Bridging multilocus species delimitation and DNA barcoding through target enrichment of UCEs: a case study with Mexican highland frogs

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Abstract

Species delimitation has been divided by two approaches: DNA barcoding that focuses on standardization of the genetic marker and multilocus methods that place a premium on genomic coverage and conceptual rigor in modeling the divergence process. Most multilocus methods fail as barcodes, however, because few assay the same marker set and are therefore not readily comparable across studies and databases. We introduce ultraconserved elements (UCEs) as potential genomic barcodes that allow rigorous species delimitation and a bridge to DNA barcoding database to allow both rigorous species delimitation and standardized identification of delimited taxa. UCEs query thousands of loci across the nuclear genome in way that is replicable across broad taxonomic groups (i.e., vertebrates). We apply UCEs to species delimitation in a species complex of frogs found in the Mexican Highlands. *Sarcohyla* contains 24 described species, many of which are critically endangered and known only from their type localities. Evidence suggests that one broadly distributed member of the genus, *S. bistincta*, might contain multiple species. We generated data from 1,891 UCEs, which contained 1,742 informative SNPs for *S. bistincta* and closely related species. We also captured mitochondrial genomes for most samples as off-target bycatch of the UCE enrichment process. Phylogenies from UCEs and mtDNA agreed in many ways, but differed in that mtDNA suggested a more complex evolutionary history perhaps influenced by reticulate processes. The species delimitation method we used identified eight putative species (which we call lineages pending further study) within *S. bistincta*. Being able to compare linked mtDNA data to existing sequences on Genbank allowed us to identify one of these lineages nested within *S. bistincta* as an already-described species, *S. pentheter*. Another lineage nested within *S. bistincta* is currently being described as a new species (referred to here as *sp. nov.*). The remaining six lineages fell into two non-sister clades, one containing the core *S. bistincta* mostly in Oaxaca and Guerrero, and another in the Transvolcanic Belt. The latter clade, at 10% divergence in mtDNA and paraphyletic with respect to other *S. bistincta*, is a clear candidate for species status. Our study

demonstrates not only that UCEs can be used as effective genomic DNA barcodes, but that combining multilocus genomic data with mtDNA is a powerful approach for both delimiting species and identifying them in poorly described and phenotypically challenging groups.

Keywords: ultraconserved elements, genomics, population genetics, phylogeography, phylogenetics, systematics, species limits, Hylinae

Introduction

Species are the fundamental biological unit and their identification and description is critical to ecology, evolution, and conservation (DeSalle and Amato 2004; Isaac et al. 2004; Mace 2004; Wiens 2007). Identifying species has always been a challenging issue in biology, especially when lineages are near the cusp of speciation (De Queiroz 2007). Yet, for the vast majority of researchers working on poorly described groups under conservation threat, conceptual debates over identifying the precise moment when one species becomes two have been superseded by the practical need for methods to quickly assess and identify the basic units of biodiversity (Hey et al. 2003; Tautz et al. 2003; Bickford et al. 2007). As a result, species delimitation methods have multiplied over the last ten years, especially those using DNA data (Pons et al. 2006; Knowles and Carstens 2007; O'Meara 2009; Yang and Rannala 2010; Fujita et al. 2012; Leaché et al. 2014).

While there are now a wide variety of methods that employ different DNA sources and algorithms to delimit species, these methods can be broadly placed into two frameworks. DNA barcoding focuses on species delimitation through the application of thresholds of sequence divergence, usually at a single mitochondrial DNA (Hebert et al. 2003) or chloroplast locus (Taberlet et al. 2007). DNA barcoding is relatively cheap and easy to implement, and by focusing on a single marker, a large database has been built by researchers, which allows for comparison of newly delimited species to already-identified species that have associated DNA. With its large user base, DNA barcoding has made substantial contributions to taxonomy and conservation of poorly known groups and geographic areas (Hebert et al. 2004; Ward et al. 2005; Witt et al. 2006; Lahaye et al. 2008). The very simplicity of DNA barcoding has made it controversial since its inception (Moritz and Cicero 2004; DeSalle et al. 2005; Ebach and Holdrege 2005; Will et al. 2005). Critics argue that it oversimplifies complex divergence processes with arbitrary thresholds that vary from one organismal group to another. Also, the history of a single gene will often fail to reflect the evolutionary history of populations and

species (Edwards and Bensch 2009; Galtier et al. 2009), thereby misleading delimitation efforts in some cases.

Multilocus species delimitation, on the other hand, addresses many of the perceived weaknesses of DNA barcoding by incorporating a process of gene inheritance (coalescence) and by using information from many genomic locations to more accurately reflect the history of lineages and how they diverged. Multilocus species delimitation, however, has been criticized on the practical grounds that large amounts of genomic data are usually not needed to delimit species, and the marginal gains more loci might afford are outstripped by the computational burden of modeling complex speciation processes with large data sets (Collins and Cruickshank 2014). Another key criticism, which we focus on specifically, is that the lack of standardized multilocus marker sets inhibits the broad adoption of multilocus delimitation methods because newly delimited taxa cannot be readily compared to an existing database to determine if the putative new species have already been identified and named and, if not, how they relate to other species in an evolutionary context.

Our goal is to introduce ultraconserved elements (UCEs) as a candidate for a standardized genomic marker set for multilocus species delimitation and identification and to show how this kind of multilocus data can be even more powerful when linked to DNA barcoding-type data (i.e., mtDNA). UCEs are appealing as genomic barcodes because the same loci are found across major branches of the tree of life, where they act as anchors for variable DNA in flanking regions (Faircloth et al. 2012). For instance, it is possible to capture the same set of 1000 or more UCEs across all mammals (McCormack et al. 2012), all reptiles (Crawford et al. 2012), or hundreds of UCEs across arachnid lineages separated by hundreds of millions of years (Starrett et al. 2016). While the power of UCEs for deep-level systematics is clear, their utility at shallower scales around the species level has only recently been demonstrated (Smith et al. 2014; McCormack et al. 2016; Zarza et al. 2016). An added benefit of the UCE enrichment process (and all so-called "sequence capture" methods) is that whole mtDNA genomes are often

97 captured as off-target "bycatch" (do Amaral et al. 2015), allowing for each individual to have
98 associated nuclear and mtDNA data (e.g., Zarza et al. 2016).

99 We apply UCEs as genomic barcodes to species delimitation in treefrogs in the genus
100 *Sarcohyla* in the northern Mexican Highlands. This genus, which was recently split from
101 *Plectrohyla* to reflect those species west of the Isthmus of Tehuantepec (Duellman et al. 2016),
102 contains 24 described species, many of them critically endangered and many that have never
103 been seen after their original discovery (references compiled in Stuart et al. 2008). Some species
104 are thought to be in serious decline or extinct (Lips et al. 2004). Species limits and relationships
105 within *Sarcohyla* are not well known because few species have associated DNA sequences, and
106 the lack of voucher material means that the tadpole stages and geographic ranges for each
107 species are not well characterized (Duellman 2001; Faivovich et al. 2009; Duellman et al. 2016).
108 We focus specifically on the *Sarcohyla bistincta* complex and closely related species. *Sarcohyla*
109 *bistincta* is one of the more broadly distributed members of the genus and might comprise
110 multiple species. Especially in its early developmental stages, *S. bistincta* looks similar to closely
111 related species, meaning that identification from tadpoles can be challenging. Thus, *Sarcohyla*
112 frogs present a good test case of whether UCE data are effective at delimiting species, and how
113 linking UCE and mtDNA data is a powerful approach for helping identify delimited taxa by
114 comparison to existing genetic databases.

115 **Methods**

116 *Sampling and Ingroup Determination*

117 MK collected tadpoles from January to June 2004 across most of the range where *Sarcohyla*
118 *bistincta* are known to exist (Duellman 2001) in the Transvolcanic Belt of Michoacán, Morelos,
119 and the state of México, the Sierra Madre del Sur of Guerrero, and the highlands of Oaxaca
120 stretching into Puebla and Veracruz (Fig. 1; Table 1). Unsampled parts of the *S. bistincta* range
121 include the far west Transvolcanic Belt in Michoacán and Jalisco, the far northwest in the Sierra

122 Madre Occidental (Nayarit, Durango, and Sinaloa), and the far northeast in Hidalgo (see Fig. S1
123 for sampled and unsampled locations and known ranges of all *Sarcohyla* species). Tadpoles
124 were targeted to improve sampling efficiency. After collection of many tadpoles from a
125 sampling location with a dip net, they were separated by species based on morphology to the
126 extent possible and reared to subadults in the laboratory prior to vouchering. Species
127 identification was based on the most recent diagnosis of *S. bistincta* and other closely related
128 species (Duellman 2001). One tadpole was chosen for the tissue voucher, while the other
129 individuals became physical vouchers with museum catalog numbers. Thus, we provide both
130 field numbers and catalog numbers in Table 1 to provide a link to both the exact genetic
131 material and the associated voucher for that genotype. Before limiting our taxonomic sampling
132 to 38 *S. bistincta* samples and two close outgroups, we ran preliminary phylogenetic analyses
133 also including samples from 45 *Sarcohyla* individuals and the outgroup genus *Exerodonta* to
134 ensure we had correctly identified the ingroup and closest outgroups (Table S1).

135 *Sequence capture and next-generation sequencing*

136 We extracted genomic DNA from tissue using a Qiagen (Valencia, CA) DNAeasy Blood and
137 Tissue extraction kit. We visualized extractions on an agarose gel to ensure fragments were
138 larger than 200 base pairs (bp) and quantified the resulting double-stranded DNA using a Qubit
139 2.0 Fluorometer (Carlsbad, CA). For each sample, we sheared 100 μl of 20ng/μl concentration
140 DNA to a size distribution with its peak between 400 and 600 bp using a Bioruptor
141 ultrasonicator (Diagenode). We prepared libraries for each sheared sample with a KAPA
142 (Boston, MA) LTP library preparation kit for the Illumina platform, attaching custom indexing
143 tags (Faircloth and Glenn 2012) to each sample to allow sample pooling.

