MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2012) doi: 10.1111/1755-0998.12054

DNA barcoding applied to *ex situ* tropical amphibian conservation programme reveals cryptic diversity in captive populations

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Abstract

Amphibians constitute a diverse yet still incompletely characterized clade of vertebrates, in which new species are still being discovered and described at a high rate. Amphibians are also increasingly endangered, due in part to disease-driven threats of extinctions. As an emergency response, conservationists have begun *ex situ* assurance colonies for priority species. The abundance of cryptic amphibian diversity, however, may cause problems for *ex situ* conservation. In this study we used a DNA barcoding approach to survey mitochondrial DNA (mtDNA) variation in captive populations of 10 species of Neotropical amphibians maintained in an *ex situ* assurance programme at El Valle Amphibian Conservation Center (EVACC) in the Republic of Panama. We combined these mtDNA sequences with genetic data from presumably conspecific wild populations sampled from across Panama, and applied genetic distance-based and character-based analyses to identify cryptic lineages. We found that three of ten species harboured substantial cryptic genetic diversity within EVACC, and an additional three species harboured cryptic diversity among wild populations, but not in captivity. *Ex situ* conservation efforts focused on amphibians are therefore vulnerable to an incomplete taxonomy leading to misidentification among cryptic species. DNA barcoding may therefore provide a simple, standardized protocol to identify cryptic diversity readily applicable to any amphibian community.

Keywords: amphibian decline, cryptic species diversity, DNA barcode of life, *ex situ* conservation, species delimitation

Received 31 August 2012; revision received 10 November 2012; accepted 21 November 2012

Introduction

Forty-one per cent of amphibian species throughout the world are threatened (Stuart *et al.* 2004; Hoffmann *et al.* 2010; Hof *et al.* 2011). The most insidious and as yet unstoppable agent of amphibian decline is a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* Longcore *et al.* 1999 that infects epidermal cells of its host and may cause death by inhibition of electrolyte transport (Longcore *et al.* 1999; Voyles *et al.* 2009). In Isthmian Central America, this pathogen seems to be advancing in an easterly-moving wave, whose detrimental effects are most severe at high elevation sites (Lips 1999; Lips *et al.* 2006). The highland frog faunas of western and central Panama have declined precipitously whereas extreme eastern Panama apparently still supports abundant and diverse faunas (Woodhams *et al.* 2008; Crawford *et al.* 2010a).

As with much of the flora and fauna of the tropical realm, Neotropical amphibian taxonomy remains regrettably incomplete, despite the fact that not only the number but even the rate of new species descriptions per year has been increasing (Glaw & Köhler 1998; Köhler *et al.* 2005). Ironically, the rate of new discoveries and the rate of species declines are increasing simultaneously (Hanken 1999). We are therefore in a race against time to both accurately characterize and conserve amphibian diversity, making current efforts at amphibian conservation in Panama

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and around the world unprecedented in their scope and urgency (Mendelson *et al.* 2006; Wake & Vredenburg 2008).

For critically endangered species, captive breeding offers our most intensive form of intervention and a short-term attempt to prevent extinction (Mendelson *et al.* 2006). Recently, an international consortium of zoos and conservation organizations has spearheaded an effort to begin captive breeding of many species of frogs from Central Panama (Gagliardo *et al.* 2008; Zippel *et al.* 2011). Collecting permits were granted and frogs were collected from various sites in an effort to capture a representative sample of conspecific genetic diversity, assuming that current taxonomy accurately reflected evolutionary diversity. To the extent that our current taxonomy belies the true diversity of independent evolutionary lineages, however, species' endangerment may be underestimated (Bickford *et al.* 2007; Angulo & Icochea 2010), species' geographical ranges may be overestimated (Wynn & Heyer 2001), and management efforts may unknowingly neglect certain species (Daugherty *et al.* 1990). If heterospecific lineages are unknowingly incorporated into an *ex situ* programme, zookeepers risk attempting to cross reproductively isolated species (Howard *et al.* 1989) or, perhaps worse, creating hybrid progeny that may be maladapted to their parents' native environment (cf. Berven 1982). Our ability to preserve these endangered species through captive breeding efforts therefore depends critically on accuracy and precision of our taxonomy.

An emerging consensus among taxonomists and evolutionary biologists affirms the importance of multiple genetic markers and independent sources of data for robust species delimitation and description (Dayrat 2005; Rissler & Apodaca 2007; Fujita *et al.* 2012). Although mitochondrial DNA (mtDNA) is just one of many sources of data that may be applied to problems of species delimitation (Sites & Marshall 2003), its standardized structure, high mutation rate and rapid sorting of ancestral polymorphism make it one of the more efficient sources (Wiens & Penkrot 2002; Avise 2004). DNA barcoding in animals refers to a global campaign to populate a public database of mtDNA and other data from vouchered specimens representing most of the world's diversity and all vertebrate species (Hebert *et al.* 2003). DNA barcoding was originally proposed as a tool for species identification, but it may also be used for rapidly appraising cryptic diversity (Floyd *et al.* 2002; Crawford *et al.* 2010a; April *et al.* 2011). Although mtDNA by itself is of limited use in delimiting or describing species (Brower 2006), it has been readily adopted as a key component of an integrative taxonomic framework (Sites & Marshall 2003; Padial & de la Riva 2007; Vieites *et al.* 2009).

The term 'cryptic species' refers to the presence of multiple distinct species grouped or 'hidden' under a single taxonomic binomen (Bickford *et al.* 2007). Our experience with evolutionary genetic and biogeographical studies of the biota of Isthmian Central America suggests that the potential is high for the existence of cryptic species and the problems they entail for conservation efforts, especially in amphibians. Phylogeographical studies of various frogs have revealed that central Panama is often a centre of cryptic diversity housing a multiplicity of previously unrecognized old lineages that were obscured by our current taxonomy (Crawford *et al.* 2007, 2010a; Wang *et al.* 2008). For example, data from the túngara frog *Engystomops* (*Physalaemus*) *pustulosus* (Cope 1864) show that even for species with geographical distributions spread across much of the Neotropics, the genetic divergences just within Panama are among the highest observed anywhere in the species' range (Weigt *et al.* 2005). Even in the absence of any obvious physical barriers to dispersal, divergences within Panama exceed that observed among conspecific populations of *E. pustulosus* separated by the Andean mountains of South America.

Here we used a DNA barcoding approach to assay mtDNA variation in the *ex situ* collection of the El Valle Amphibian Conservation Center (EVACC) located in central Panama and managed as part of the Panama Amphibian Rescue and Conservation (PARC) project, which is a conservation consortium of zoos and institutions including the Houston Zoo and the Smithsonian Tropical Research Institute (for more information see http://amphibianrescue.org/). We obtained genetic data from 10 of the 11 species of the highest conservation priority maintained in EVACC, along with data from wild populations of these same species, to assess whether any of the 10 threatened species may harbour cryptic lineages in the wild and whether any cryptic lineages are being housed in EVACC currently (Table 1). This information will provide a basis for subsequent taxonomic studies and possible description of candidate species uncovered here, as well as inform *ex situ* conservation actions.

