methods). We were also interested in the performance of both types of methods under scenarios with divergent rates of coalescence across species. We tested a total of four tree-based methods on the data, including the single-threshold GMYC (sGMYC; Pons *et al.* 2006; Fujisawa & Barraclough 2013), multiple-threshold GMYC (mGMYC; Monaghan *et al.* 2009), bPTP (Zhang *et al.* 2013), and mPTP (Kapli *et al.* 2016). All data were aligned using MUSCLE v.3.8.31 (Edgar 2004) implemented in AliView v.1.17.1 (Larsson 2014). The total alignment consisted of 871 bp, although sequence lengths for the *P. douglasii* complex were shorter (alignments available on Dryad). Prior to species-delimitation analyses, we used RAxML v.8.0.0 (Stamatakis 2014) to remove duplicate haplotypes from a matrix of 368 sequences. This left a total of 220 haplotypes for species delimitation. Although identical sequences should generally be removed prior to tree-based methods of species delimitation (J. Zhang, pers. comm.), we also performed a series of duplicate analyses using all 368 sequences for comparison. We used MEGA7 (Kumar *et al.* 2016) to calculate average within-species genetic distances using the Tamura-Nei model with gamma distributed rate heterogeneity to account for multiple substitutions. In addition, we used the R-packages APE (Paradis et al. 2004) and PEGAS (Paradis 2010) to calculate Watterson's estimator of theta ($\theta = 4N_e\mu$) as an indicator of effective population sizes.

We used BEAST v.2.4.3 (Bouckaert *et al.* 2014) to generate ultrametric gene trees under a strict clock and constant-size coalescent tree prior following the relative performance of clock models in previous studies (Monaghan *et al.* 2009; Satler *et al.* 2013; Talavera *et al.* 2013). A GTR+I+Γ model of substitution was used as estimated using BIC in jModelTest2 (Darriba *et al.* 2012). We calibrated the rate of mtDNA substitution by specifying a normal prior with a mean of 0.00805 substitutions/site/million years and sigma of 0.001 (Bryson *et al.* 2012), a rate initially estimated using vicariant scenarios in geckos (Macey *et al*. 1999). Similar substitution rates have been applied in numerous studies to estimate divergence times in several vertebrate groups (see Macey *et al*. 1999; Bryson *et al*. 2012 for examples), and our specified prior distribution accommodated uncertainty in the estimate. Analyses were run for 20 million generations, sampling every 2,000. Convergence and mixing were monitored in Tracer v.1.6 (Rambaut *et al.* 2014), and ESS values >200 indicated adequate sampling of the posterior. TreeAnnotator v.2.4.3 (Bouckaert *et al.* 2014) was used to create a maximum clade credibility (MCC) tree using mean heights for node annotation. Maximum-likelihood (ML) phylogenetic analyses were implemented in RAxML under a GTRGAMMA model by first implementing a rapid bootstrap search (Stamatakis *et al.* 2008) with autoMRE bootstopping followed by a full ML search (-f a option). Trees were rooted using *P. asio* (Leaché & Linkem 2015).

*Species delimitation analyses*

Among the multiple methods of single-locus species delimitation currently available, the most popular is the GMYC model (Pons *et al.* 2006; Fujisawa & Barraclough 2013). Previous studies indicate that the GMYC model is fairly robust (Fujisawa & Barraclough 2013), especially if applied to an ultrametric tree constructed using BEAST (Tang *et al.* 2014), and that the choice of

clock model and tree prior has a relatively low impact on the results (Talavera *et al.* 2013). We used the R package SPLITS (Ezard *et al.* 2009) to fit both the single- and multiple-threshold models to the data. We initially included haplotypes only for species containing multiple sequences (*P. mcallii*, *P. platyrhinos*, *P. orbiculare*, *P. hernandesi*, *P. douglasii* complex). However, sGMYC results were not significantly different from the null model of coalescence. Thus, we added singletons of *P. cornutum*, *P. coronatum*, *P. solare*, *P. asio*, and *P. taurus* to increase the Yule portion of the tree and better fit the model to the data (Talavera *et al.* 2013).

bPTP analyses were performed using the online server (http://species.h-its.org/) and the ML trees from RAxML. We ran the analyses for 500,000 generations with a thinning of 500 and burnin of 0.1. Convergence was assessed by visualizing plots of MCMC iteration versus log-likelihood. We ran analyses both with and without the outgroup taxon (*P. asio*). As results were qualitatively similar (not shown), all subsequent comparisons were made with the outgroup to negate taxonomic discrepancy among analyses. We compared the results from bPTP to the recently developed mPTP model that accommodates different rates of coalescence within clades (Kapli *et al.* 2016). Discordant coalescent patterns could be due to uneven sampling intensity among species or varying degrees of genetic structure arising from differences in evolutionary processes and effective population sizes ($N_e$). We performed both ML and MCMC analyses on RAxML ML trees using the standalone mPTP software (v.0.1.1). MCMC analyses were run for 100 million generations, sampling every 10,000. The first 2 million generations were discarded as burnin and analyses started from the ML species delimitation estimate (identical results were obtained when starting from both random and null delimitations). Convergence was again assessed by monitoring the plot of generation versus log-likelihood. Both ML and MCMC analyses utilized the *--multi* option to incorporate differences in rates of coalescence among

species and used a minimum branch length of 0.0001. We compared results among multiple MCMC runs (10) to assess congruence.