144 We enriched pools of eight samples using a set of synthetic RNA probes that target 5,060
145 tetrapod UCEs (MYbaits_Tetrapods-UCE-5K kit, Mycroarray) following the standard UCE
146 enrichment protocol (Faircloth et al. 2012) with one modification. Amphibians have large and
147 variable genome sizes with a high percentage of repetitive DNA (Olmo 1991). While we do not

148 have information about the genome size and composition of *Sarcohyla* specifically, we wanted to
149 decrease the potential risk of the probes hybridizing to repetitive elements (McCartney-Melstad
150 et al. 2016). We thus increased by 6X the amount of the Cot-1 blocker, a synthetic DNA derived
151 from chicken that binds to repetitive regions. After enrichment and recovery PCR, we verified
152 the library size range with an Agilent 2100 Bioanalyzer (Palo Alto, CA). We quantified the
153 enriched pools using qPCR and combined them in equimolar ratios before sequencing on an
154 Illumina HiSeq 2000 lane (100-bp paired-end cycle) at the University of California Santa Cruz
155 Genome Technology Center.

156 *Bioinformatics of next-generation sequencing data*

157 We demultiplexed the Illumina raw reads and converted them to FASTQ format with the
158 program bcl2fastq v.1.8.4 (Illumina, Inc.). We created quality control reports with FASTQC
159 v0.10.1 to detect overrepresented sequences and adapter contamination. To eliminate adapter
160 contamination and low quality bases, we trimmed the FASTQC output using illumiprocessor
161 (Faircloth 2012). We trimmed and assembled these reads into contigs with Trinity (Haas et al.
162 2013) and ABySS (Simpson et al. 2009), both of which are built into the PHYLUCE pipeline
163 (Faircloth 2015). PHYLUCE uses LASTZ (Harris 2007) to align all assembled contigs to UCE
164 probe sequences in order to isolate only UCE contigs and to identify and eliminate any paralogs
165 (MATCH_CONTIGS_TO_PROBES script). We then turned this filtered set of UCE contigs into a
166 data matrix describing which UCE loci are present in which samples (GET_MATCH_COUNTS
167 script).

168 *Phylogenetic trees from concatenated UCE data*

169 We extracted contigs into a single FASTA file (GET_FASTAS_FROM_MATCH_COUNTS script)
170 and aligned the output for each locus (SEQCAP_ALIGN script) using MAFFT (Katoh et al.
171 2005). We then required that 75% of the samples needed to have data for a given locus to be
172 included in the final concatenated matrix (GET_LOCI_WITH_MIN_TAXA script). We

173 converted the NEXUS file into PHYLIP format (FORMAT_NEXUS_FILES_FOR_RAxML script)
174 and constructed a maximum-likelihood (ML) tree in RAxML v8.0.19 (Stamatakis 2014) under
175 the GTRGAMMA model of evolution with 100 bootstrap searches, followed by a search for the
176 tree with the highest likelihood.

177 *Mitochondrial DNA assembly and analysis*

178 We identified and assembled mtDNA genomes from off-target, trimmed Illumina reads using
179 the reference genome of a closely related species, *Hyla annectans* (Genebank accession number
180 KM271781; Ye et al. 2016). We used MITObim 1.7 (Hahn et al. 2013), a Perl wrapper for MIRA
181 4.0.2 (Chevreux et al. 1999), that takes a baiting and iterative mapping approach for assembly.
182 We conducted *de novo* annotation of the assembled mtDNA regions with the MITOchondrial
183 genome annotation Server, MITOS (Bernt et al. 2013). We selected for phylogenetic analysis
184 only those individual genomes with MIRA quality score grater than 30. We aligned each
185 protein-coding region separately in Geneious vR8 (Kearse et al. 2012) using the integrated
186 MUSCLE (Edgar 2004) plugin. We corrected the alignments manually when necessary and
187 constructed a concatenated mtDNA matrix, which we also ran in RAxML v8.0.19.

188 Additionally, we melded our mtDNA data with existing *Sarcohyla* and *Plectrohyla*
189 mtDNA data on Genbank to determine whether any of the lineages we uncovered in *S. bistincta*
190 relate to already-described species. We determined that *cytochrome b* is the best-represented on
191 Genbank in this group. We downloaded all existing *cytochrome b* sequences from *Sarcohyla* and
192 *Plectrohyla* taxa. We combined these sequences with those from a subset of our *S. bistincta*
193 samples, choosing the individual with the most raw reads from each major genetic lineage in
194 the UCE tree. We used *Exerodonta* as an outgroup. Here, we aligned the trimmed, filtered reads
195 for each individual to a *Sarcohyla cytochrome b* reference sequence. We formed a consensus
196 sequence for each individual from the mapped reads. We then created an alignment and
197 generated a phylogeny using BEAST v2.4.2 (Bouckaert et al. 2014). Because we were particularly

198 interested in how *S. pentheter* related to our lineages, but there are no *cytochrome b* sequences for
199 *S. pentheter* on Genbank, we also compared our samples to Genbank samples using the *16S*
200 gene.

201 *Calling SNPs from UCE loci*

202 We called SNPs from UCE loci so that we could run genetic clustering tests and infer a species
203 tree. Calling SNPs requires a reference sequence, and we chose the sample with the most UCE
204 contigs recovered within the ingroup (UMMZ 239727). We then used BWA (Li and Durbin
205 2009) to map the reads of each sample to this reference. We used SAMtools (Li et al. 2009) to
206 sort the reads, and Picard (available at http://broadinstitute.github.io/picard) to identify and
207 remove PCR duplicates. We realigned the mapped reads to minimize mismatched bases due to
208 indels, and we removed indels using the Genome Analysis Toolkit 3.2 (GATK; McKenna et al.
209 2010) and a custom script (indelrealigner.sh), as suggested by the Best Practices workflow
210 (DePristo et al. 2011; van der Auwera et al. 2013).

211 There is no SNP database available for treefrogs, so we followed best practices for base
212 recalibration for non-model organisms suggested by GATK (McKenna et al. 2010). This consists
213 of (1) doing an initial round of calling SNPs on the original, uncalibrated data, (2) selecting the
214 SNPs with the highest confidence (a minimum emission and call quality of 40 or more), and (3)
215 using these SNPs as the database of known SNPs. We executed four rounds of base
216 recalibration on the original data to filter out systematic error using a custom script (genotype-
217 recal.sh). We called genotypes on the last recalibrated BAM file. We used vcf-tools (Danecek et
218 al. 2011) to select one SNP per UCE and produce two data sets, one allowing 25% missing data
219 for STRUCTURE v 2.3.4 (Pritchard et al. 2000), and one with no missing data, which is a
220 requirement for SNAPP (Bryant et al. 2012) species tree analysis, implemented in BEAST v2.2.1
221 (Bouckaert et al. 2014).

222 *STRUCTURE analyses*

223 The species delimitation method we used requires some *a priori* information about possible
224 clustering of individuals into species. We used STRUCTURE v2.3.4 as an unbiased way to
225 assess the limits of fine-scale genetic structure in our data. Thus, our intent was not to
226 determine the single most likely number of genetic clusters. Rather, our goal was to determine
227 the maximum number of genetic clusters in our data. We began by analyzing all individuals of
228 *S. bistincta* plus two outgroup species *S. chryses* and *S. hazelae* under *K*=4, reasoning that this
229 would likely split out the two outgroups as well as revealing one division within the ingroup.
230 After this, each identified genetic cluster was further analyzed at *K*=2 until no further structure
231 was evident.

232 *SNAPP tree and species delimitation*

233 We generated a species tree from the SNP matrix using SNAPP 1.1.10 (Bryant et al. 2012). This
234 analysis included all putative *S. bistincta* samples and one outgroup, *S. chryses.* For this run, we
235 made no a priori assumptions about how individuals grouped into species and allowed each
236 individual to be considered its own "species" (i.e., terminal tip). We ran two instances of
237 SNAPP for seven million generations using default priors. We combined tree and parameter
238 files from both runs with LogCombiner 2.1.3 and displayed the full set of likely species trees
239 with Densitree v2.2.1 (Bouckaert et al. 2014).

240 We then applied Bayes Factor Species Delimitation (BFD; Grummer et al. 2014; Leaché et
241 al. 2014) to SNAPP analyses to determine the most likely number of species. We used the
242 Structure results to generate hypotheses for how individuals might cluster into species. We
243 tested five scenarios with BFD that split or lumped populations identified in the Structure
244 analysis, including the hypothesis that all putative *S. bistincta* group into a single species. We
245 assessed which scenario (2, 6, 7, 9, or 10 species) was favored by comparing Bayes factors using
246 marginal likelihoods from the SNAPP analysis.

247 **Results**

248 *NGS summary statistics*

249 Detailed summary statistics for each of the 38 ingroup samples and two outgroups are
250 described in Table 1. ABySS produced longer contigs than Trinity, and a higher number of UCE
251 loci, so we used ABYSS contigs in all downstream analyses. Reads per sample ranged from
252 17,052 to 3,423,330 with an average of 1,185,165 reads. The number of identified UCEs ranged
253 from 381 to 2,444 with an average of 1,976 UCEs. The mean length of individual UCE loci per
254 individual ranged from 222 to 717 bp with an average of 522 bp. On average, 18% of the
255 assembled contigs corresponded to unique UCE loci.

256 For SNP calling, across 40 samples of *S. bistincta* and outgroups, 9% of the trimmed
257 reads mapped to our designated reference individual. The SNP read depth ranged from 2.4 to
258 35.0 with an average depth of 21.2. The recalibration and quality control steps resulted in an
259 initial matrix of 16,578 SNPs. After removing non-biallelic loci, selecting one SNP for every
260 UCE, and allowing 25% missing data, there were 1,742 SNPs in the STRUCTURE data set, while
261 the 100% complete data matrix for SNAPP contained 399 SNPs.