Materials and methods

Statement of compliance

Animal Care and Use Protocols were approved by the Houston Zoo and Smithsonian Tropical Research Institute. Field samples were collected with the kind permission of the *Autoridad Nacional del Ambiente* of Panama, under permit numbers 20-2000, SE/A-88-05, SE/A-51-06, SE/A-37-07, SE/A-66-07, SC/A-4-08, SE/A-73-08 and SE/A-128-10.

Table 1 Summary by original taxonomy of sampling from *ex situ* and wild populations of Panamanian frogs along with conspecific genetic divergence in captive samples and in all samples. COI and 16S refer to number of mitochondrial DNA sequences of each gene included in the analysis

Family	Genus species	EVACC COI & 16S data (proportion of samples with both genes)	Wild COI and 16S data (proportion of samples with both genes)	π_B MRCA (SE)			
				EVACC COI	EVACC 16S	All samples COI	All samples 16S
Bufonidae	*Atelopus limosus*	18 & 19 (16 of 21)	4 & 3 (3 of 4)	0.0075 (0.0026)	0.0010 (0.0007)	0.0077 (0.0027)	0.0009 (0.0006)
Centrolenidae	**Centrolene* sp.	2 & 3 (1 of 4)	N/A	0.0677 (0.0107)	0.0136 (0.0052)	N/A	N/A
Craugastoridae	†*Pristimantis museosus*	38 & 15 (14 of 39)	36 & 40 (24 of 52)	0.1951 (0.0195)	0.1087 (0.0150)	0.2195 (0.0179)	0.1319 (0.0135)
Craugastoridae	‡*Craugastor punctariolus*	2 & 14 (2 of 14)	18 & 15 (14 of 19)	0.0000 (0.0000)	0.0000 (0.0000)	0.1602 (0.0178)	0.0933 (0.0133)
Craugastoridae	*Craugastor tabasarae*	2 & 9 (2 of 9)	3 & 5 (3 of 5)	0.0015 (0.0015)	0.0277 (0.0069)	0.0076 (0.0025)	0.0266 (0.0067)
Strabomantidae	*Strabomantis bufoniformis*	0 & 6 (0 of 6)	26 & 29 (24 of 37)	N/A	0.0000 (0.0000)	0.1138 (0.0128)	0.0492 (0.0081)
Dendrobatidae	*Colostethus panamansis*	2 & 1 (1 of 2)	35 & 30 (34 of 43)	0.1175 (0.0147)	N/A	0.1129 (0.0130)	0.0432 (0.0078)
Hemiphractidae	*Gastrotheca cornuta*	34 & 84 (25 of 93)	5 & 5 (5 of 5)	0.0615 (0.0091)	0.0175 (0.0049)	0.0619 (0.0092)	0.0172 (0.0048)
Hemiphractidae	*Hemiphractus fasciatus*	45 & 38 (26 of 57)	7 & 9 (6 of 10)	0.0928 (0.0115)	0.0333 (0.0059)	0.0932 (0.0114)	0.0370 (0.0065)
Hylidae	*Ecnomiohyla* spp.	1 & 7 (0 of 8)	2 & 1 (1 of 2)	N/A	0.0000 (0.0000)	0.0086 (0.0033)	0.1205 (0.0155)

N/A, indicates that genetic distances were not calculable, as <2 samples were available.

**Centrolene* sp. samples were identified as belonging to the confamilial taxon, *Rulyrana* cf. *flavopunctata*, by BLAST search to GenBank, and may constitute an unnamed taxon new to Panama. These samples were not compared to wild-caught Panamanian samples as neither of these genera is known from Panama.

†*Pristimantis museosus* data from wild populations included the closely related *P. cruentus* and an unnamed candidate species, *P.* aff. *museosus* (Crawford *et al.* 2010a), whereas EVACC data included a candidate species referred to as *P.* aff. *latidiscus*. For comparisons of ‡*Craugastor punctariolus* with wild populations, we included samples belonging to the newly described species, *P. evanesco*, as individuals may have been selected for *ex situ* conservation before the latter species was described, as well as samples from Costa Rica of the closely related *C. ranoides* that also occurs in Panama. π_B refers to the mean divergence between all pairs of haplotypes whose most recent common ancestor (MRCA) is the same as the MRCA of all samples, with standard errors (SE) in parentheses, and is proportional to the expected coalescent time (Hudson 1990; Slatkin 1991).

Sampling

From the EVACC captive facility we obtained 277 genetic samples comprised mostly of skin swabs (Mendoza *et al.* 2012; Prunier *et al.* 2012) along with some frozen samples of liver, toe clips or blood. These samples represented 10 of 11 nominal frog species of special conservation concern (Table 1). To place the genetic data from the captive populations into a wider geographical context, we added 177 additional samples (2–52 samples per species) of conspecific or closely related Panamanian amphibians obtained from frozen tissue collections of the *Círculo Herpetológico de Panamá* (CH), published data from specimens deposited in the National Museum of Natural History's Division of Amphibians and Reptiles (USNM), and other ancillary sources. The numbers of DNA sequences obtained per taxon in EVACC and from the wild are given in Table 1. Specimen numbers, locality information and GenBank accession numbers are provided in Supplementary Table S1. DNA sequences, chromatograms and specimen data are also publicly available on BoLD (Ratnasingham & Hebert 2007) under project codes 'EVACC' and 'EVACW'. A map of collecting sites is provided in Fig. 1. For three EVACC species we included suspected or known heterospecific DNA barcode data to facilitate identifications. Because *Pristimantis museosus* (Ibáñez *et al.* 1994) and *P. cruentus* (Peters 1873) may be involved in a cryptic species complex (Savage 1981; Miyamoto 1984; Crawford *et al.* 2010b), we included samples of both species along with previously uncovered mtDNA lineages closely allied to these species (Crawford *et al.* 2010a). *Craugastor punctariolus* (Peters 1863) founders were moved to EVACC prior to the description of a close relative, *C. evanesco* Ryan *et al.* 2010; therefore we included samples of both taxa along with another close relative, *C. ranoides* (Cope 1886) from Costa Rica, given that the latter occurs in Panama and these taxa are known to show low genetic and morphological divergence (Miyamoto 1983; Campbell & Savage 2000; Crawford & Smith 2005). EVACC samples included *Ecnomiohyla rabborum* Mendelson *et al.* 2008 and a sample initially labelled *E.* sp. We therefore included previously published data from *E. miliaria* (Cope 1886) for comparison.