Because our taxonomic sampling for the four target taxa was highly uneven (i.e., *P. platyrhinos* was represented by ~3x the number of haplotypes), we re-ran all of the above analyses after pruning haplotypes from *P. platyrhinos* to determine the potential influence of highly heterogeneous sampling intensity on species delimitation. Haplotypes were selected (40 of 111 total) based on the previous phylogenetic analyses to maximize diversity within the species. A new model of substitution was then calculated (HKY+I+Γ) and all phylogenetic and species delimitation analyses were repeated as described above. However, only unique haplotypes (149) were included in this set of analyses.

To compare the results from the tree-based methods above, we ran Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.* 2012a) on both the full and pruned data sets. ABGD is a computationally efficient distance-based method of species delimitation that has been shown to perform well when compared to tree-based coalescent methods (Puillandre *et al.* 2012b; Kekkonen & Hebert 2014; Kapli *et al.* 2016) and other threshold techniques (Ratnasingham & Hebert 2013). The method seeks to quantify the location of the barcode gap that separates intra- from interspecific distances. As the presence of singletons may bias the analysis (Puillandre *et al.* 2012a), ABGD analyses were restricted to *P. mcallii*, *P. orbiculare*, *P. platyrhinos*, and the *P. douglasii* complex. Default settings were used for the prior range for maximum intraspecific divergence (0.001, 0.1). Results were compared using both JC69 and K80 corrected distances and minimum slope increase (*X*) of 1.5 (default) and 1.0.

For all analyses, we reported the number of delimited species inferred by each method along with the corresponding confidence intervals. In addition, we used current horned lizard

taxonomy (Leaché & Linkem 2015; Montanucci 2015), to compare the proportion of delimited species matching taxonomic species, the proportion of taxonomic species lumped into a delimited species, and the number of taxonomic species splits. We note that large values for species lumps and splits may not necessarily indicate poor performance of methods, but they do provide evidence that horned lizard taxonomy might be in need of revision. Next, we reported the match ratio (following Ahrens *et al*. 2016) using the following formula:

$$match\ ratio = 2 * \frac{N_{match}}{(N_{delimited} + N_{morph})}$$

where $N_{match}$ is the number of delimited species exactly matching taxonomic species, $N_{delimited}$ is the total number of delimited species, and $N_{morph}$ is the number of taxonomic, morphologically defined species. Finally, we quantified performance of methods using the recently developed Relative Taxonomic Resolving Power Index ($R_{tax}$) and the Taxonomic Index of Congruence ($C_{tax}$) following Miralles & Vences (2013). The $R_{tax}$ index quantifies the relative power of a method to infer all estimated speciation events present in a data set (large $R_{tax}$ = small Type-II error), but does not necessarily imply correct delimitations (i.e. can lead to oversplitting). $R_{tax}$ metrics were calculated as follows:

$$Rtax(A) = \frac{nA}{n(A \cup B \cup C \cup D \cup E)}$$

where A,B,C,D,E represent the five species delimitation methods tested, the numerator ($nA$) represents the number of speciation events inferred by method $A$, and the denominator represents the cumulative number of speciation events inferred by all methods. An $R_{tax}$ value of 1 would indicate that the method recovered all speciation events present across methods.

The $C_{tax}$ index is a measure of congruence in species assignments among two methods, with a value of 1 indicating complete congruence. $C_{tax}$ metrics were calculated as follows:

$$Ctax(AB) = \frac{n(A \cap B)}{n(A \cup B)}$$

where $A \cap B$ represents the number of speciation events shared by methods *A* and *B*, and $A \cup B$ represents the total number of speciation events inferred by method *A* and/or *B*. We refer the reader to the original publication for additional descriptions of these metrics (Miralles & Vences 2013).

## Results and Discussion

*Uneven sampling*

Inferred genealogies were congruent with previous studies based on mtDNA (Reeder & Montanucci 2001; Hodges & Zamudio 2004; Leaché & McGuire 2006) and discordant from nuclear phylogenies (Leaché & Linkem 2015), presumably due to historical introgression (Leaché & McGuire 2006; Leaché & Linkem 2015). Our inferred divergence times based on an assumed mtDNA substitution rate of 0.00805 substitutions per site per million years were congruent with times previously inferred using secondary calibration information for the crown age of phrynosomatids (Leaché & Linkem 2015). Average pairwise sequence divergence varied considerably among species, from relatively low levels in *P. mcallii* (0.94%) to high divergence within the *P. douglasii* complex (7.7%; Table 1). Similarly, effective population sizes varied by an order of magnitude.

All MCMC species delimitation analyses indicated adequate convergence based on visualizing plots of generation versus likelihood score. Because no differences were detected when using a single run versus 10 independent MCMC runs, we present results from single runs

only. The number of horned lizard species inferred by each delimitation method was substantially different between the full (220 haplotypes) and reduced (149 haplotypes) data sets.