262 *UCE phylogeny from concatenated data*

263 Our more taxonomically inclusive data set with all available *Sarcohyla* and outgroup *Exerodonta*
264 *xera* (Table S1) contained 1,866 UCE loci and 1,030,450 bp for a concatenated analysis. The
265 resulting ML tree (Fig. S2) showed strong support for monophyly of *Sarcohyla*, and identified *S.*
266 *arborescandens* and *S. cyclada* as sister species that together form a clade sister to the rest of the
267 *Sarcohyla* included in the study. We thus limited further analyses to a smaller data set of 40
268 samples with *S. hazelae* as the outgroup (Table 1). This focal data set contained 1,891 UCE loci
269 and 1,038,600 bp. The ML tree of these 40 samples found strong support for many clades within
270 the species currently described as *S. bistincta*, conforming to distinct geographic areas (Fig. 2a).
271 In brief, there were three clades on relatively long branches: one distributed across the

272 Transvolcanic Belt (blue clade); one inhabiting two disjunct areas along the coastal slopes of the
273 Sierra Madre del Sur in Guerrero and Oaxaca (gray clade); and one broadly distributed in the
274 Sierra Madre del Sur (red and pink clades), the Oaxaca Highlands (yellow + orange clade), and
275 one individual in the southern portion of the Transvolcanic Belt (purple). One individual that
276 nested within *S. bistincta* was labeled as a different species, *S. mykter*, from Guerrero. We
277 suspect that this sample was mislabeled and is actually a duplicate of an *S. bistincta* sample
278 already included in the study because their field numbers are similar (last two digits
279 transposed) and the two samples grouped together in all analyses. We have left this sample in
280 all analyses, but labeled it as a duplicate of *S. bistincta* UMMZ 239749.

281 *mtDNA tree*

282 Our final concatenated mtDNA matrix was 11,269 base pairs including gaps, as coverage of the
283 mtDNA genome varied from sample to sample in accordance with the non-targeted nature of
284 the DNA collection (Table 1). Relationships in the ML tree (Fig. 2b) among the 29 individuals
285 with high quality scores were similar to the concatenated UCE tree with two key differences
286 within the broadly distributed clade in Guerrero and Oaxaca: (1) in the mtDNA tree,
287 individuals from Eastern and Western Guerrero (pink and red) formed a clade, whereas they
288 were more divergent in the UCE tree; (2) in the mtDNA tree, individual UMMZ 239731 (purple)
289 was still on a relatively long branch, but that branch was nested within the Guerrero clade
290 described above instead of being sister to a much more expansive clade, as in the UCE tree.

291 *Structure analysis*

292 The first run of STRUCTURE at K=4 split the two outgroup species into distinct clusters and
293 split the remaining individuals into two clusters. Further analysis of each cluster at K=2
294 revealed nine genetic clusters (Fig. 1b), which are concordant with clades observed in the UCE
295 and mtDNA phylogeny, as well as with the delimited species (lineages) described below.

296 *SNAPP species tree and species delimitation*

297 The cloudogram of posterior species trees obtained with SNAPP (Fig. 3) is consistent with the
298 genetic clusters in the Structure analysis and with relationships in phylogenies resulting from
299 the UCE and mtDNA concatenated analyses. With respect to the discrepancies between the two,
300 the SNAPP tree agreed with the mtDNA tree that eastern and western Guerrero individuals
301 form a clade, but agreed with UCE tree in the placement of individual UMMZ 239731. Bayes
302 factor analysis of SNAPP runs with varying number of species suggested the best model
303 consists of nine species. The model with nine species was supported by a factor of 10 over other
304 models with more and fewer species (Table 2). These putative species are labeled as lineages in
305 Fig. 3 and elsewhere, pending further study.

306 *mtDNA phylogeny combining new data with Genbank sequences*

307 Using 16S sequences, we determined that our delimited Lineage 8 matched an *S. pentheter*
308 sequence on Genbank. Lineage 8 individual UMMZ 239772 had one of the lowest read counts of
309 any samples and very few mtDNA reads. However, five reads mapped to 16S covering 421 bp
310 of the 681 bp reference sequence (Genbank *S. pentheter* accession number DQ055825). Over this
311 stretch, UMMZ 239772 was identical to the reference. As a point of comparison, UMMZ 239679
312 (a member of the blue *S. bistincta* Lineage 1 in the Transvolcanic Belt) had 70 differences across
313 the 681 bp (10.3% divergence). This DNA identification of UMMZ 239772 as *S. pentheter* was
314 confirmed by re-examining the subadult specimen.

315 After confirming UMMZ 239679 as *S. pentheter*, we generated a Bayesian tree of
316 *cytochrome b* combining our samples with Genbank sequences (Fig. 4). This tree revealed not
317 only that Lineage 8 (*S. pentheter*) was nested within the current *S. bistincta*, but so was another
318 species not included in our sampling, *S. calthula*. Relationships within *S. bistincta* were
319 otherwise similar to the more taxonomically inclusive mtDNA phylogeny. For example UMMZ
320 239731 (purple) was still nested within the Guerrero samples (red and pink). The combined
321 phylogeny also helped clarify relationships outside of *S. bistincta* by supporting *S. chryses* + *S.*
322 *mykter* to be sister to the *S. bistincta* + *S. pentheter* + *S. calthula* complex.

323 **Discussion**

324 One limitation of current multilocus species delimitation methods is the lack of a standardized
325 marker set that would allow for data from delimited species to be combined with already-
326 existing data both to identify species and place them into broader evolutionary context (Collins
327 and Cruickshank 2014; Coissac et al. 2016). Here, we show that UCEs not only are a good
328 candidate for this standardized "genomic barcode" for multilocus species delimitation, but also
329 that they are especially powerful when they can be linked to mtDNA data, which provides a
330 bridge to well-developed mtDNA databases for species identification. Having linked mtDNA
331 data allowed us to query our lineages, delimited with multilocus genomic data, against
332 GenBank to see if any of them corresponded to already-described species and to place them in
333 as broad a phylogenetic context as possible. Doing so revealed that *S. bistincta* is paraphyletic,
334 with two already-described species and one species in the process of description nested within
335 its current species limits (Fig. 4). Our approach of delimiting species with multilocus data and
336 identifying them with the help of mtDNA data will be especially powerful in groups where
337 geographic ranges and subadult phenotypes are not well described or where species are
338 phenotypically cryptic.

339 It was not our intention with this study to compare species delimitation methods, as this
340 has been examined elsewhere (Camargo et al. 2012; Carstens et al. 2013; Miralles and Vences
341 2013). We employed one, rather new delimitation method (Leaché et al. 2014), but there are
342 others that would surely suggest somewhat higher or lower numbers of delimited species. We
343 are not advocating that species be delimited and described solely based on DNA. There are
344 cases where both DNA barcoding and multilocus species delimitation will under-split species
345 (Hime et al. 2016) and cases where coalescent methods will confound population structure with
346 speciation, thereby recommending over-splitting species (Sukumaran and Knowles 2017). We
347 discuss the results of our delimitation analysis below, but by calling the putative species
348 "lineages" we acknowledge that new species descriptions should await integrative taxonomy

349 including, at minimum, careful study of phenotypic differences in museum specimens. Finally,
350 although we discuss discrepancies between the mtDNA and nuclear DNA phylogenies below,
351 we did not set out explicitly to compare multilocus species delimitation to DNA barcoding. As
352 mentioned, we feel our results demonstrate how mtDNA and nuclear data are most powerful
353 when used together. Despite a few notable differences, discussed below, our UCE and mtDNA
354 phylogenies are broadly congruent, suggesting that, if we carried out such a comparison, our
355 results would be in line with prior work showing that multilocus species delimitation and DNA
356 barcoding produce similar results (Collins and Cruickshank 2014; Dowton et al. 2014).

357 Rather, in addition to showing the power of linking nuclear genomic data with mtDNA
358 data, another goal of our study was to demonstrate that UCEs adhere to the concept of a
359 "extended genomic barcode" (sensu Coissac et al. 2016) and produce data sets with enough
360 resolving power for the necessarily recent divergences involved in species delimitation.
361 Another recent study applied UCEs to species delimitation in two other frog genera,
362 *Melanophryniscus* and *Brachycephalus* (Pie et al. 2017). This study used a more restrictive cut-off
363 for including loci and produced 800 loci (in a 100% complete data matrix) with sufficient
364 resolving power for species delimitation, compared to the roughly 1,900 loci (in a 75% complete
365 data matrix) in our study. UCE probe sets are now available for many taxonomic groups
366 (Faircloth et al. 2013; Faircloth et al. 2015; Starrett et al. 2016). They capture a discrete and
367 replicable portion of the genome, in this case a set of around 2,000 loci in frogs (from a larger set
368 of ~5,000 vertebrate loci) that query approximately 1,000,000 base pairs, or 0.02% of the frog
369 genome. The replicable nature of UCEs sets them apart from other types of genomic markers,
370 like RAD loci, which can vary from experiment to experiment (DaCosta and Sorenson 2014) and
371 find fewer orthologs with increasing phylogenetic distance (Cruaud et al. 2014).

372 Bayes factor species delimitation identified nine lineages within the current *S. bistincta*
373 and a close outgroup, *S. chryses*. Apart from *S. chryses*, which was correctly delimited, the other
374 eight lineages fell into three clades (Fig. 3). One lineage turned out to be the already-described

375 *S. pentheter* of Oaxaca, which we discovered by querying its 16S mtDNA data against existing
376 Genbank sequences and reexamining the subadult specimen. Thus, both *S. pentheter* and *S.*
377 *calthula* are nested within the currently-described *S. bistincta*. Below, we describe the three
378 clades containing the eight lineages. Where warranted, formal species descriptions will follow
379 in later publications.

380 **Clade 1** – Members of this clade are found in the Transvolcanic Belt of central Mexico. They are
381 sister to the rest of the current *S. bistincta* + *S. pentheter* + *S. calthula*. This clade is a
382 clear candidate for species recognition and might itself contain multiple species, as
383 delimited below. Based on geography, unsampled populations of *S. bistincta* in the
384 Sierra Madre Occidental (Fig. S1) are most likely related to this clade, but should be
385 included in future studies, as they might comprise their own lineage.

386 Lineage 1 (light blue in Fig. 1) – Michoacán to western Mexico state. There is fine-scale
387 genetic structure across this range, and the presence of a geographic and genetic
388 intermediate hints at continuity of gene flow along the distribution from sites 1 to 4
389 in Figure 1. Some populations in far western Michoacán (Fig. S1) are as yet
390 unsampled and could reveal further genetic structure.