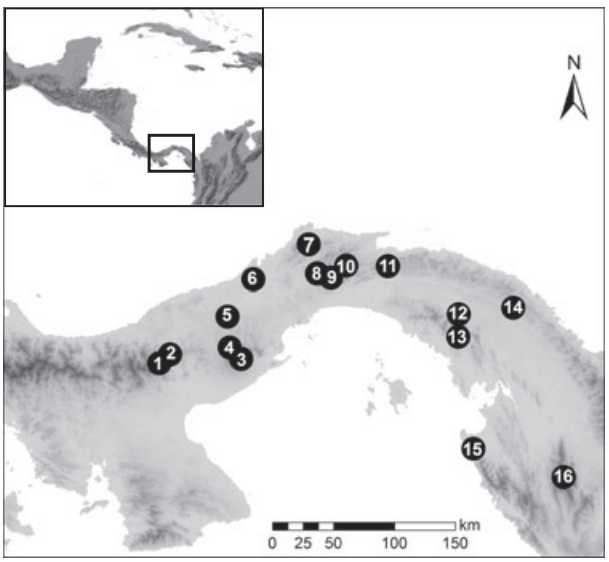

Fig. 1 Map of central and eastern Panama showing sampling locations for genetic samples included in this study. Number localities refer to 1) Río Blanco, 2) El Copé, 3) Altos del María, 4) San Miguel, 5) Río Indio, 6) Fort Sherman, 7) Cerro Bruja, 8) Río Chico, 9) Cerro Azul, 10) Cerro Brewster, 11) Nusagandi, 12) Wacuco (Majé), 13) Cerro Chucantí, 14) Nurra, 15) Cerro Sapo, 16) Cana. The Panama Canal connecting the two oceans lies between localities 6 and 7. Locality details and a list of samples per site are found in Supplementary Table S1.

Molecular genetic protocols

Prior to genomic DNA (gDNA) extraction, swabs were cut to fit in 1.5 mL microcentrifuge tubes with 180 μL of lysis buffer and subjected to 1 min of agitation on a Mini-Beadbeater-8 (BioSpec) with no beads added. All samples (swabs and tissues) were then treated with proteinase K and digested overnight. For EVACC samples, gDNA was extracted using a BioSprint 96 (QIAGEN) robotic extractor based on magnetic beads. Field-collected CH and AJC tissues were extracted on an AutoGenprep 965 (AutoGen) robotic extractor that implements a standard phenol-choloform protocol.

We collected mtDNA sequence data from two genes, the animal Barcode of Life (Hebert *et al.* 2003), also known as the Folmer fragment of cytochrome oxidase I (COI), and a fragment of the 16S ribosomal RNA gene (16S). The COI marker was PCR-amplified using the primer pair dgHCO2198 (5′-TAA ACT TCA GGG TGA CCA AAR AAY CA-3′) and dgLCO1490 (5′-GGT CAA CAA ATC ATA AAG AYA TYG G-3′) (Folmer *et al.* 1994; Meyer *et al.* 2005) and 0.25 μg/μL of bovine serum albumin. The 16S marker was amplified using 16SB-H (aka, 16Sbr-H) (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) and 16SA-L (aka, 16Sar-L) (5′-CGC CTG TTT ATC AAA AAC AT-3′) (Kessing *et al.* 2004). For both markers, PCR contained 2.0 mM of Mg^{2+}, utilized an annealing temperature of 49 °C, with all additional reaction and cycling conditions standard (Kessing *et al.* 2004). PCR products were cleaned using *Exo*I and SAP enzymes (Werle *et al.* 1994), with Sanger sequencing reactions run on ABI 3130 automated sequencers. All enzymatic and sequencing reactions, including trouble-shooting, were performed in a high-throughput 96-well format. Failed

samples were not submitted to individualized trouble-shooting procedures.

COI sequences were aligned by eye (no length variation was observed) and checked for inferred mis-sense mutations using MacClade (Maddison & Maddison 2005) and the Barcode of Life Database (BoLD) platform (Ratnasingham & Hebert 2007). 16S sequences were aligned using *ClustalX* (Thompson *et al.* 1997) including only congeneric samples to avoid introducing excessive numbers of gaps in alignments. For each gene and for the combined two-gene data set we inferred a neighbour-joining (NJ) tree (Saitou & Nei 1987) using HKY + Γ distances (Hasegawa *et al.* 1985; Yang 1994), i.e., correcting for increased transition rates, unequal nucleotide frequencies and among-site heterogeneity in rates of substitution, all known characteristics of animal mtDNA (Kocher *et al.* 1989). Although most DNA barcoding studies have utilized Kimura 2-parameter (K2P) distances (Kimura 1980), we prefer the HKY model as it accounts for nucleotide frequency bias and performs as well as, or superior to, the K2P model in DNA barcoding analyses (Collins *et al.* 2011; Srivathsan & Meier 2012).

Within each named species and for each gene we calculated the mean divergence between pairs of haplotypes since the most recent common ancestor, that is π_B (Charlesworth 1998) across the root node as inferred from the NJ trees (see above). π_B is proportional to the expected coalescent time (Hudson 1990; Slatkin 1991). Mean HKY distances with standard errors were obtained using MEGA 5 (Tamura *et al.* 2011) that implements the equivalent model under the name Tamura 3-parameter model (Tamura 1992).

To evaluate possible 'unconfirmed candidate species' (Vieites *et al.* 2009) or 'primary species hypotheses' we applied the *Automatic Barcode Gap Discovery* (*ABGD*) algorithm (Puillandre *et al.* 2011). In a recent comparison of barcoding algorithms, *ABGD* was found to be efficient as well as robust to variation in sampling design (Paz & Crawford 2012), a potential issue in this study of captive populations. Rather than presume a single threshold of genetic divergence to identify potential candidate species, *ABGD* evaluates a range of thresholds suggested by the data themselves, and through an iterative refinement procedure may suggest slightly different thresholds for different clades within the same data set. At present, *ABGD* implements Jukes–Cantor (Jukes & Cantor 1969) and K2P, or accepts distance matrices produced by *MEGA* or *dnadist* in the *PHYLIP* package (Felsenstein 2005). To estimate genetic distances we sought to apply evolutionary models that were justifiable by the data, yet no more complex than the HKY + Γ model (Collins *et al.* 2011; Srivathsan & Meier 2012). We estimated genetic distance with the program *dnadist* in the PHYLIP package, in which the most complex model implemented is the F84 + Γ model (Felsenstein & Churchill 1996), equivalent to HKY + Γ. To select best-fit nucleotide substitution models for a given species data set (Supplemental Table S2), we first used *jModeltest* version 0.1.1 (Posada 2008) and the Bayesian Information Criterion, which may select simpler models than the Akaike Information Criterion depending on sample and effect sizes (Burnham & Anderson 2004). Model and parameter values were used as input for calculations in *dnadist*, with the resulting genetic distance matrix input into *ABGD*. Two-gene analyses included only samples with data from both genes. Prior maximum intraspecific divergences included 15 thresholds between 1% and 15%, although the exact values are dictated by the data such that thresholds are not distributed uniformly across this interval. *ABGD* was applied to the combined EVACC plus wild-caught data, as we were looking for candidate species in the named taxon as a whole, not just within EVACC.