For the full data set, sGMYC was the most conservative method, inferring a total of 10 species with wide confidence intervals (Table 1; Fig. 1). The comparatively small number of species inferred with sGMYC was likely a result of the method having difficulty in locating the threshold point in the data (Supplementary Fig. S1A). sGMYC is expected to work well when there is a clear demarcation in branching rates between versus within species (Pons *et al.* 2006; Reid & Carstens 2012; Esselstyn *et al.* 2012; Fujisawa & Barraclough 2013), which was not the case in the horned lizard data as the threshold was placed relatively deep in the genealogy. Conversely, mGMYC suggested an unrealistically large number of species (81), a high proportion of splits (0.95), and low match ratio (0.13), and appeared to have an equally difficult time placing transition points between inter- and intraspecific branching processes (Supplementary Fig. S1B). mGMYC suggested up to 31 species within *P. platyrhinos* alone (Fig. 1), which is unlikely given the low levels of divergence within the species (Table 1). bPTP also suggested an unrealistically high number of horned lizard species (52) with wide confidence intervals from MCMC analyses. However, unlike mGMYC, bPTP inferred only four species within *P. platyrhinos* (Fig. 1). Both mGMYC and bPTP inferred a large number of species within the *P. douglasii* complex (29 and 26 species, respectively). mPTP analyses suggested an intermediate number of total species (18) that was the most congruent with the relative levels of structure in the inferred genealogies (Table 1; Fig. 1). These results were consistent with previous findings that the original PTP model tends to oversplit, whereas mPTP is more conservative and likely to represent true species clusters (Kapli *et al.* 2016).

ABGD analysis suggested a total of 12 species based on initial partitioning over a range of prior values for maximum intraspecific divergence (Table 1; Supplementary Fig. S2). Results based on JC69 and K80 corrected distances were identical. The number of species decreased to five with a maximum intraspecific divergence prior value (*P*) of 0.021554, and to one species with a value of 0.035938. Although there is still a lack of consensus of how to interpret discordant ABGD results (Kekkonen & Hebert 2014), previous studies advocate using a P-value of ~0.01 (Puillandre *et al.* 2012a), which in our data would result in the recognition of 12 species of horned lizards (excluding the singletons). The relatively low value for the proportion of species matches (0.17) and match ratio (0.20) was likely a result of both excluding singletons from the analysis and the likely presence of multiple undescribed species in the data. Applying the recursive algorithm resulted in a maximum of 20 species when $X = 1.5$ (Supplementary Fig. S2A). This value increased to a maximum of 36 species when $X = 1.0$ (Supplementary Fig. S2B). To be conservative, we focus on the results from the initial partitioning as this scheme has been shown to be more stable across parameter settings and congruent with other species delimitation methods (Puillandre *et al.* 2012a; Puillandre *et al.* 2012b; Kekkonen & Hebert 2014), including those examined in this study.

$R_{tax}$ values for the full 220 unique haplotype data set ranged from 0.05 for sGMYC to 0.96 for mGMYC (Table 2), further illustrating the tendency of mGMYC to delimit a large number of (likely erroneous) species. Congruence among methods, based on $C_{tax}$ values, was highest between mPTP and ABGD (0.64) and lowest between sGMYC and mGMYC (0.05). mPTP and ABGD also showed the largest mean $C_{tax}$ among methods (Table 2).

Results of the full 368 sequence data sets (including identical sequences) revealed varying degrees of sensitivity of methods to the presence of duplicates, with most algorithms

316 suggesting additional species (Supplementary Table S2). mGMYC was by far the most sensitive
317 of the methods compared, inferring a total of 164 species versus 81 species in the unique 220
318 haplotype data set. In general, the performance of all methods (except bPTP) was reduced based
319 on match ratios. Interestingly, mPTP failed to distinguish between *P. cornutum* and *P. solare*
320 even though these species are distantly related (Leaché & Linkem 2015). The performance of
321 ABGD also seemed to be impacted by the inclusion of identical sequences, concordant with
322 other recent findings (Ahrens *et al*. 2016). Thus, although previous studies suggest that the
323 GMYC model may be robust to the inclusion of identical sequences (Talavera et al. 2013), our
324 results suggest that additional bias may be introduced when duplicate haplotypes are not
325 removed.
326
327 *Even sampling*
328 Results from our analyses using a relatively even sampling scheme (after pruning the number of
329 *P. platyrhinos* haplotypes to 40) revealed varying levels of sensitivity among the species
330 delimitation methods to uneven sampling among species. sGMYC results were virtually identical
331 to the full analysis due to the same position of the inflection point (Supplementary Fig. S3A), but
332 the confidence interval was substantially reduced. In contrast, mGMYC was extremely sensitive
333 to sampling regime (Figs. 1,2; Table 3), and placed the four thresholds at different times in the
334 evenly sampled genealogy (Supplementary Fig. S3B). The proportion of species splits inferred
335 by mGMYC was substantially reduced in the pruned data set (0.71 vs. 0.95) and the match ratio
336 was increased from 0.13 to 0.39. For example, only a single species within *P. platyrhinos* was
337 inferred by mGMYC for the reduced data set (Fig. 2), compared to 31 species for the full data
338 (Fig. 1). These results were surprising since we pruned *P. platyrhinos* haplotypes evenly

throughout the original genealogy. mGMYC also inferred different numbers of species within *P. orbiculare* and the *P. douglasii* complex. Thus, in contrast to sGMYC, mGMYC appears quite sensitive to sampling conditions, which may further limit the utility of the method (Esselstyn *et al.* 2012; Fujisawa & Barraclough 2013; Talavera *et al.* 2013). Conversely, bPTP and mPTP appear less sensitive to sampling issues as the number of inferred species, proportion of matches, lumps and splits and match ratios were similar between both sets of analyses (Tables 1,3). mPTP was the most consistent, with 18 species inferred from the full data set and 17 in the reduced data set (Figs. 1,2). The discrepancy in the single species arose from a slightly different gene tree for the reduced data set within the *P. platyrhinos* clade. Zhang et al. (2013) also tested for the influence of sampling evenness on species delimitation results and found that PTP outperformed GMYC with even sampling, whereas GMYC was slightly more accurate with uneven sampling. Due to the extreme sensitivity of mGMYC to sampling conditions, the largest $R_{tax}$ value was obtained from bPTP (1.00 vs. 0.59 for mGMYC) in the pruned data set (Table 4). Similar to the full 220 haplotype analysis, pairwise $C_{tax}$ was highest between mPTP and ABGD (0.69) and in this case lowest between sGMYC and bPTP (0.11; Table 4).