391 Lineage 2 (dark blue in Fig. 1) – Morelos. Further sampling between sites 4 and 5 could
392 help determine whether the genetic distinctness of this individual is a true genetic
393 discontinuity or the result of a sampling gap.

394 **Clade 2** – Members of this clade form the core *S. bistincta* and occur from Guerrero to Puebla
395 and Veracruz and south through Oaxaca.

396 Lineage 3 (red in Fig. 1) – Central and eastern Guerrero. Members of this lineage are
397 distinct from the pink lineage below and are monophyletic in the UCE trees (though
398 not in the mtDNA tree). Further sampling in between site 6 and site 7 would clarify

399 whether the genetic discontinuity between Lineages 3 and 4 results from a sampling
400 gap.

401 Lineage 4 (pink in Fig. 1) – Western Guerrero. This lineage is distinct and monophyletic
402 in all trees, although only a few individuals were sampled only from one locality.

403 Lineage 5 (orange and yellow in Fig. 1) – Puebla, Veracruz, and Oaxaca. The geographic
404 range of this group contains the type locality for *S. bistincta*. Central and southern
405 Oaxaca individuals (orange) are genetically distinct from individuals to the north
406 (yellow). One genetic intermediate in central Oaxaca suggests genetic continuity
407 across this range. One individual from southern Oaxaca (site 25 in Fig. 1) has an
408 unexpectedly northern genetic profile given its geographic location, suggesting a
409 more complex geographic structuring within this group than a simple cline from
410 north to south. An unsampled northern population of *S. bistincta* in Hidalgo is most
411 likely related to this lineage, and should be included in future studies.

412 Lineage 6 (purple in Fig. 1) – far northern Guerrero. Represented by a single individual,
413 this lineage is distinct and on a relatively long branch. However, in the mtDNA
414 tree, this individual is nested within Lineages 4 and 5 above. Sampling more
415 individuals is needed to determine how distinct this lineage might be.

416 **Clade 3** – Pacific slope of Guerrero and Oaxaca. Sister to Clade 2, members of this clade
417 comprise two species, one already described and one currently being described.

418 Lineage 7 (dark gray in Fig. 1) – Pacific slope of Guerrero. This lineage was being
419 described as a new species (we call it *sp. nov.*) on the basis of phenotypic differences
420 before this genetic study was begun. Thus, our analysis lends support to species
421 status for this lineage.

422 Lineage 8 (light gray in Fig. 1) – Pacific slope of Oaxaca. Genbank 16S mtDNA data and
423 the phenotype of the voucher reveal this lineage to be the already-described species,
424 *S. pentheter*, of southern and central Oaxaca.

425 The three clades and nearly all of the lineages were distinct in the mtDNA tree as well as
426 the UCE tree (Fig. 2). The mtDNA tree, however, supports a more complicated history for
427 Lineages 3, 4, and 6 in Clade 1 from Guerrero. It is unclear why UCE and mtDNA results
428 differed in this regard, but some reticulate processes might have influenced the mtDNA
429 genomes of these lineages, perhaps, given their close geographic proximity, mtDNA capture of
430 one lineage by another through ancient hybridization (e.g., Bryson Jr et al. 2010).

431 The phylogeny combining our mtDNA data with Genbank data (Fig. 4) is the most
432 taxonomically complete phylogeny of *Sarcohyla* and its sister genus *Plectrohyla* to date and
433 demonstrates the power of linking multilocus species delimitation with mtDNA data by
434 allowing delimited taxa to be placed in a broader context. This revealed that two already-
435 described species are nested within the current *S. bistincta*, lending support to species status of
436 the divergent Clade 1 from the Transvolcanic Belt (which was ~10% divergent in mtDNA from
437 *S. pentheter*). Additional insights afforded by the combined data mtDNA tree include support
438 for a previously hypothesized close relationship between *S. hazelae* and *S. thorectes* (Faivovich et
439 al. 2009), and a sister relationship between *S. mykter* and *S. chryses*. As the genus *Sarcohyla* is
440 very poorly represented by voucher specimens and DNA sequences (Fig. S1), a complete
441 understanding of the history of this genus must await more complete taxonomic and genomic
442 sampling. Unfortunately, there appear to be some microendemic *Sarcohyla* that might have
443 already gone extinct (Lips et al. 2004), especially in the Oaxacan highlands, although recent
444 resurveys give some cause for hope of rediscoveries (Delia et al. 2013).

445 By introducing UCEs as extended DNA barcodes, we are not advocating abandoning
446 traditional mtDNA barcoding and moving solely toward multilocus species delimitation. Even

447 though next-generation sequencing techniques are becoming more standardized and
448 widespread, they still pose a significant financial barrier and require more technical expertise
449 and equipment compared to mtDNA sequencing. However, there is no reason that nuclear
450 genomic DNA barcodes like UCEs cannot develop their own user community and databases in
451 parallel with mtDNA barcoding. As our study shows, mtDNA barcoding and multilocus
452 species delimitation are powerful when deployed together. Where they agree in this study is
453 that there is significant frog diversity in the Mexican Highlands that is currently unrecognized.
454 To the extent that these distinct lineages can be rigorously delimited and compared against
455 existing species, they can be added to biodiversity lists, allowing for their recognition and
456 protection.

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Tables

Table 1. Specimen information and summary statistics for *Sarcohyla bistincta* and closely related species.

Map Number[1]	Field Number[2]	Catalog Number[3]	Current Taxonomy	State	Latitude	Longitude	Trimmed Paired Reads	UCEs	Average UCE Length	SNP Read Depth	mtDNA Reads	mtDNA Average Coverage	mtDNA Average Quality
1	MK 618	UMMZ 239796	*S. bistincta*	MICHOACAN	19.7911	-100.6605	1,889,670	2,323	529.7	31.8	3,381	20.8	86
2	MK 627-31	UMMZ 239683	*S. bistincta*	MICHOACAN	19.4266	-102.0736	1,933,019	2,254	370.9	21.6	3,415	20.8	85
3	MK 666	UMMZ 239745	*S. bistincta*	MICHOACAN	19.3452	-100.3128	2,186,871	2,350	621.3	33.9	3,900	23.4	83
4	MK 600	UMMZ 239679	*S. bistincta*	MEXICO	19.1501	-100.1469	932,448	2,174	500.8	24.4	2,377	15.7	83
4	MK 600 (1)	UMMZ 239678	*S. bistincta*	MEXICO	19.1501	-100.1469	1,217,987	2,267	532.8	21.9	2,570	16.4	84
5	MK 645	UMMZ 239749	*S. bistincta*	MORELOS	18.9224	-99.2442	2,217,054	2,406	532.5	34.2	3,494	21.8	86
5	MK 645 dup	UMMZ 239749	*S. bistincta*	MORELOS	18.9224	-99.2442	548,545	1,964	513.0	17.7	841	7.4	60
6	MK 759	UMMZ 239701	*S. bistincta*	GUERRERO	18.0013	-101.1716	1,224,835	2,199	557.6	25.0	2,522	16.2	84
6	MK 760	UMMZ 239705	*S. bistincta*	GUERRERO	18.0013	-101.1716	1,074,315	2,203	559.4	24.3	743	6.9	58
6	MK 760 (2)	UMMZ 239704	*S. bistincta*	GUERRERO	18.0013	-101.1716	927,774	2,246	579.2	26.3	906	9.4	6
7	MK 691 (5)	UMMZ 239744	*S. bistincta*	GUERRERO	17.5324	-99.8994	2,084,203	2,353	672.5	27.2	1,678	11.9	80
8	MK 650 (1)	UMMZ 239725	*S. bistincta*	GUERRERO	17.6843	-99.8034	941,101	2,124	556.6	22.6	1,278	9.7	74
8	MK 650 (2)	UMMZ 239726	*S. bistincta*	GUERRERO	17.6843	-99.8034	2,224,898	2,394	506.4	28.4	1,737	12.2	79
8	MK 652	UMMZ 239727	*S. bistincta*	GUERRERO	17.6843	-99.8034	3,423,330	2,444	526.0	29.8	1,396	10.4	74
9	MK 671 (4)	UMMZ 239733	*S. bistincta*	GUERRERO	17.6407	-99.6797	1,012,300	2,107	557.0	22.5	842	7.4	62
9	MK 672	UMMZ 239738	*S. bistincta*	GUERRERO	17.6407	-99.6797	297,782	1,667	439.8	11.9	400	5.1	41
10	MK 656 (1)	UMMZ 239729	*S. bistincta*	GUERRERO	17.5526	-99.6626	580,194	1,950	497.6	18.4	547	5.9	42
11	MK 674 (1)	UMMZ 239690	*S. bistincta*	GUERRERO	17.5087	-99.1258	659,418	1,941	565.4	18.8	1,086	8.7	68
11	MK 675 (2)	UMMZ 239879	*S. bistincta*	GUERRERO	17.5087	-99.1258	336,474	1,565	519.1	10.4	227	4.2	24
12	MK 662	UMMZ 239731	*S. bistincta*	GUERRERO	18.6359	-99.6491	1,137,742	2,130	548.7	23.5	796	7.2	56
13	MK 697 (3)	UMMZ 239789	*S. bistincta*	VERACRUZ	18.6585	-97.1574	1,524,182	2,214	625.6	24.3	2,479	16.0	82
13	MK 699 (1)	UMMZ 239791	*S. bistincta*	VERACRUZ	18.6477	-97.1574	1,342,010	2,206	610.9	24.4	1,769	12.3	79
14	MK 700 (2)	UMMZ 239750	*S. bistincta*	PUEBLA	18.3220	-97.0285	2,203,360	2,298	697.8	27.6	4,155	24.8	86
15	MK 705 (1)	UMMZ 239862	*S. bistincta*	OAXACA	18.1576	-96.8684	2,529,703	2,417	644.6	30.3	3,164	19.6	85
16	MK 715	UMMZ 239755	*S. bistincta*	OAXACA	17.2390	-97.0032	347,152	1,712	437.7	12.1	254	4.3	31
17	MK 716 (1)	UMMZ 239758	*S. bistincta*	OAXACA	17.3036	-96.7930	52,461	594	267.4	2.7	8	3.0	1
18	MK 718 (2)	UMMZ 239765	*S. bistincta*	OAXACA	17.4211	-96.6876	606,978	2,014	568.6	19.7	366	4.9	37
19	MK 755 (1)	UMMZ 239786	*S. bistincta*	OAXACA	17.4153	-96.5671	871,034	2,062	563.2	22.0	1,022	8.4	69
20	MK 751	UMMZ 239785	*S. bistincta*	OAXACA	17.3160	-96.4435	1,577,190	2,342	621.5	28.5	2,376	15.4	84
22	MK 748 (2)	UMMZ 239780	*S. bistincta*	OAXACA	16.9791	-96.1364	91,912	1,009	302.5	4.2	23	3.1	3
22	MK 748 (4)	UMMZ 239782	*S. bistincta*	OAXACA	16.9791	-96.1364	1,058,426	2,170	553.8	23.7	499	5.7	42
22	MK 767	UMMZ 239788	*S. bistincta*	OAXACA	16.9859	-96.1358	1,083,058	2,206	524.7	24.3	617	6.3	52
23	MK 721	UMMZ 239767	*S. bistincta*	OAXACA	16.7377	-97.0384	146,471	1,215	376.0	6.1	62	3.3	10
24	MK 766	UMMZ 239794	*S. bistincta*	OAXACA	16.2522	-97.1536	994,521	2,166	577.4	23.3	467	5.5	43
25	MK 730 (2)		*P. bistincta*	OAXACA	16.0186	-96.5301	2,967,630	2,404	716.9	31.1	3,581	21.7	84
26	MK 685 (2)	UMMZ 239739	*S. sp. nov.*	GUERRERO	17.3812	-100.2009	269,729	1,645	450.6	10.8	184	4.0	20
27	MK 689 (2)	UMMZ 239740	*S. sp. nov.*	GUERRERO	17.3000	-100.2792	67,313	502	221.6	2.6	32	3.1	6
28	MK 727 (2)	UMMZ 239772	*S. pentheter*	OAXACA	16.1916	-97.0958	17,052	381	247.1	2.4	10	3.0	2
29	MK 691 (3)	UMMZ 239651	*S. chryses*	GUERRERO	17.5324	-99.8994	2,267,946	2,347	663.6	35.0	4,919	33.4	83
30	MK 770	UMMZ 239802	*S. hazelae*	OAXACA	17.2216	-96.5839	538,500	2,066	526.2	19.8	557	5.9	44