For those species or species groups that showed evidence of cryptic diversity (see Results) based on the phenetic or distance-based criterion implemented in ABGD, we also evaluated lineage diversity using a character-based phylogenetic approach (Samadi & Barberousse 2006; De Queiroz 2007). We inferred a maximum likelihood (ML) molecular phylogeny (Felsenstein 1981) using the software *GARLI* version 2.0.1019 (Zwickl 2006). The character matrix was partitioned by gene (COI vs 16S) and the best-fit nucleotide substitution model (see above) for each gene was applied independently to each partition. As relatively simple models were sufficient (see below) no further partitioning alternatives were pursued. Search parameters employed default values. Clade support was assessed by non-parametric bootstrapping (Felsenstein 1985) involving 200 re-sampling replicates and unpartitioned data and fixed parameter values to speed tree searches and optimization.

DNA barcode gap analyses such as *ABGD* are based on genetic distances whereas species are diagnosed and described using derived character state changes (Goldstein & DeSalle 2011). As a complement to the ML phylogenetic analyses, we therefore asked whether the COI barcode data provided any potential autapomorphies that could be used to diagnose or describe potential candidate species in the future. Diagnostic nucleotide sites for candidate species were inferred using the Character Attribute Organization System (CAOS) software (Sarkar *et al.* 2008; Bergmann *et al.* 2009). We limited this analysis to the COI data because this marker is more quickly evolving and shows no evidence of length variation among Panamanian amphibians (Crawford *et al.* 2010a), thus providing more variable sites of less ambiguous homology relative to 16S (Xia *et al.* 2012). We report only homogeneous sPu characters, i.e., nucleotide sites showing fixed differences between two lineages, for ease of interpretation.

Results

Success in obtaining DNA sequence data from EVACC samples varied widely between genes and among taxa. COI and 16S data were obtained from 52% and 73% of all EVACC samples respectively. For samples from EVACC, within-taxon genetic divergences (π_B) ranged from zero for *Craugastor punctariolus* (two samples with COI, 14 samples of 16S) and *Strabomantis bufoniformis* (Boulenger 1896; six samples of 16S), up to a maximum of 20% at COI and 11% at 16S for *Pristimantis museosus* (38 samples with COI, 15 samples of 16S; Table 1). Note, however, this latter taxon included EVACC samples preliminarily identified based on morphology as a possible cryptic species, *P.* aff. *latidiscus* (Fig. 2). Other species with noteworthy 'conspecific' divergence included *Hemiphractus fasciatus* (Peters 1862; Fig. 3) with 9.3% divergence at COI (45 samples) and 3.3% at 16S (38 samples), and *Colostethus panamansis* (Dunn 1933) with 12% divergence at COI (two samples; Table 1). Two other taxa showed modest within-species divergence, *Centrolene* Jiménez de la Espada 1872 sp. and *Gastrotheca cornuta* (Boulenger 1898), whereas two more species, *Atelopus limosus* Ibáñez *et al.* 1995 and *Craugastor tabasarae* (Savage *et al.* 2004), showed nucleotide variability, but very low divergence (Table 1). The final taxon, *E. fimbrimembra*, showed no divergence among the eight EVACC samples at either gene (Table 1), but in point of fact one highly divergent sample identified as *Ecnomiohyla* sp. was found within EVACC. This sample was sequenced successfully only for COI whereas the other samples yielded only 16S. Only by comparison with wild-caught samples could these sequences be compared (see below).

For most species, the level of within-taxon genetic divergence observed among EVACC samples matched that observed for the combined EVACC plus wild-caught data set. For example, *A. limosus* showed little divergence within EVACC or among all samples, and the addition of wild-caught *P. museosus* (Fig. 2) or *H. fasciatus* (Fig. 3) samples to conspecific EVACC data increased divergence only slightly (Table 1). Two exceptions to this trend were presented by *S. bufoniformis* (Fig. 3) and *C. punctariolus* (Fig. 4), which showed no genetic variation within EVACC, but substantial genetic divergence among wild-caught samples. Recall that *C. punctariolus* samples were combined with two additional named species to facilitate identification of EVACC samples (Fig. 4). Combining EVACC plus wild-caught samples across these three nominal taxa revealed 16% divergence at COI (18 samples) and 9.3% at 16S (15 samples). EVACC plus wild-caught samples of *S. bufoniformis* together showed 11% divergence at COI (24 samples) and 4.9% at 16S (29 samples). Samples of *E. fimbrimembra* from EVACC versus *E. miliaria* from the wild showed a remarkable 12% divergence at the 16S gene (eight samples; Table 1, Supplementary Fig. S1).

Applying the *ABGD* algorithm to the COI, 16S and two-gene data sets for each of the 10 focal taxa revealed no evidence for cryptic lineages in two cases (*A. limosus* and *Centrolene* sp., although the latter appears to be a new species or new record for the country) and, not surprisingly, strong evidence of additional lineages within the three taxa already known or suspected to contain additional species, *P. museosus*, *C. punctariolus* and *Ecnomiohyla* spp. (Table 2). *ABGD* identified the '*E.* sp.' sample from EVACC as *E. miliaria* (Supplementary Fig. S1). Among the remaining five taxa, the evidence was rather weak for additional species hypotheses within *C. tabasarae*, *S. bufoniformis*, *C. panamansis* and *G. cornuta*, whereas *H. fasciatus* could potentially harbour candidate species (Table 2, Fig. 3). For example, in *S. bufoniformis* a barcode gap threshold of 10% at COI or 2.7% at 16S implied no cryptic species were present, whereas in *H. fasciatus* a barcode gap threshold of 10% at COI supported the presence of three species (i.e. two additional candidate species) and for 16S a threshold of 4.7% supported two species. Results for combined two-gene analyses are not usually reported in the literature, but we provided them here for an additional, perhaps intermediate, perspective (Table 2).

Even though the barcode gap thresholds are higher with COI than with 16S (Vences *et al.* 2005a; Xia *et al.* 2012), *ABGD* analyses of each species gave largely consistent results among data sets (Table 2), with two exceptions. Samples related to *P. museosus* contained six primary species hypotheses according to the COI data when the prior assumptions of maximum intraspecific divergence ranged as high as 15%. Two of the six groups were comprised of 'singletons,' that is primary species hypothesis consisting of a single specimen (Table 2). Using the combined COI + 16S data including only samples with both genes (Table 1), *ABGD* identified just four groups among *P. museosus* and related specimens. Two candidate species inferred from COI-only data were missing in the two-gene analyses as one of the COI 'singleton' candidate species was grouped with other samples and a second singleton COI candidate lacked 16S data. Using the 16S data alone, the same four primary species hypotheses were recovered, across prior thresholds of 5% to 10%, although a threshold of 12% collapsed all samples into a single putative taxon (Table 2). The second taxon that appeared to give inconsistent results among data sets was *Ecnomiohyla* spp., with minimal divergence at COI yet high divergence at 16S (Tables 1 and 2). This contrast is a simple artefact caused by the one divergent 16S sample (likely heterospecific to the other specimens) not amplified for COI.