Collectively, these results suggest mGMYC and bPTP were more sensitive to sampling regime, and that the large difference in the inferred number of species between bPTP and mPTP is likely due to the latter fitting multiple exponential branch length distributions to species to account for different rates of coalescence in heterogeneous data sets containing species with different $N_e$ and demographic histories. Thus, our results provide further empirical evidence that mPTP may be a good choice for single-locus species delimitation based on accuracy, consistency, and speed (Kapli *et al.* 2016).

ABGD analyses on the evenly sampled (pruned) data set also indicated 12 species for most values of *P* using the initial partition (Table 3; Supplementary Fig. S4A,B). Once again, the recursive partition suggested a substantially higher number of species, particularly when P < 0.0129. There were also slight differences in the recursive partition between K80 and JC69 corrected distances (Supplementary Fig. S4A,B). However, K80 distances indicated the same number of groups (12) among the initial and recursive partition when P = 0.0129.

*Performance of methods*

Many studies have examined the GMYC model in detail using both simulated and empirical data (Reid & Carstens 2012; Esselstyn *et al.* 2012; Fujisawa & Barraclough 2013; Talavera *et al.* 2013; Tang *et al.* 2014; Ahrens *et al*. 2016). Tang et al. (2014) quantified the influence of gene tree reconstruction method and rate smoothing technique on the performance of both GMYC and PTP and found that the former was generally more sensitive to the selected model. They found that most of the sensitivity was likely due to errors during the smoothing step and subsequently advocated the use of BEAST to generate ultrametric gene trees. Talavera et al. (2013) used a large butterfly data set to test the performance of GMYC and suggested that the model is highly stable under a variety of conditions including tree reconstruction method, the number of singletons included, the number of species included in the gene tree, and sampling coverage. They provided a summary table and chart with recommendations for running GMYC on empirical data sets. Interestingly, their analysis suggested that sGMYC often overestimates the number of species, in contrast to our analysis where the model may be underestimating true diversity. However, our results were concordant in the sense that species delimitations did not change significantly with different sample coverage, although it was slightly impacted by the

384 inclusion of identical sequences. Studies have also indicated that GMYC may be sensitive to
385 general phylogenetic history, sampling intensity, DNA sequence length, speciation rate, $N_e$, and
386 differences in $N_e$ among species (Reid & Carstens 2012; Esselstyn *et al.* 2012), and may
387 sometimes underestimate (sGMYC) or overestimate (mGMYC) the true number of species
388 (Esselstyn *et al.* 2012). This is a likely explanation for our sGMYC results that estimated only 10
389 species of horned lizards, as there was no abrupt change in branching rates between versus
390 within species. More recently, Ahrens *et al*. (2016) used both simulated and empirical data to
391 better understand potential biases in GMYC due to sampling and population genetic artifacts.
392 Their results suggested that the majority of bias is introduced by variation in $N_e$ among species,
393 which can be exacerbated by uneven species abundance/sampling. In these cases, sGMYC tends
394 to lump species and return wide confidence intervals, which is consistent with our results for
395 horned lizards. To help overcome these issues, they suggest increasing the number of clades
396 examined to balance out the large skew in $N_e$ among species. Although this solution may
397 alleviate some of the issues with sGMYC, mPTP may be more reliable in such cases as the
398 method can explicitly account for differences in $N_e$ and rates of coalescence among species.
399 To our knowledge, few studies have examined the potential influence of methods for
400 summarizing node height information in BEAST analyses for subsequent species delimitation
401 using GMYC. To provide preliminary data on this issue, we performed a suite of additional
402 sGMYC and mGMYC analyses on both the full (368 sequences) and unique haplotype (220
403 sequences) data sets. Three methods of summarizing node heights were compared: mean heights,
404 median heights, common ancestor heights. Different results were detected depending on whether
405 the single- or multiple-threshold model was used (Table 5). Using common ancestor node
406 heights with sGMYC dramatically increased the number of delimited species, due to the

407 threshold point be pushed closer to the present (Supplementary Fig. S5). This effect was
408 negligible with mGMYC, which appeared to be more affected by the inclusion of identical
409 sequences (Supplementary Fig. S6) resulting in a doubling of the number of inferred species
410 (Table 5). Thus, there appears to be additional nuances of GMYC that should be considered
411 when utilizing these methods on empirical data. Based on relative concordance with our mPTP
412 and ABGD analyses, using sGMYC with mean or median node heights may be a good approach.
413 Additional simulation studies will be needed to test this prediction further.