[1] Map number in Figure 1
[2] The first three-digit number corresponds to a sampling location. If there is a second number in parentheses, this corresponds to different aquaria where tadpoles were sorted by species before
[3] All specimens are from the University of Michigan Museum of Zoology

Table 2. Bayes factor species delimitation results.

Model	Species	MLE	Rank	*ln*(BF)
runA	9	-2832.33	1	10.52
runB	10	-2837.59	2	132.52
runC	7	-2903.85	3	69.36
runD	6	-2938.53	4	3822
runE	2	-4849.53	5	-

Figures

Figure 1. (A) Map of central Mexico showing sampling sites for *S. bistincta* and close outgroups, with numbers matching localities listed in Table 1 and colors matching Structure results below. Unsampled parts of the distribution of *S. bistincta* are shown in Fig. S1; (B) *S. bistincta* individual from near site 1; (C) Composite results of repeated Structure runs at $K = 2$ showing the finest detectable structure in the genetic data. Each vertical line represents an individual labeled with its UMMZ catalog number above and, in descending order below, the site number and the lineages indicated by the species delimitation analysis.

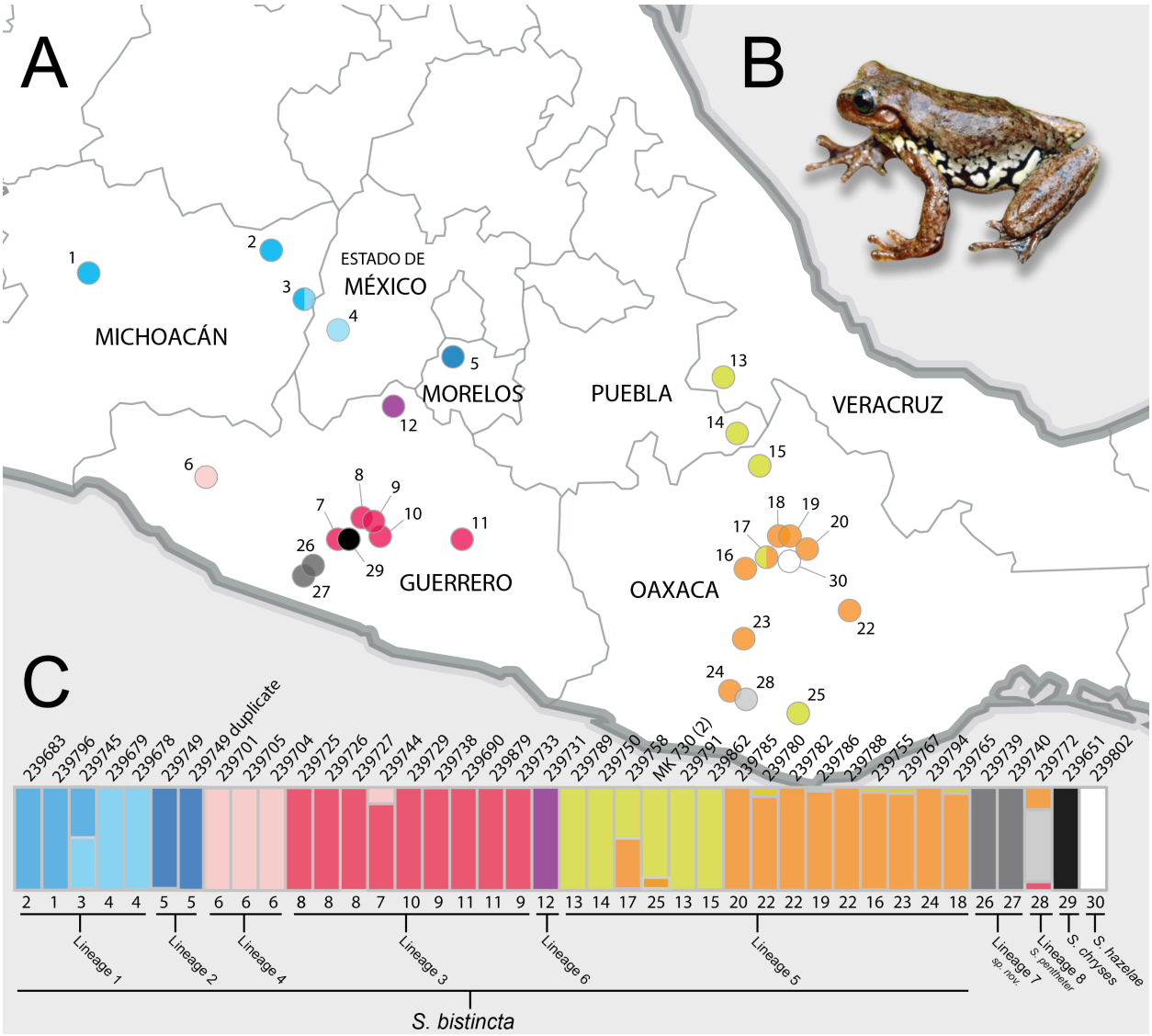
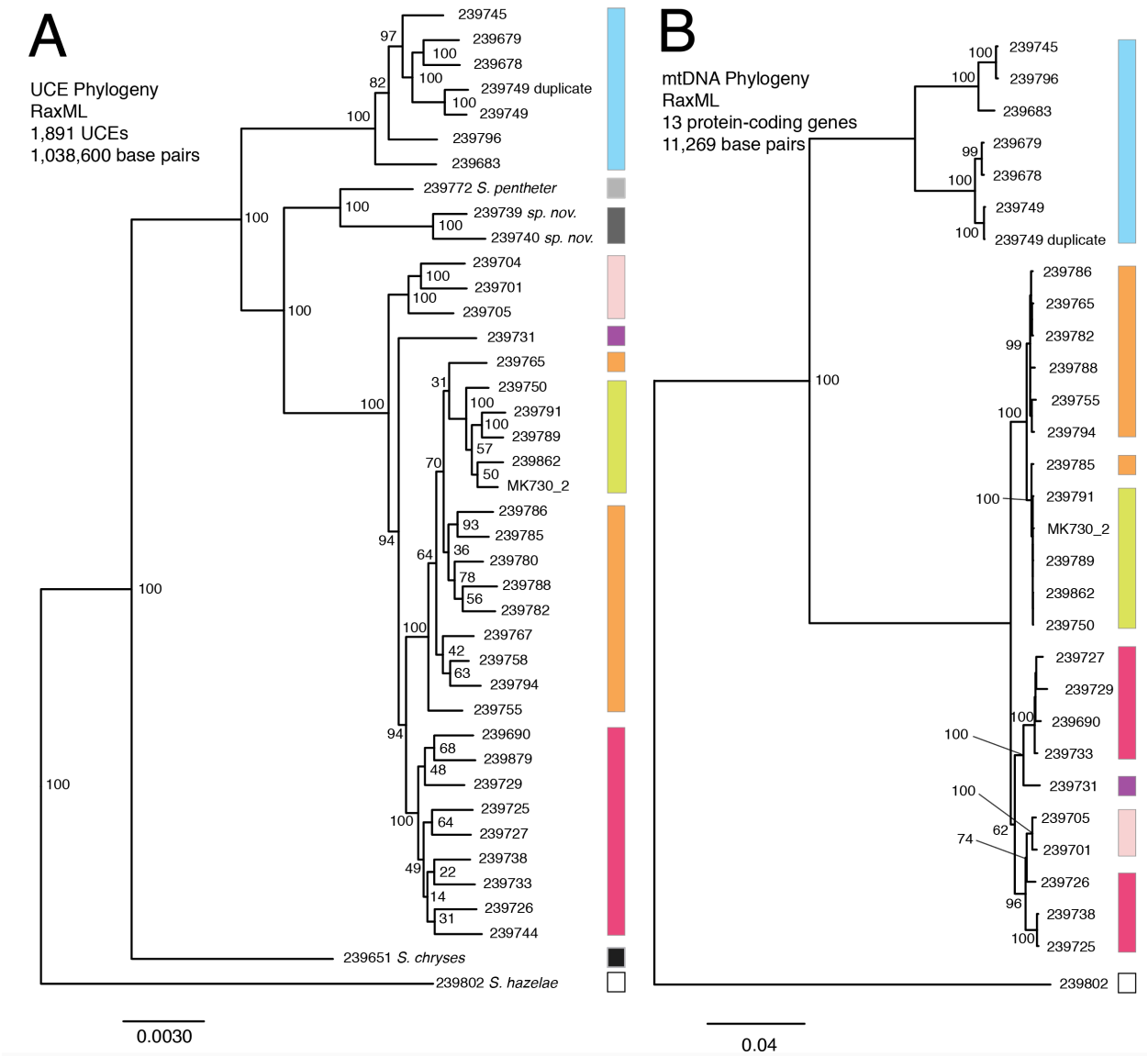
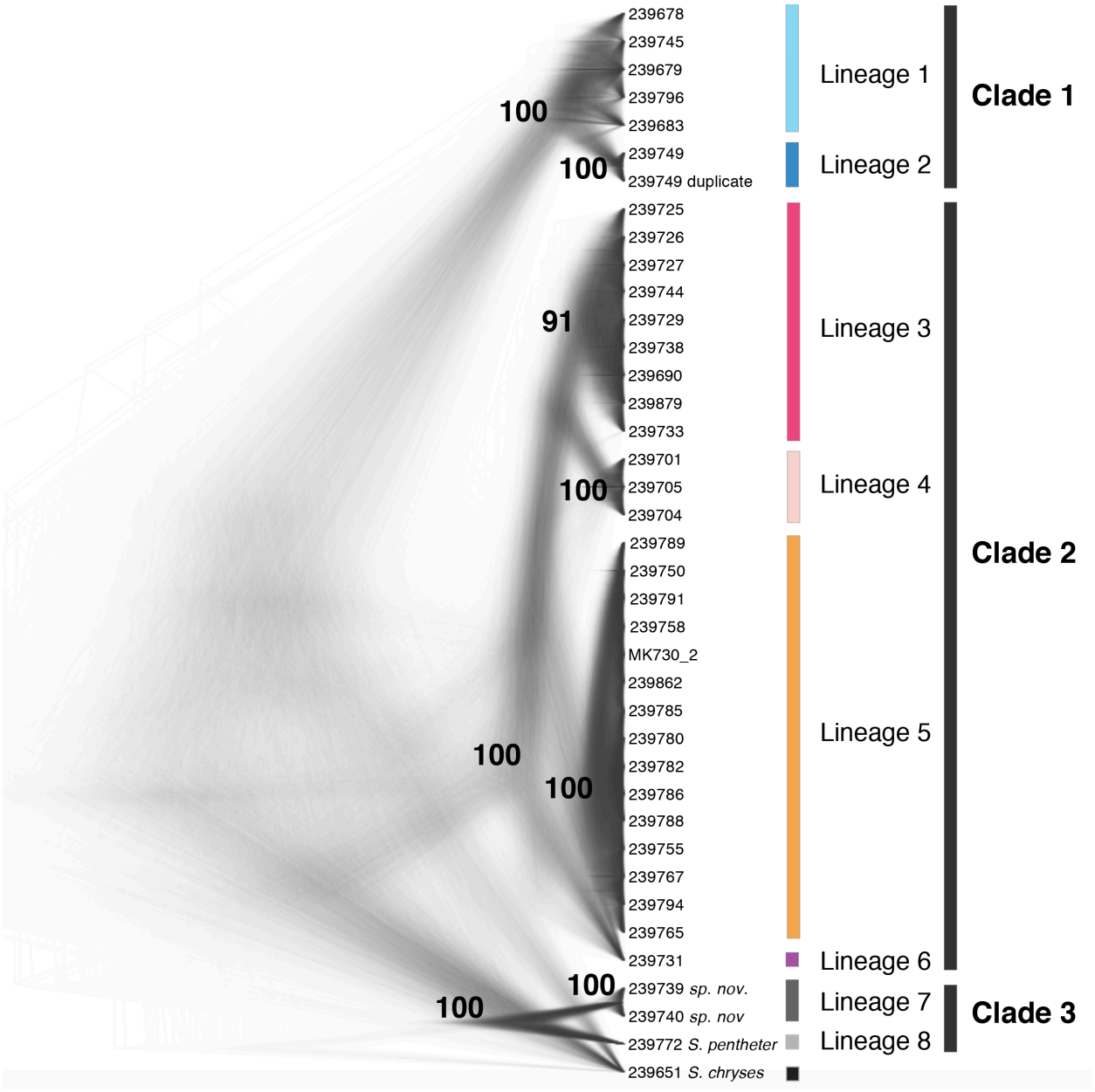