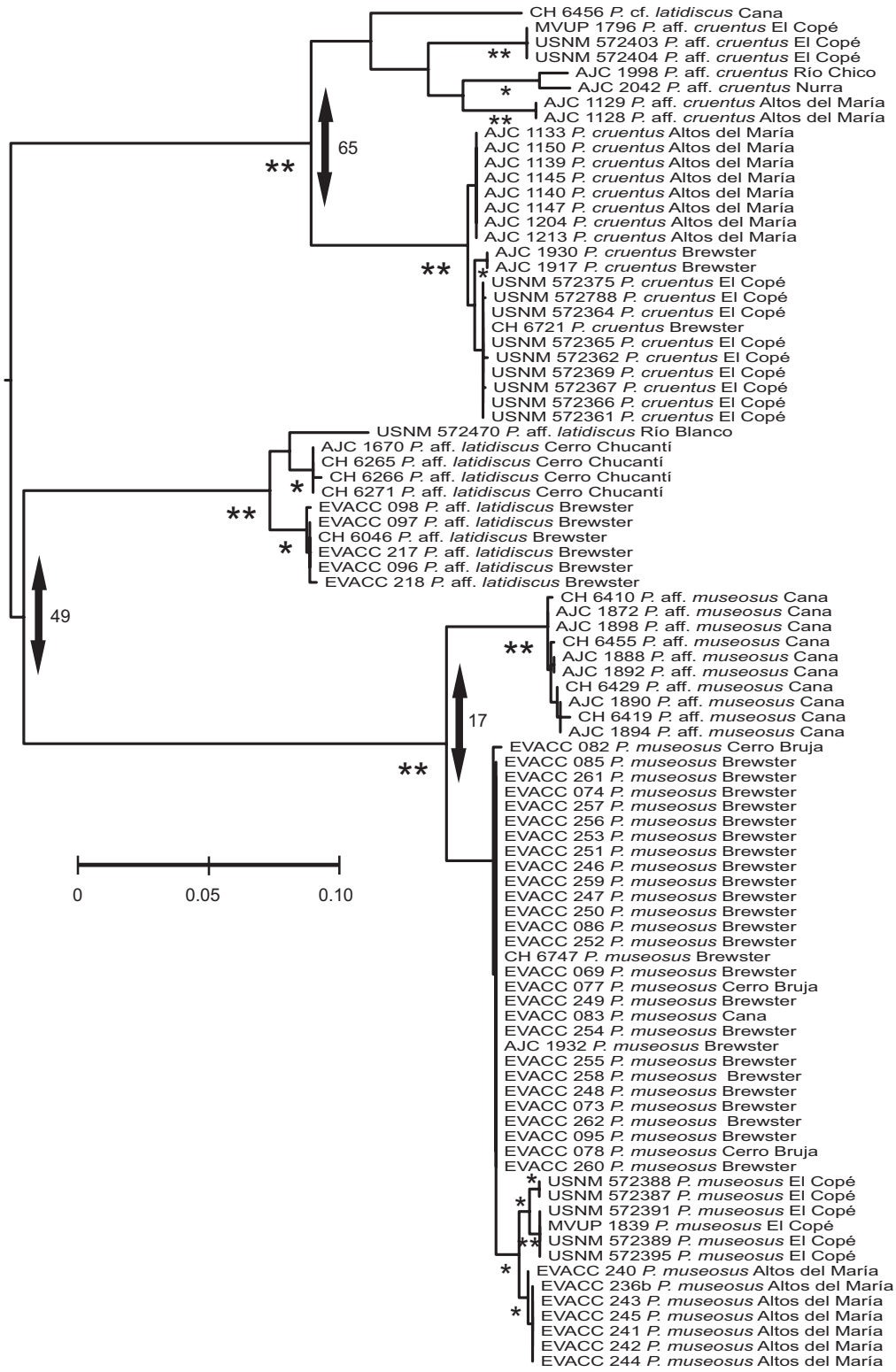

Fig. 2 Maximum likelihood phylogeny based on a partitioned analysis of COI and 16S mitochondrial DNA gene fragments from *Pristimantis museosus* and closely related taxa, inferred using the software GARLI 2.0 and rooted at mid-point. Single asterisk (*) indicates nodes with 80% to 94% bootstrap support and double asterisks (**) indicate support $\geq$ 95%. Numbers by double-headed arrows reflect the number of sites at the COI gene showing fixed nucleotide differences between the indicated sister lineages, as inferred from the *CAOS* analysis. Scale bar indicates inferred patristic distance.