414 Given the high levels of discordance observed among the methods tested, how should
415 researchers use these algorithms to discover and delimit diversity? As detailed above, mPTP has
416 numerous advantages over other methods. The consistency of mPTP to delimit putative species
417 in our study despite varied sampling depths and effective population sizes provides additional
418 evidence suggesting that the model may be appropriate for a wide variety of empirical data sets
419 (Kapli *et al.* 2016). Further, mPTP may be a good choice in data sets such as ours where sGMYC
420 has difficulty in placing the threshold point due to a more gradual slope in branching times,
421 possibly as a result of sampling a low species-to-individual ratio or due to large differences in $N_e$
422 among species (Ahrens *et al*. 2016). However, we agree with previous authors in that taxonomic
423 changes should not be made solely on the results of these methods (Lohse 2009; Puillandre *et al.*
424 2012a; Esselstyn *et al.* 2012; Zhang *et al.* 2013; Talavera *et al.* 2013), although concordance
425 using multiple analyses does tend to increase reliability (Puillandre *et al.* 2012b; Carstens *et al.*
426 2013; Satler *et al.* 2013). Rather, robust single-locus approaches should be used to form primary
427 taxonomic hypotheses that are subsequently tested with other types of data as part of an
428 integrative taxonomic framework (Fujita *et al.* 2012). Although ABGD has the potential to offer
429 a rapid and robust framework for assessing concordance (Puillandre *et al.* 2012a; Puillandre *et*

*al.* 2012b; Ahrens *et al*. 2016), additional work is needed to determine optimal parameter settings and whether the recursive partition tends to oversplit. Moreover, additional studies are needed to compare the performance of RESL through BOLD against some of the newer tree-based methods (e.g., bPTP and mPTP).

The adoption of single-locus species delimitation methods to biodiversity research seems particularly relevant to large metabarcoding studies (including microbial 16S rRNA sequencing) as a rapid and cost-effective means to target groups for additional investigation. High-throughput, multiplex amplicon sequencing using next-generation sequencing platforms allows for the rapid generation of single-locus data from a large number of samples for primary species delineation. Due to its speed and accuracy, mPTP seems to be an ideal analytical tool for these large heterogeneous data sets consisting of species with different coalescent histories. We anticipate that empiricists will continue to explore the utility of single-locus methods as sequencing technologies improve and new analytical tools are developed.

*Taxonomy of horned lizards*

Based on the performance and consistency of single-locus species delimitation methods tested in this study, species-level diversity within *Phrynosoma* might be underestimated. Analyses of the mitochondrial gene ND4 provide evidence to suggest at least 11 species could be present: *P. mcallii* (1 spp.), *P. platyrhinos* (2 spp.), *P. orbiculare* (5 spp.) and *P. douglasii* complex (3 spp.). We note that this interpretation is conservative and based on congruence between mPTP and ABGD analyses. Within *P. orbiculare*, both methods suggest the presence of one species in the northern Sierra Madre Occidental of Chihuahua, one species in the southern Sierra Madre Occidental, one in the northern Sierra Madre Oriental and Central Mexican Plateau, one in the

453 southern Sierra Madre Oriental and adjacent Trans-Mexican Volcanic Belt (Veracruz and
454 Puebla), and one in the central Trans-Mexican Volcanic Belt (Estado de México and Distrito
455 Federal). mPTP also suggested an additional species in the southern Sierra Madre Oriental in
456 Hidalgo (SMOr-H; Fig. 3).
457 Species delimitation scenarios for the *P. douglasii* complex varied among the methods. In
458 our RAxML gene trees, a strongly supported *P. douglasii* clade (bootstrap=93%) was nested
459 deep within *P. hernandesi* sensu lato (s.l.), albeit with weak support (<50% bootstrap; Fig. 3).
460 However, this relationship resulted in non-monophyly of *P. hernandesi* in RAxML gene trees. In
461 contrast, BEAST analyses reconstructed two major clades, one consisting of haplotypes from *P.*
462 *hernandesi* s.l. and one containing haplotypes from *P. douglasii*. This is one reason why the tree-
463 based species delimitation methods failed to differentiate the two species. In all phylogenetic
464 analyses, however, a single divergent haplotype was found that may represent a third species
465 within this clade (PDU71587 from Arizona), a finding that warrants further evaluation in future
466 studies. ABGD further split *P. hernandesi* into two putative species that correspond to the "SER"
467 and "GB/CP" lineages of Zamudio et al. (1997). There was weak evidence to support the
468 recently proposed taxonomy of Montanucci (2015), who resurrected the names *P. brevirostris*
469 and *P. ornatissimum* and described two new taxa (*P. bauri* and *P. diminutum*) based on
470 morphological comparisons. *Phrynosoma bauri* and *P. ornatissimum* were represented in our
471 data by one haplotype each, which were both strongly nested within *P. hernandesi* sensu stricto
472 (s.s.) in all phylogenetic analyses. In addition, nearly all species delimitation analyses (including
473 tree-based and distance-based) suggested that these taxa are not distinct from *P. hernandesi*.
474 Only bPTP and mGMYC analyses suggested that these species may be valid, but these analyses
475 generally tended to split *P. hernandesi* s.l. into a large number of singletons that are not