Figure 2. (A) UCE tree; (B) mtDNA tree. Colors match Structure groups identified in Fig. 1. Tips are labeled with their UMMZ catalog number.



683 Figure 3. Cloudogram of the posterior distribution of SNAPP trees from 399 high-quality SNPs
684 mined from UCE loci. Tip labels are UMMZ catalog numbers. Colored bars show the eight
685 lineages identified by the species delimitation analysis in addition to the outgroup *S. chryses*.
686 Clades are discussed in text. Colors match genetic clusters from Figure 1.



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688 Figure 4. Phylogeny of mtDNA for a subset of the individuals from this study (labeled with red
689 UMMZ catalog numbers) combined with existing sequences from Genbank (labeled with
690 accession numbers). Colored boxes relate to genetic lineages in prior figures. The tree was
691 rooted with *Exerodonta xera*. Photo credit: Peter Heimes.

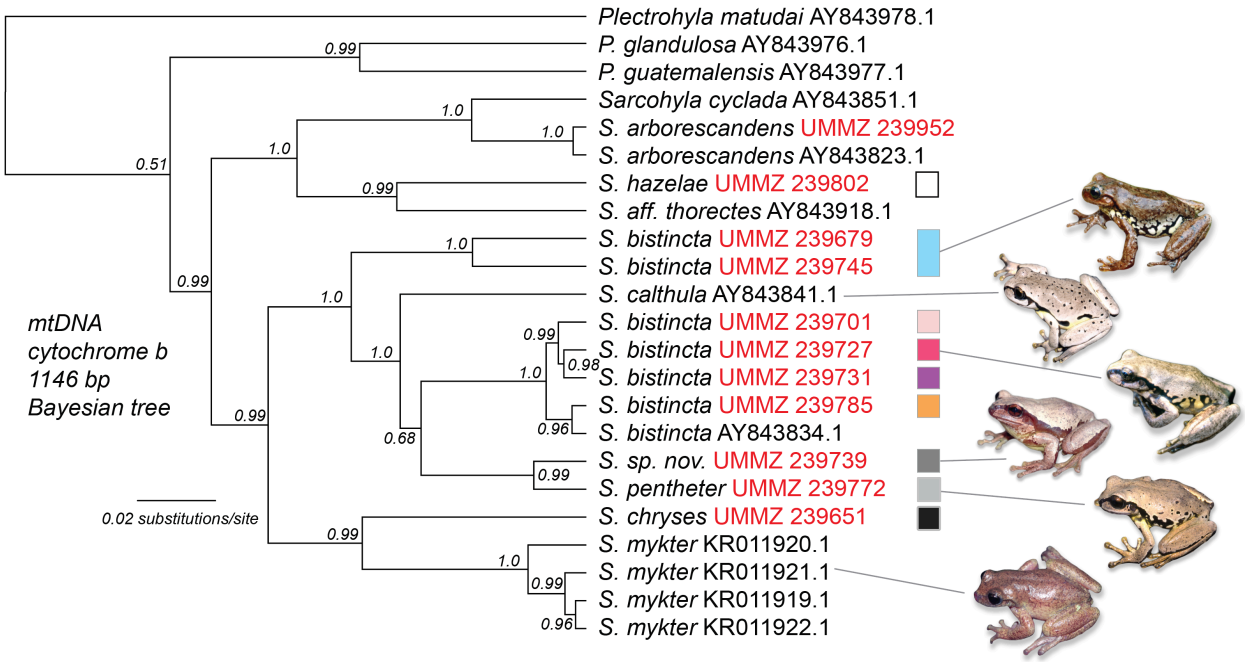

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Table S1. Information and summary statistics on all 45 samples used to determine the ingroup for this study.