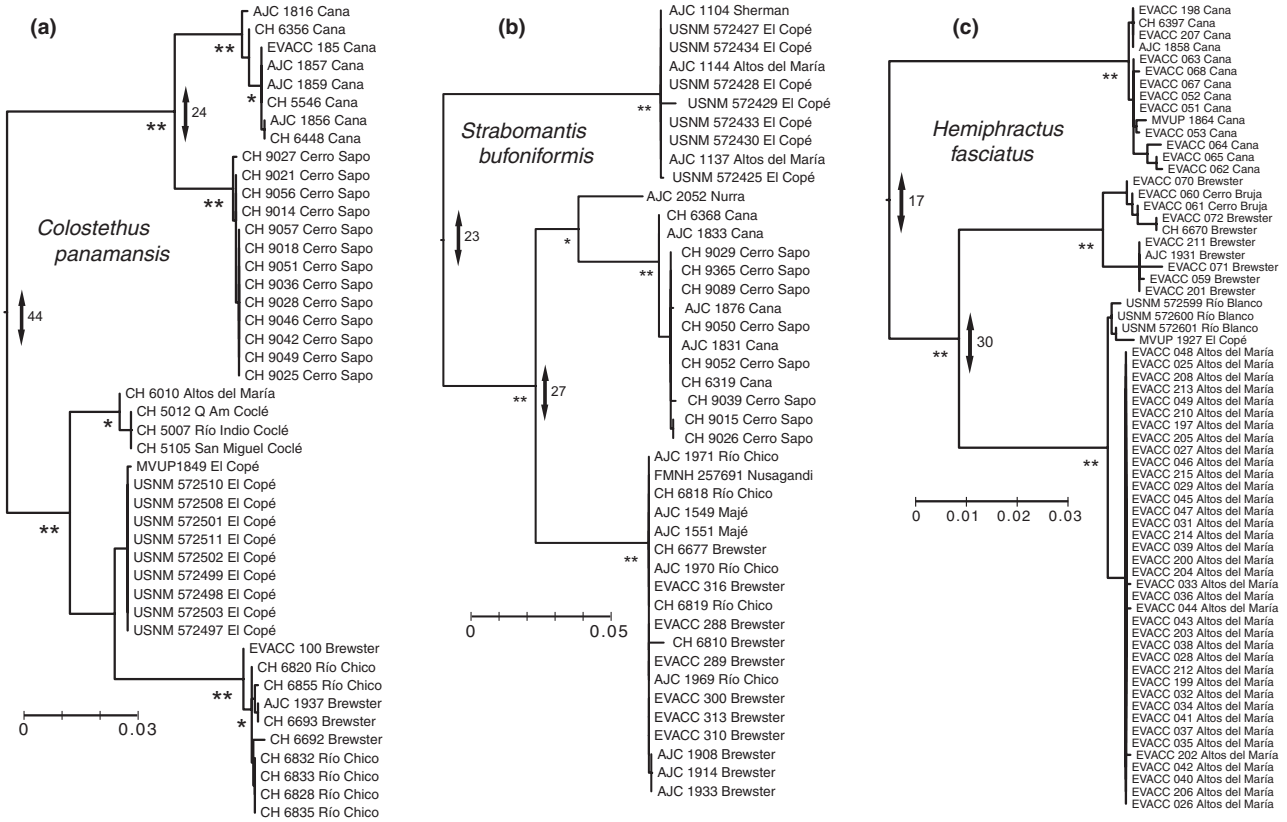

Fig. 3 Three maximum likelihood phylogenies based on a partitioned analysis of COI and 16S mitochondrial DNA gene fragments from three species inferred independently using the software GARLI 2.0 and rooted at mid-point. Asterisks and arrows are as in Fig. 2. Scale bars indicate inferred patristic distance separately for each tree. The three species are (a) *Colostethus panamansis*, (b) *Strabomantis bufoniformis*, (c) *Hemiphractus fasciatus*.

Phylogenetic inference using ML confirmed the existence of multiple divergent and statistically supported monophyletic lineages, and revealed that within-species divergence is largely structured geographically. The samples related to *P. museosus* and *P. cruentus* are divided into three major mtDNA lineages and a total of four to six potential species (see *ABGD* results). The locality Brewster (Fig. 1) hosts all three of these principle lineages in sympatry, and other sites such as Altos del María and El Copé host at least two lineages (Fig. 2). Phylogenetic analyses of three additional species with notable conspecific diversity, *C. panamansis*, *S. bufoniformis* and *H. fasciatus*, revealed shared patterns of spatial genetic structure. In each species, specimens from eastern Panama (Cana and Cerro Sapo) formed distinct clades (Fig. 3) relative to all other samples. Specimens from central Panama were further subdivided into two distinctive groups occupying either side of the Panama Canal. In *S. bufoniformis* and *H. fasciatus* the areas east and west of the Canal formed reciprocally monophyletic clades (Figs 1 and 3). *Craugastor ranoides* of Costa Rica grouped with (or within) a closely related *C. evanesco* relative to a deeply diverged *C. punctariolus* (Fig. 4).

The CAOS analysis of COI gene sequences revealed 17–44 fixed nucleotide differences between pairs of clades even for moderately diverged populations (Fig. 3). Character-based DNA barcoding supports the above distance-based inferences, although clades showing greater patristic distances do not necessarily show greater numbers of fixed differences in character states. The COI gene therefore offers a wealth of molecular-based autapomorphies, should taxonomists want to use such information to support species descriptions as well as diagnoses (Goldstein & DeSalle 2011).

Discussion

We argue the parallel races to characterize and conserve amphibian diversity as mutually interdependent: the success of one depends upon the success of the other (Dubois 2003). Conservation planning and action are often based on lists of species and knowledge of their distribution, implying that incomplete taxonomy may be an impediment to achieving the goals of biological conservation (Mace 2004). What is not described cannot be protected (Daugherty *et al.* 1990; May 1990). Genetic

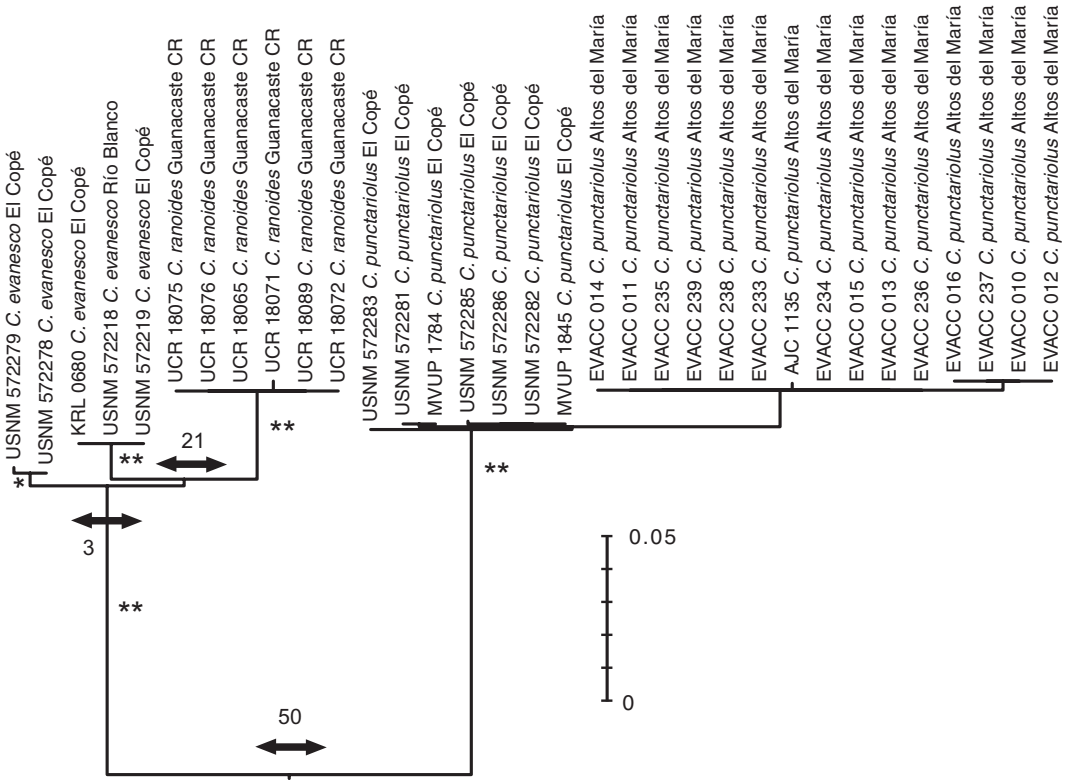

Fig. 4 Maximum likelihood phylogeny based on a partitioned analysis of COI and 16S mitochondrial DNA gene fragments from *Craugastor punctariolus* and two related species, inferred using the software GARLI 2.0 and rooted at mid-point. Asterisks and arrows are as in Fig. 2. Scale bar indicates inferred patristic distance.

analyses, therefore, offer information vital to successful intervention and conservation, especially for species representing poorly known taxonomic groups or from biogeographically complex regions (Allendorf & Luikart 2007). Phylogeographical studies and DNA barcoding efforts built around a solid systematic and taxonomic framework can reveal sympatric cryptic species, provide a measure of relatedness between allopatric populations, and provide more accurate estimates of species' ranges (Moritz 1994; Rocha *et al.* 2007). In this study, we used mtDNA data as an assay of previously unrecognized lineage diversity that could hamper captive breeding efforts if ignored.