476 consistent with the recovered genealogies, and thus we view these results as unlikely.
477 *Phrynosoma brevirostris* was recovered as a clade deeply nested within *P. hernandesi* s.s. in all
478 the genealogies. Once again, only bPTP and mGMYC suggested that *P. brevirostris* is a distinct
479 species. Interestingly, mGMYC on the full 220 haplotype data set split *P. brevirostris* into
480 multiple species, whereas mGMYC on the evenly sampled data set lumped all haplotypes into a
481 single entity. Much more taxonomic work remains for the *P. douglasii* complex, and it is likely
482 that nuclear data will be required to resolve the discrepancies between morphology and mtDNA
483 data.
484 Within *P. platyrhinos*, our results suggested between two (ABGD) and four (mPTP)
485 species, with strong support from mPTP. Concordance among results suggests two samples from
486 Yuma Proving Ground in La Paz County, Arizona (LVT9951 and LVT9952) may represent a
487 new species, consistent with previous suggestions (Mulcahy *et al.* 2006; Jezkova *et al.* 2016).
488 mPTP results also suggested that sample LVT818 was a separate taxon as was a clade containing
489 samples DGM478 and DGM481, although the latter clade was weakly placed in alternative
490 positions in all of our ML and Bayesian analyses and unlikely to represent a distinct species.
491 Finally, the majority of analyses suggested that *P. mcallii* consists of a single species, concordant
492 with the low level of genetic diversity in the species (Mulcahy *et al.* 2006). However, mGMYC
493 on the full 368 sequence data set suggested a highly unrealistic estimate of 18 species within *P.*
494 *mcallii* (Supplementary Table S2), further illustrating the propensity of this method to oversplit.
495 We note that although many of the tree-based methods we tested are robust under a
496 variety of scenarios, poorly supported nodes and/or non-monophyly in gene trees may render
497 results unreliable until further data are collected—a broad limitation of the single-locus approach
498 (Esselstyn *et al.* 2012; Fujisawa & Barraclough 2013; Kapli *et al.* 2016). Thus, when delimiting

499 putative species based on single-locus data researchers should consider using both tree- and
500 threshold-based methods like ABGD to account for the shortcomings of each type of method
501 (Hamilton *et al.* 2011; Puillandre *et al.* 2012b). Prior to formal taxonomic changes, results should
502 be subsequently tested with additional types of data and analyses, such as morphological and/or
503 ecological data and multilocus coalescent-based methods (Puillandre *et al.* 2012b; Satler *et al.*
504 2013). This multiple-lines-of-evidence approach can therefore account for gene tree/species tree
505 discordance before formal taxonomic changes are implemented (Fujita *et al.* 2012; Carstens *et*
506 *al.* 2013). Nevertheless, single-locus methods such as mPTP have the potential to quickly and
507 accurately target lineages that warrant additional investigation.

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## Author Contributions

Designed study (CB), performed statistical analyses (CB), contributed to manuscript writing and figure preparation (CB, RWB).

## Data Accessibility

All multiple sequence alignments and gene trees can be found on the Dryad Digital Repository (doi:10.5061/dryad.r7989).

**Table 1**. Number of horned lizard species (*Phrynosoma*) inferred by each single-locus species delimitation method tested on the full data set of unique haplotypes. Results from GMYC and PTP are from genealogies containing 220 ND4 haplotypes (n) with highly heterogeneous sampling intensity of target taxa. All bPTP and mPTP results are from Bayesian MCMC analyses. Confidence intervals for totals are in parentheses. ABGD results are based on the initial partitioning scheme with a maximum intraspecific diversity value of 0.012915 (K80 distances). Singletons were pruned prior to ABGD analysis. Taxonomy for *P. douglasii* complex follows Montanucci (2015) and includes *P. douglasii*, *P. hernandesi*, *P. bauri*, *P. brevirostris*, and *P. ornatissimum.* Also shown are average corrected pairwise distances (substitutions/site) for each species containing multiple haplotypes and Watterson's theta ($\theta = 4N_e\mu$). NA= Not applicable. 'Species matched' refers to the proportion of delimited species matching defined taxonomic species; 'Species lumped' indicates the proportion of taxonomic species classified within a delimited species; 'Species splits' represents the proportion of taxonomic species splits by each delimitation method.

| Taxon | n | Mean Tamura-Nei distance | Watterson's theta | GMYC single | GMYC multiple | bPTP | mPTP | ABGD |
|---|---|---|---|---|---|---|---|---|
| *P. douglasii* complex | 41 | 0.0774 | 115.6936 | 2 | 29 | 26 | 2 | 4 |
| *P. orbiculare* | 34 | 0.0633 | 56.985 | 1 | 14 | 11 | 6 | 5 |
| *P. mcallii* | 29 | 0.0094 | 12.4772 | 1 | 2 | 1 | 1 | 1 |
| *P. platyrhinos** | 111 | 0.0211 | 35.2124 | 1 | 31 | 4 | 4 | 2 |
| *P. asio* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. cornutum* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. coronatum* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. solare* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. taurus* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| Total | 220 | 0.1258¶ | 121.1346† | 10 (7-98)[a] | 81 (69-103)[b] | 52.40 (37-84)[c] | 18 (16-21)[d] | 12 |
| Species matched (percentage of total) | | | | 0.80 | 0.07 | 0.15 | 0.33 | 0.17 |
| Species lumped (percentage of total) | | | | 0.50 | 0.02 | 0.04 | 0.28 | 0.42 |
| Species splits (percentage of total) | | | | 0.20 | 0.95 | 0.74 | 0.67 | 0.83 |
| Match ratio | | | | 0.70 | 0.13 | 0.24 | 0.39 | 0.20 |

*a = likelihood ratio versus null model 10.90506, p = 0.0043; b = likelihood ratio versus null model 23.85364, p < 0.001; c = mean number of species; d = Central Credible Interval; * = ~3x number of haplotypes versus* P. douglasii *complex*, P. orbiculare, P. mcallii; † *= theta for all 220 sequences;* ¶ *= mean Tamura-Nei distance for all 220 sequences.*