Field Number	UMMZ Number	Current Taxonomy	State	Locality	Latitude	Longitude	Trimmed Paired Reads	UCEs	Average UCE length	mtDNA reads	mtDNA average coverage	mtDNA average quality	Fig. 1 Map Number	Accession number
MK 618	UMMZ 239796	*Sarcohyla bistincta*	MICHOACAN	LOS AZUFRES / SAN PEDRO ROAD	19.791051	-100.660542	1,889,670	2323	529.7	3381	20.8	86	1	
MK 627-31	UMMZ 239683	*Sarcohyla bistincta*	MICHOACAN	PARQUE BARRANCA DEL CUPATITZIO URUAPAN	19.426646	-102.073574	1,933,019	2254	370.9	3415	20.8	85	2	
MK 666	UMMZ 239745	*Sarcohyla bistincta*	MICHOACAN	13 KMS ROAD ZITACUARO MACHERO (LITTLE TOWN WERE THERE IS A MONARCH BUTTERFLY SANCTUARY)	19.3452	-100.3128	2,186,871	2350	621.3	3900	23.4	83	3	
MK 600	UMMZ 239679	*Sarcohyla bistincta*	MEXICO	CARRETERA VALLE DE BRAVO-SAN PEDRO TENEYAC ARROYO 1 (6860 FEET) (POR ALBARRADA) BOSQUE BIEN CONSERVADO Y HUMEDO	19.150083	-100.1469	932,448	2174	500.8	2377	15.7	83	4	
MK 600 (1)	UMMZ 239678	*Sarcohyla bistincta*	MEXICO	CARRETERA VALLE DE BRAVO-SAN PEDRO TENEYAC ARROYO 1 (6860 FEET) (POR ALBARRADA) BOSQUE BIEN CONSERVADO Y HUMEDO	19.150083	-100.1469	1,217,987	2267	532.8	2570	16.4	84	4	
MK 645	UMMZ 239749	*Sarcohyla bistincta*	MORELOS	2 KMS OUT OF CUERNAVACA ON THE CUERNAVACA CHALMA RD. (PASSING CONJUNTO CERRADO) OF LA BARRANCA WHERE THE SALTO SAN ANTONIO IS	18.922402	-99.244151	2,217,054	2406	532.5	3494	21.8	86	5	
MK 645 dupe	UMMZ 239749	*Sarcohyla bistincta*	MORELOS	2 KMS OUT OF CUERNAVACA ON THE CUERNAVACA CHALMA RD. (PASSING CONJUNTO CERRADO) OF LA BARRANCA WHERE THE SALTO SAN ANTONIO IS	18.922402	-99.244151	548,585	1964	513.0	841	7.4	60	5	
MK 759	UMMZ 239701	*Sarcohyla bistincta*	GUERRERO	CA 100 DEG KMS FROM CIUDAD ALTAMIRANO VIA IXTAPA ZIHUATANEJO	18.0013	-101.1716	1,224,835	2199	557.6	2522	16.2	84	6	
MK 760	UMMZ 239705	*Sarcohyla bistincta*	GUERRERO	CA 100 DEG KMS FROM CIUDAD ALTAMIRANO VIA IXTAPA ZIHUATANEJO	18.0013	-101.1716	1,074,315	2203	559.4	743	6.9	58	6	
MK 760 (2)	UMMZ 239704	*Sarcohyla bistincta*	GUERRERO	CA 100 DEG KMS FROM CIUDAD ALTAMIRANO VIA IXTAPA ZIHUATANEJO	18.0013	-101.1716	927,774	2246	579.2	906	9.4	6	6	
MK 691 (5)	UMMZ 239744	*Sarcohyla bistincta*	GUERRERO	2-3 KMS IN THE ROAD TO JALEACA FROM POINT WHERE ROAD TRIFURCATE TO PTO. DEL GALLO / YEXTLA / ANDJALEACA.COMMING FROM CARRIZAL DE BRAVO / IN RIVER UNDER THE BRIDGE	17.5324	-99.8994	2,084,203	2353	672.5	1678	11.9	80	7	
MK 650 (1)	UMMZ 239725	*Sarcohyla bistincta*	GUERRERO	MOUNTAINS W. OF CHILPANCINGO / TOWN LOS MORROS	17.6843	-99.80339	941,101	2124	556.6	1278	9.7	74	8	
MK 650 (2)	UMMZ 239726	*Sarcohyla bistincta*	GUERRERO	MOUNTAINS W. OF CHILPANCINGO / TOWN LOS MORROS	17.6843	-99.80339	2,224,898	2394	506.4	1737	12.2	79	8	
MK 652	UMMZ 239727	*Sarcohyla bistincta*	GUERRERO	MOUNTAINS W. OF CHILPANCINGO / TOWN LOS MORROS	17.6843	-99.80339	3,423,330	2444	526.0	1396	10.4	74	8	
MK 671 (4)	UMMZ 239733	*Sarcohyla bistincta*	GUERRERO	BEHIND CHICHIHUALCO / ON ROAD TO CARRIZAL DE BRAVO / 2 KMS FROM ENTRONQUE	17.6407	-99.6797	1,012,300	2107	557.0	842	7.4	62	9	
MK 672	UMMZ 239738	*Sarcohyla bistincta*	GUERRERO	BEHIND CHICHIHUALCO / ON ROAD TO CARRIZAL DE BRAVO / 2 KMS FROM ENTRONQUE	17.6407	-99.6797	297,782	1667	439.8	400	5.1	41	9	
MK 656 (1)	UMMZ 239729	*Sarcohyla bistincta*	GUERRERO	3 KMS FROM THE TOWN OF OMILTEMI IN THE ROAD OMILTEMI CHILPANCINGO	17.552603	-99.662569	580,194	1950	497.6	547	5.9	42	10	
MK 674 (1)	UMMZ 239690	*Sarcohyla bistincta*	GUERRERO	ON ATZACUALOYA HUEYCATENANGO RD.	17.5087	-99.1258	659,418	1941	565.4	1086	8.7	68	11	
MK 675 (2)	UMMZ 239879	*Sarcohyla bistincta*	GUERRERO	ON ATZACUALOYA HUEYCATENANGO RD.	17.5087	-99.1258	336,474	1565	519.1	227	4.2	24	11	
MK 662	UMMZ 239731	*Sarcohyla bistincta*	GUERRERO	1.6 KMS FROM THE TOWN OF TETIPAC ON THE TETIPAC TAXCO ROAD (MAYBE ARROYO LAS DAMAS)	18.635895	-99.6491	1,137,742	2130	548.7	796	7.2	56	12	
MK 697 (3)	UMMZ 239789	*Sarcohyla bistincta*	VERACRUZ	ON ATZOMPA XOXOCOTLA RD. 1.5 KMS FROM XOXOCOTLA	18.6585	-97.1574	1,524,182	2214	625.6	2479	16.0	82	13	
MK 699 (1)	UMMZ 239791	*Sarcohyla bistincta*	VERACRUZ	ON STREAM CROSSING THE TOWN OF XOXOCOTLA	18.6477	-97.1574	1,342,010	2206	610.9	1769	12.3	79	13	
MK 700 (2)	UMMZ 239750	*Sarcohyla bistincta*	PUEBLA	IN THE STREAM LOCATED AFTER THE TOWN OF ZOQUITLAN TURNING DOWN AT THE CENTRO DE SALUD	18.322	-97.0285	2,203,360	2298	697.8	4155	24.8	86	14	
MK 705 (1)	UMMZ 239862	*Sarcohyla bistincta*	OAXACA	56 KMS FROM TEOTITLAN VIA HUAUTLA	18.1576	-96.8684	2,529,703	2417	644.6	3164	19.6	85	15	
MK 715	UMMZ 239755	*Sarcohyla bistincta*	OAXACA	NEAR "EL TEJOCOTE" (FRENTE AL KINDER)	17.239	-97.0032	347,152	1712	437.7	254	4.3	31	16	
MK 716 (1)	UMMZ 239758	*Sarcohyla bistincta*	OAXACA	ON ROAD BETWEEN THE TOWNS OF SAN JUAN DEL ESTADO AND SAN MIGUEL ALOAPAN	17.3036	-96.793	52,461	594	267.4	8	3.0	1	17	
MK 718 (2)	UMMZ 239765	*Sarcohyla bistincta*	OAXACA	PASSING SAN MIGUEL ALOAPAN	17.4211	-96.6876	606,978	2014	568.6	366	4.9	37	18	
MK 755 (1)	UMMZ 239786	*Sarcohyla bistincta*	OAXACA	ON ROAD SAN JUAN ATEPEC SAN MIGUEL ABEJONES	17.4153	-96.5671	871,034	2062	563.2	1022	8.4	69	19	
MK 751	UMMZ 239785	*Sarcohyla bistincta*	OAXACA	3.8 KMS PASSING "RANCHO TEXAS" ON THE ROAD FROM THE TOWN OF IXTLAN DE JUAREZ	17.316	-96.4435	1,577,190	2342	621.5	2376	15.4	84	20	
MK 748 (2)	UMMZ 239780	*Sarcohyla bistincta*	OAXACA	CA 37 KMS FROM MITLA ON THE ROAD MITLA AYUTLA	16.9791	-96.1364	91,912	1009	302.5	23	3.1	3	22	
MK 748 (4)	UMMZ 239782	*Sarcohyla bistincta*	OAXACA	CA 37 KMS FROM MITLA ON THE ROAD MITLA AYUTLA	16.9791	-96.1364	1,058,426	2170	553.8	499	5.7	42	22	
MK 767	UMMZ 239788	*Sarcohyla bistincta*	OAXACA	9.2 KMS E STA MARIA ALBARRADAS / SIERRA MIXE	16.985888	-96.135816	1,083,058	2206	524.7	617	6.3	52	22	
MK 721	UMMZ 239767	*Sarcohyla bistincta*	OAXACA	2 KMS N. THE TOWN OF STA. MARIA LAXICHIO VIA THE TOWN OF SAN SEBASTIAN RIO DULCE	16.7377	-97.0384	146,471	1215	376.0	62	3.3	10	23	
MK 766	UMMZ 239794	*Sarcohyla bistincta*	OAXACA	CERRO DE VIDRIO VIA A PUERTO ESCONDIDO	16.25216	-97.15359	994,521	2166	577.4	467	5.5	43	24	
MK 730 (2)		*Sarcohyla bistincta*	OAXACA	SLIGHTLY NORTH OF THE TOWN JALATENGO ON HWY 175	16.0186	-96.5301	2,967,630	2404	716.9	3581	21.7	84	25	
MK 685 (2)	UMMZ 239739	*Sarcohyla sp. nov.*	GUERRERO	ON ATOYAC PTO DE GALLO RD / BETWEEN 10 - 20 KMS NORTH OF THE TOWN "EL PARAISO"	17.3812	-100.2009	269,729	1645	450.6	184	4.0	20	26	
MK 689 (2)	UMMZ 239740	*Sarcohyla sp. nov.*	GUERRERO	ON ATOYAC PTO DEL GALLO RD. / 500 M. NORTH OF THE TOWN OF SAN VICENTE	17.3	-100.2792	67,313	502	221.6	32	3.1	6	27	
MK 727 (2)	UMMZ 239772	*Sarcohyla pentheter*	OAXACA	RIO "EL SALADO" / 8 KMS N SAN JUAN LACHAO ON HWY 135	16.1916	-97.0958	17,052	381	247.1	10	3.0	2	28	
MK 691 (3)	UMMZ 239651	*Plectrohyla chryses*	GUERRERO	2-3 KMS IN THE ROAD TO JALEACA FROM POINT WHERE ROAD TRIFURCATE TO PTO. DEL GALLO / YEXTLA / ANDJALEACA.COMMING FROM CARRIZAL DE BRAVO / IN RIVER UNDER THE BRIDGE	17.5324	-99.8994	2,267,946	2347	663.6	4919	33.4	83	29	
MK 770	UMMZ 239802	*Plectrohyla hazelae*	OAXACA	EL PUNTO SIERRA JUAREZ	17.22156	-96.58386	538,500	2066	526.2	557	5.9	44	30	
MK 667	UMMZ 239952	*Sarcohyla arborescandens*	VERACRUZ	PUERTO DEL AIRE (ARRIBA DE ALCUTZINGO)	18.6787	-97.3485	923,330	2116	511.8	1154	9.0	69		
MK 700 (1)	UMMZ 239813	*Sarcohyla cyclada*	PUEBLA	IN THE STREAM LOCATED AFTER THE TOWN OF ZOQUITLAN TURNING DOWN AT THE CENTRO DE SALUD	18.322	-97.0285	2,490,616	2385	661.4	2961	18.4	82		
MK 701	UMMZ 239814	*Sarcohyla cyclada*	OAXACA	24 KMS FROM THE TOWN OF TEOTITLAN DE FLORES MAGON VIA HUAUTLA DE JIMENEZ	18.1781	-97.0054	1,166,574	2129	565.5	1061	8.7	69		
MK 742 (1)	UMMZ 239954	*Sarcohyla arborescandens*	VERACRUZ	LEFT ROAD BIFURCATING FROM ROAD TO THE TOWN OF "LAS MINAS"	19.6758	-97.1751	1,074,225	2240	553.8	1750	12.0	79		
MK 768	UMMZ 239833	*Exerodonta xera*	PUEBLA	5 KM SW ZAPOTITLAN DE SALINAS	18.311958	-97.51266	1,257,938	2288	581.5	3506	21.4	84		