Using DNA barcoding we have identified three nominal taxa currently being maintained at EVACC that show substantial divergence within the captive population (*P. museosus*, *C. panamansis* and *H. fasciatus*; Figs 2 and 3), along with two other nominal taxa that harbour cryptic diversity among wild populations within Panama that was not captured among our EVACC samples (*S. bufoniformis* and *C. punctariolus*; Figs 3 and 4). This result is alarming for *ex situ* conservation efforts, yet it may not be that surprising given that cryptic diversity may still be the rule rather than the exception among amphibians (Meegaskumbura *et al.* 2002; Vieites *et al.* 2009; Funk *et al.* 2011). DNA barcoding was also useful in identifying the EVACC sample *Ecnomiohyla* sp. as belonging to *E. miliaria* as it matched previously published COI barcodes (Crawford *et al.* 2010a).

Although measuring genetic divergence is relatively straightforward, determining whether divergent lineages represent distinct species is not. We suggest that DNA barcoding provides an excellent 'first pass' assay for cryptic diversity, yet determining specific status of each mtDNA lineage should be accomplished through an integrative approach to taxonomy (Will *et al.* 2005; Padial *et al.* 2009). Robust species delimitation and description of new species should integrate information from multiple sources, such as morphology, ecology and, in the case of frogs, male advertisement calls when possible (Angulo & Reichle 2008; Jansen *et al.* 2011). Among the species studied here, however, half call very rarely or have no known calls (*e.g.*, *C. tabasarae*, *C. punctariolus*, *S. bufoniformis*, *P. museosus* and *H. fasciatus*). Pending further taxonomic studies, therefore, we can use the mtDNA data in hand to identify what are known as 'unconfirmed candidate species' (Vieites *et al.* 2009; Padial *et al.* 2010), that is we can flag divergent lineages for

Table 2 Number of primary species hypotheses per taxon inferred by the *Automatic Barcode Gap Discovery* (*ABGD*) algorithm (Puillandre *et al*. 2011) applied to three mitochondrial DNA sequence data sets: the COI 'Barcode of Life' fragment, the 16S ribosomal gene, and a combination of COI and 16S sequence data. Numbers in parentheses indicate the maximum value of the *a priori* threshold for conspecific divergence that yielded the given number of primary species hypotheses. For each taxon-by-gene combination, the first entry provides the smallest number >1 of primary candidate hypotheses and its corresponding threshold, whereas the second entry provides the minimum threshold that yielded a single inferred taxon (i.e. no candidate species). Cells have only one entry when a threshold of 15% (the maximum value considered) still supports the presence of multiple taxa, or when a threshold <1% is consistent with a single inferred taxon. N/A indicates insufficient number of sequences for *ABGD* analysis. Note, the *Craugastor punctariolus* data set includes three named taxa, yet the ABGD algorithm recovers >2 (i.e. four) candidate species only with low thresholds of $\leq$2.2% divergence with COI data, $\leq$1.0% with 16S data or $\leq$1.5% with the combined data set

Genus species	COI	16S	Two-gene
Atelopus limosus	1 (0.19%)	1 (0.10%)	1 (0.13%)
**Centrolene* sp.	N/A	1 (0.10%)	N/A
†*Pristimantis museosus*	6 (15%)	4 (10%)	4 (15%)
		1 (12%)	
‡*Craugastor punctariolus*	2 (15%)	2 (15%)	2 (15%)
Craugastor tabasarae	1 (0.10%)	2 (1.9%)	1 (0.10%)
		1 (2.7%)	
Strabomantis bufoniformis	3 (8.4%)	10 (2.3%)	3 (5.2%)
	1 (10%)§	1 (2.7%)	1 (6.1%)§
Colostethus panamansis	3 (5.7%)	5 (2.2%)	3 (4.7%)
	1 (6.9%)	1 (2.6%)	1 (5.7%)
Gastrotheca cornuta	2 (2.6%)	1 (0.23%)	1 (0.72%)
	1 (3.2%)		
Hemiphractus fasciatus	3 (10%)	2 (4.7%)	3 (8.4%)
	1 (12%)	1 (5.7%)§	1 (10%)§
Ecnomiohyla spp.	1 (0.10%)	2 (10%)	N/A
		1 (12%)	

N/A, indicates that genetic distances were not calculable, as <2 samples were available.

**Centrolene* sp. samples were identified as belonging to the confamilial taxon, *Rulyrana* cf. *flavopunctata*, by BLAST search to GenBank, and may constitute an unnamed taxon new to Panama. These samples were not compared to wild-caught Panamanian samples as neither of these genera is known from Panama.

†*Pristimantis museosus* data from wild populations included the closely related *P. cruentus* and an unnamed candidate species, *P.* aff. *museosus* (Crawford et al. 2010a), while EVACC data included a candidate species referred to as *P.* aff. *latidiscus*. For comparisons of ‡*Craugastor punctariolus* with wild populations, we included samples belonging to the newly described species, *P. evanesco*, as individuals may have been selected for *ex situ* conservation before the latter species was described, as well as samples from Costa Rica of the closely related *C. ranoides* that also occurs in Panama.

§Γ-shape parameter (α) increased to 0.5 when maximum likelihood parameter estimates from *jModeltest* yielded very low values (e.g., $\alpha = 0.03$) resulting in genetic distances that were much too high given the data. By increasing α, genetic distance estimates are lower (more conservative).

further study. Thus, mtDNA may serve a practical role in the planning and emergency implementation of captive assurance colonies of tropical amphibians and other endangered yet poorly known animals.

To quantify lineage diversity in the absence of a complete taxonomy, amphibian taxonomists have suggested thresholds of 16S or COI divergence that appear to be associated with specific status in frogs. Vences *et al*. (2005b) assayed genetic divergence at the 16S gene and found that presumably heterospecific lineages could show as little as 2% divergence whereas presumably conspecific populations could show as much as 6% divergence, arguing against a 'one size fits all' threshold for species delimitation. As a first approximation, however, 5% divergence at 16S and 10% divergence at COI were suggested as thresholds to identify potential candidate species from mtDNA surveys (Vences *et al*. 2005a). Subsequent work on Neotropical frogs suggested a 'more inclusive' threshold of 3% at the 16S marker (Fouquet *et al*. 2007). If we apply these latter thresholds (3% at 16S and 10% at COI) to our data, then in addition to the *P. museosus* and *C. punctariolus* cryptic species, we also find one or two candidate species within *H. fasciatus* (Table 2; Fig. 3). If we focus instead on the raw genetic divergences (Table 1) rather than the barcode gap analysis, and apply the above thresholds, then *S. bufoniformis* and *C. panamansis* also contain candidate species that need to be evaluated with integrated taxonomic data (cf. Fig. 3).