**Table 2**. Calculation of the Taxonomic Index of Congruence ($C_{tax}$) and Relative Taxonomic Resolving Power Index ($R_{tax}$) for all species delimitation methods compared based on the full 220 unique haplotype data set.

| | | | $C_{tax}$ | | | Mean $C_{tax}$ | $R_{tax}$ | # species |
|---|---|---|---|---|---|---|---|---|
| | sGMYC | mGMYC | bPTP | mPTP | ABGD | | | |
| sGMYC | - | - | - | - | - | 0.22 | 0.05 | 5 |
| mGMYC | 0.05 | - | - | - | - | 0.22 | 0.96 | 76 |
| bPTP | 0.12 | 0.50 | - | - | - | 0.3 | 0.53 | 42 |
| mPTP | 0.33 | 0.16 | 0.29 | - | - | 0.36 | 0.15 | 13 |
| ABGD | 0.36 | 0.15 | 0.27 | 0.64 | - | 0.36 | 0.14 | 12 |

*Total number of speciation events across all methods (excluding singletons) = 78; Mean $C_{tax}$ = average value for method across all pairwise comparisons. Singletons were excluded from calculations to allow fair comparisons (all singletons were pruned prior to ABGD analyses). See text for additional details.*

**Table 3**. Number of horned lizard species (*Phrynosoma*) inferred by each single-locus species delimitation method tested on the pruned data set. Results from GMYC and PTP are from genealogies containing 149 ND4 haplotypes (n) with approximately even sampling intensity of target taxa. All bPTP and mPTP results are from Bayesian MCMC analyses. Confidence intervals for totals are in parentheses. ABGD results are based on the initial partitioning scheme with a maximum intraspecific diversity value of 0.012915 (K80 distances). Singletons were pruned prior to ABGD analysis. Taxonomy for *P. douglasii* complex follows Montanucci (2015) and includes *P. douglasii*, *P. hernandesi*, *P. bauri*, *P. brevirostris*, and *P. ornatissimum*. Also shown are average corrected pairwise distances (substitutions/site) for each species containing multiple haplotypes and Watterson's theta ($\theta = 4N_e\mu$). NA= Not applicable. 'Species matched' refers to the proportion of delimited species matching defined taxonomic species; 'Species lumped' indicates the proportion of taxonomic species classified within a delimited species; 'Species splits' represents the proportion of taxonomic species splits by each delimitation method.

| Taxon | n | Mean Tamura-Nei distance | Watterson's theta | GMYC single | GMYC multiple | bPTP | mPTP | ABGD |
|---|---|---|---|---|---|---|---|---|
| *P. douglasii* complex | 41 | 0.0774 | 115.6936 | 2 | 11 | 22 | 2 | 4 |
| *P. orbiculare* | 34 | 0.0633 | 56.985 | 1 | 10 | 12 | 6 | 5 |
| *P. mcallii* | 29 | 0.0094 | 12.4772 | 1 | 1 | 1 | 1 | 1 |
| *P. platyrhinos* | 40 | 0.0256 | 34.7945 | 1 | 1 | 3 | 3 | 2 |
| *P. asio* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. cornutum* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. coronatum* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. solare* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| *P. taurus* | 1 | NA | NA | 1 | 1 | 1 | 1 | - |
| Total | 149 | 0.1468¶ | 129.0831† | 10 (3-19)[a] | 28 (9-66)[b] | 46.74 (34-68)[c] | 17 (11-19)[d] | 12 |
| Species matched (percentage of total) | | | | 0.80 | 0.29 | 0.19 | 0.35 | 0.17 |
| Species lumped (percentage of total) | | | | 0.50 | 0.14 | 0.00 | 0.29 | 0.42 |
| Species splits (percentage of total) | | | | 0.20 | 0.71 | 0.73 | 0.65 | 0.83 |
| Match ratio | | | | 0.70 | 0.39 | 0.30 | 0.40 | 0.20 |

*a = likelihood ratio versus null model 10.02172, p = 0.0067; b = likelihood ratio versus null model 12.4399, p = 0.002; c = mean number of species; d = Central Credible Interval*; † *= theta for all 149 sequences;* ¶ *= mean Tamura-Nei distance for all 149 sequences.*

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**Table 4**. Calculation of the Taxonomic Index of Congruence ($C_{tax}$) and Relative Taxonomic Resolving Power Index ($R_{tax}$) for all species delimitation methods compared based on the pruned 149 unique haplotype data set.

| | | | $C_{tax}$ | | | Mean $C_{tax}$ | $R_{tax}$ | # species |
|---|---|---|---|---|---|---|---|---|
| | sGMYC | mGMYC | bPTP | mPTP | ABGD | | | |
| sGMYC | - | - | - | - | - | 0.25 | 0.11 | 5 |
| mGMYC | 0.18 | - | - | - | - | 0.40 | 0.59 | 23 |
| bPTP | 0.11 | 0.59 | - | - | - | 0.33 | 1.00 | 38 |
| mPTP | 0.36 | 0.38 | 0.30 | - | - | 0.43 | 0.30 | 12 |
| ABGD | 0.36 | 0.43 | 0.30 | 0.69 | - | 0.45 | 0.30 | 12 |