Fig. S1. Sampled and unsampled parts of *S. bistincta* range in relation to known distributions (or localities, where distributional information is lacking) of other *Sarcohyla* species.

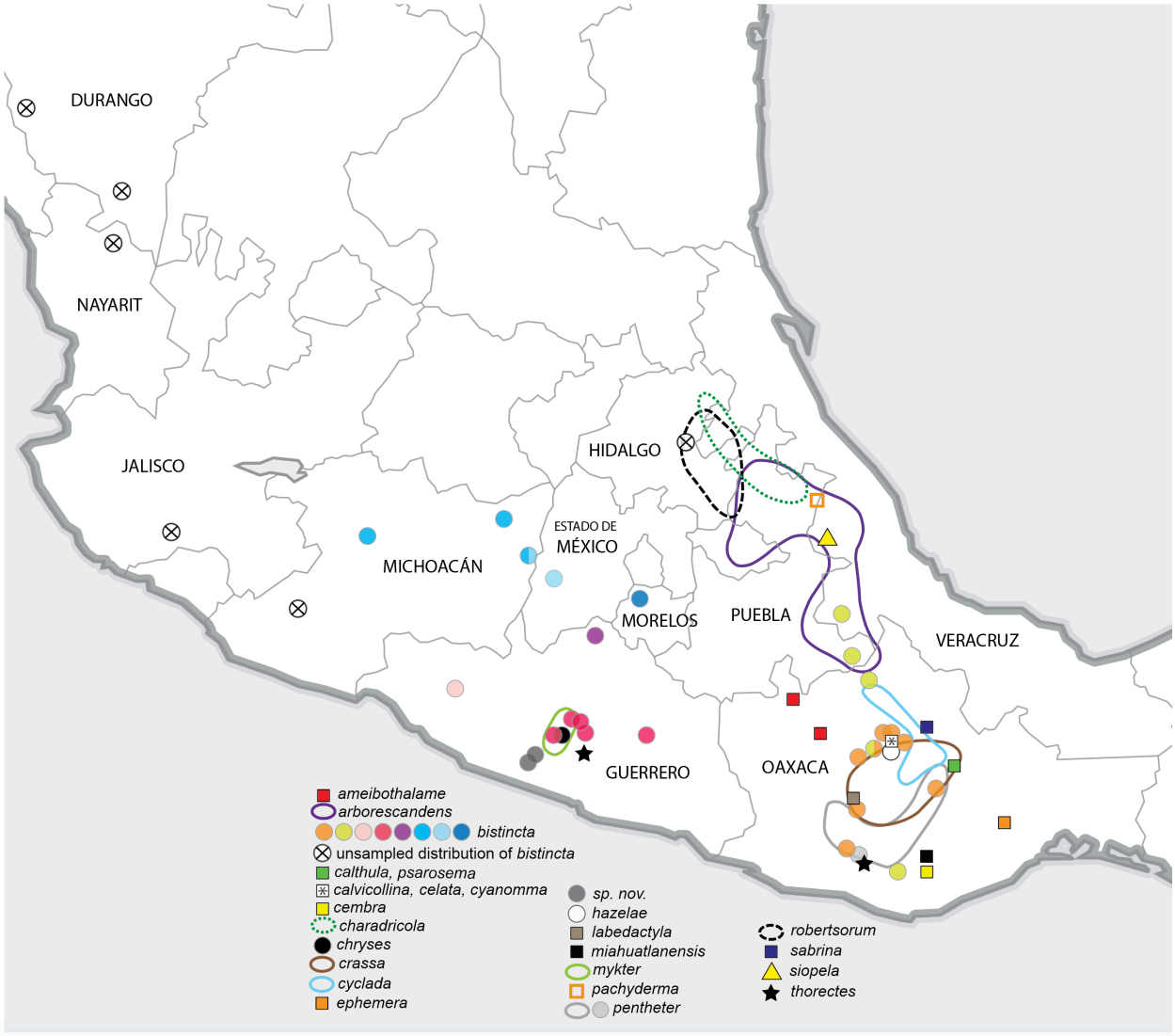
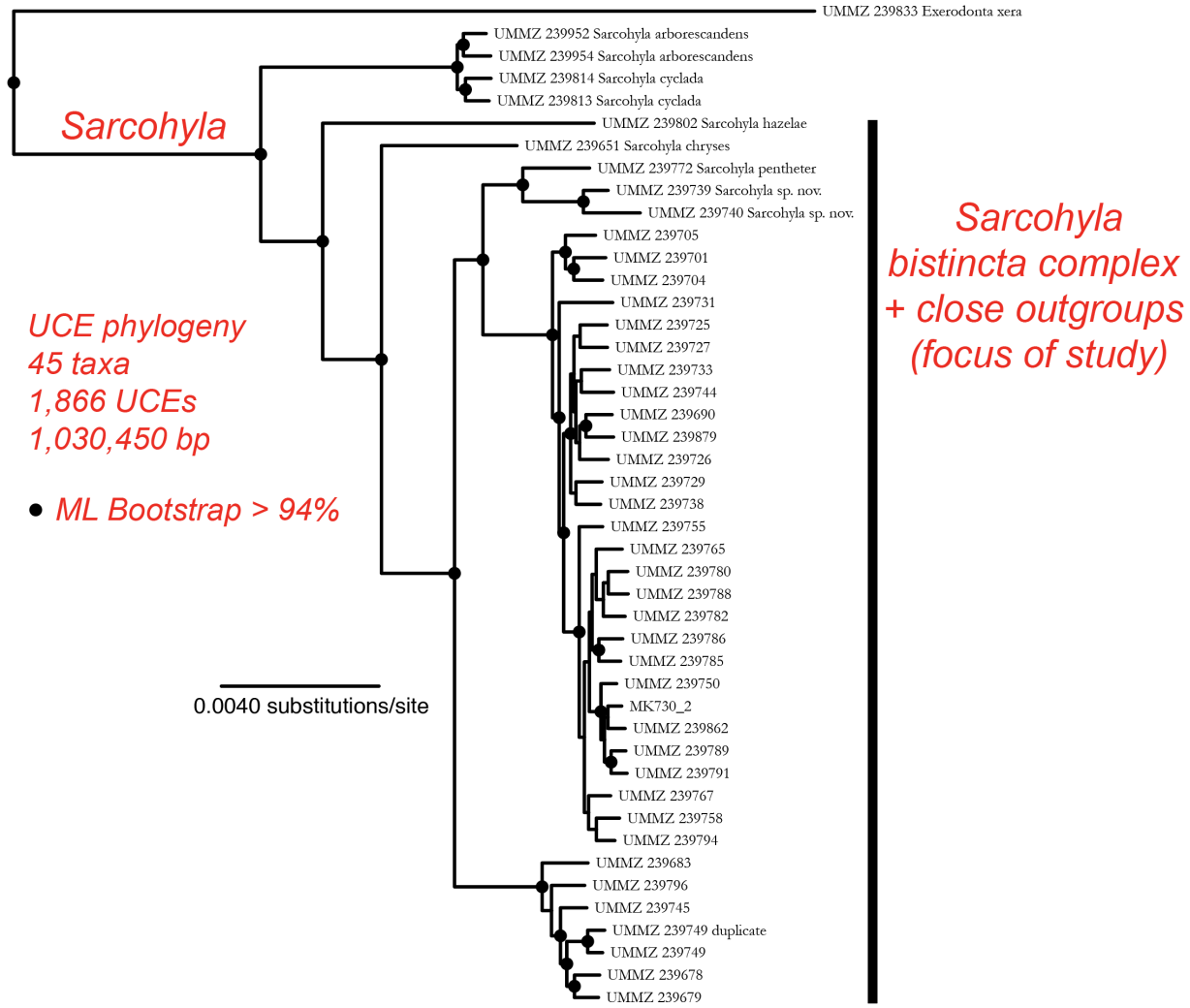
Fig. S2. UCE tree of 45 samples of *Sarcohyla* and outgroup *Exerodonta xera* used to determine the ingroup.