*Total number of speciation events across all methods (excluding singletons) = 37; Mean* $C_{tax}$ *= average value for method across all pairwise comparisons. Singletons were excluded from calculations to allow fair comparisons (all singletons were pruned prior to ABGD analyses). See text for additional details.*

**Table 5**. Comparison of the number of delimited horned lizard (*Phrynosoma*) species by the single- (sGMYC) and multiple-threshold (mGMYC) GMYC models based on different methods of annotating node height and sampling regimes. 'Unique' = only unique haplotypes (220); 'All' = all sequences (368). Three ways to summarize node heights on BEAST maximum clade credibility trees were evaluated ('Mean heights', 'Common ancestor heights', 'Median heights'). Values represent the number of ML entities with confidence intervals.

| Data set | Node heights | sGMYC | mGMYC |
|---|---|---|---|
| Unique | Mean heights | 10 (7-98)** | 81 (69-103)*** |
| Unique | Common ancestor heights | 64 (58-72)*** | 66 (61-66)*** |
| Unique | Median heights | 9 (7-69)** | 76 (68-103)*** |
| All | Mean heights | 13 (8-138)* | 164 (157-164)*** |
| All | Common ancestor heights | 79 (76-87)*** | 100 (83-100)*** |
| All | Median heights | 9 (7-16)* | 191 (13-299)*** |

* = P < 0.05; ** = P < 0.005; *** = P < 0.001

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670

671 **Figure Legends**

672

673 **Fig. 1.** Comparison of species delimitation results of horned lizards (*Phrynosoma*) based on
674 analysis of 220 unique ND4 haplotypes and highly heterogeneous taxonomic sampling. Each
675 coloured bar represents a species delimited by each method tested. Gene tree is from a BEAST
676 analysis under a strict clock and constant size coalescent tree prior. Node height was determined
677 using mean heights across the posterior distribution. Node values represent Bayesian posterior
678 probabilities (>0.95) for major clades. sGMYC = single threshold GMYC; mGMYC = multiple
679 threshold GMYC; bPTP = single-rate Poisson Tree Processes model (MCMC analysis of a
680 RAxML gene tree) fitting a single branch length distribution to coalescent events; mPTP =
681 multi-rate Poisson Tree Processes model (MCMC analysis of a RAxML gene tree) fitting
682 multiple branch length distributions to coalescent events across distinct species. ABGD =
683 Automatic Barcode Gap Discovery. Note that singletons (i.e. *P. asio, P. taurus, P. cornutum, P.*
684 *solare, P. coronatum*) were removed prior to ABGD analysis. * = OTUs (PDU71587,
685 PDU71589) clustered together as a single species based on mPTP analysis of a RAxML gene
686 tree, which yielded a slightly different topology than the BEAST genealogy shown. See Fig. 3
687 for additional details. Refer to online version for a full colour representation of figure.

688

689 **Fig. 2.** Comparison of species delimitation results of horned lizards (*Phrynosoma*) based on
690 analysis of 149 unique ND4 haplotypes with relatively even intraspecific sampling. Each
691 coloured bar represents a species delimited by each method tested. Gene tree is from a BEAST
692 analysis under a strict clock and constant size coalescent tree prior. Node height was determined
693 using mean heights across the posterior distribution. Node values represent Bayesian posterior

probabilities (>0.95) for major clades. sGMYC = single threshold GMYC; mGMYC = multiple threshold GMYC; bPTP = single-rate Poisson Tree Processes model (MCMC analysis of a RAxML gene tree) fitting a single branch length distribution to coalescent events; mPTP = multi-rate Poisson Tree Processes model (MCMC analysis of a RAxML gene tree) fitting multiple branch length distributions to coalescent events across distinct species. ABGD = Automatic Barcode Gap Discovery. Note that singletons (i.e. *P. asio*, *P. taurus*, *P. cornutum*, *P. solare*, *P. coronatum*) were removed prior to ABGD analysis. * = OTUs (PDU71587, PDU71589) clustered together as a single species based on mPTP analysis of a RAxML gene tree, which yielded a slightly different topology than the BEAST genealogy shown. See Fig. 3 for additional details. Refer to online version for a full colour representation of figure.

**Fig. 3.** Species delimitation of horned lizards (*Phrynosoma*) based on MCMC mPTP analysis of a RAxML gene tree constructed with 220 ND4 sequences under a GTRGAMMA model. Branches are colour-coded to represent speciation (black) or coalescence (red) events. Values at nodes indicate probability of a speciation event based on mPTP MCMC analysis (first value) and maximum likelihood bootstrap proportions using the autoMRE bootstopping criterion in RAxML (second value). YPG = samples LVT9951 and LVT9952 from Yuma Proving Ground, Arizona. SMOcN = northern Sierra Madre Occidental; SMOcS = southern Sierra Madre Occidental; SMOrS = southern Sierra Madre Oriental; TMVB = Trans-Mexican Volcanic Belt; TMVBc = central Trans-Mexican Volcanic Belt; SMOr-H = Sierra Madre Oriental-Hidalgo; SMOrN = northern Sierra Madre Oriental; CMP = Central Mexican Plateau. *PSS* = *Phrynosoma* singleton species (*P. cornutum*, *P. coronatum*, *P. solare*, *P. taurus*). *P. hernandesi* s.l. = *P. hernandesi*, *P.*

716 *bauri*, *P. brevirostris*, *P. ornatissimum*. Horned lizard shown is a *P. orbiculare* from Coahuila,

717 Mexico. Refer to online version for a full colour representation of figure.