The animal mitochondrial genome does not always reflect genetic diversity or divergence at the nuclear gen-

ome, so conservation planners should bear in mind the limits of inferences based solely on mtDNA (Moritz 1994). Within-population variability at mitochondrial loci may or may not predict polymorphism at nuclear loci (Nabholz *et al.* 2008; Piganeau & Eyre-Walker 2009). Divergence at mtDNA may underestimate nuclear genome divergence due to introgression (e.g. Hailer *et al.* 2012) or may overestimate nuclear divergence due to sex-biased dispersal (e.g. Turmelle *et al.* 2011). Thus, the ideal survey of genetic variation would include data from both genomes. As a tool for standardized initial surveys of genetic divergence, mtDNA does have its advantages, however. Significant conflict between mitochondrial and nuclear markers tends to be rare, although the former may offer a more sensitive indicator of population structure (Zink & Barrowclough 2008) due to higher mutation rates and fourfold smaller effective population size (Avise 2004). Finally, animal mtDNA in general and DNA barcoding in particular offer a standardized, high-throughput methodology that may be applied to nearly all taxa, regardless of previous genetic data (Borisenko *et al.* 2009), and these data can be applied to species discovery and delimitation as well as identification (Padial & de la Riva 2007; Goldstein & DeSalle 2011).

Regardless of whether divergent lineages should or should not be recognized and described as distinct species, even conspecific divergence is important in *ex situ* conservation programmes. With the exceptions of the *P. museosus* and *C. punctariolus* lineages, all other nominally conspecific yet divergent lineages represent allopatric populations (Fig. 3). These populations may have genetic variants that represent incompatibilities (Howard *et al.* 1989) or local adaptations (e.g., Phillimore *et al.* 2010; Lind *et al.* 2011) and perhaps cannot or should not be interbred in captivity. DNA barcoding may provide a rapid and standardized assay of population divergence that could inform *ex situ* planning and implementation for species without prior genetic information, such as tropical frogs in assurance colonies.

As amphibian populations continue to be decimated, conservationists must conduct increasingly ambitious efforts to preserve remnants of biodiversity, often utilizing *ex situ* techniques, with limited resources and on species for which very little basic information is available. We suggest that genetic considerations be an integral part of any amphibian conservation response, especially for those projects that involve captive breeding, translocations and releases of amphibians, and funding for such components be allocated during the project's inception (Zippel *et al.* 2006). As argued, DNA barcoding fulfils this objective well. Confirming the existence of cryptic species or any confounding population substructure within a putative amphibian species that is a conservation target early on in a programme can only increase the likelihood of a forming a successful captive assurance colony and, ultimately, a re-established wild population.

Acknowledgements

EVACC was supported by the Houston Zoo, the PARC project partners, and numerous other institutions. Fieldwork was supported by an Association of Zoos and Aquariums (AZA) Conservation Endowment Fund grant to EVACC and a National Geographic Committee for Research and Exploration grant to AJC, KRL and RI. Analysis of EVACC samples was supported by a grant from the Bay and Paul Foundation to PC, with laboratory assistance from Sandra Flechas, Marta Vargas and Maribel González. Wild-caught specimens were analysed at the LAB with the help of Andrea Ormos. Figure 1 was created by Lucas Barrientos. We would like to thank Editor Tim Vines for inviting us to submit this contribution.

References

Allendorf FW, Luikart G (2007) *Conservation and the Genetics of Populations*. Blackwell, Malden, MA.

Angulo A, Icochea J (2010) Cryptic species complexes, widespread species and conservation: lessons from Amazonian frogs of the *Leptodactylus marmoratus* group (Anura: Leptodactylidae). *Systematics and Biodiversity*, **8**, 357–370.

Angulo A, Reichle S (2008) Acoustic signals, species diagnosis, and species concepts: the case of a new cryptic species of Leptodactylus (Amphibia, Anura, Leptodactylidae) from the Chapare region, Bolivia. *Zoological Journal of the Linnean Society*, **152**, 59–77.

April J, Mayden RL, Hanner RH, Bernatchez L (2011) Genetic calibration of species diversity among North America's freshwater fishes. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 10602–10607.

Avise JC (2004) *Molecular Markers, Natural History, and Evolution*, 2nd edn. Sinauer Associates, Inc., Sunderland, Massachusetts.

Bergmann T, Hadrys H, Breves G, Schierwater B (2009) Character-based DNA barcoding: a superior tool for species classification. *Berliner und Münchener Tierärztliche Wochenschrift*, **122**, 446–450.

Berven KA (1982) The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*. I. An experimental analysis of life history traits.. *Evolution*, **36**, 962–983.

Bickford D, Lohman DJ, Sodhi NS *et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, **22**, 148–155.

Borisenko AV, Sones JE, Hebert PDN (2009) The front-end logistics of DNA barcoding: challenges and prospects. *Molecular Ecology Resources*, **9**, 27–34.

Boulenger GA (1896) Descriptions of new reptiles and batrachians from Colombia. *Annals and Magazine of Natural History Series*, **6**, 16–21.

Boulenger GA (1898) An account of the reptiles and batrachians collected by Mr. W. F. H. Rosenberg in Western Ecuador. *Proceedings of the Zoological Society of London*, **1898**, 107–126.

Brower AVZ (2006) Problems with DNA barcodes for species delimitation: 'Ten species' of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). *Systematics and Biodiversity*, **4**, 127–132.

Burnham KP, Anderson DR (2004) Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods and Research*, **33**, 261–304.

Campbell JA, Savage JM (2000) Taxonomic reconsideration of middle American frogs of the *Eleutherodactylus rugulosus* group (Anura: Leptodactylidae): a reconnaissance of subtle nuances among frogs. *Herpetological Monographs*, **14**, 186–292.

Charlesworth B (1998) Measures of divergence between populations and the effect of forces that reduce variability. *Molecular Biology and Evolution*, **15**, 538–543.

Collins R, Boykin L, Cruickshank R, Armstrong K (2011) Barcoding's next top model: an evaluation of nucleotide substitution models for specimen identification. *Methods in Ecology and Evolution*, **3**, 457–465.

Cope ED (1864) Contributions to the herpetology of tropical America. *Proceedings of the Academy of Natural Sciences of Philadelphia*, **16**, 166–181.

Cope ED (1886) Thirteenth contribution to the herpetology of tropical America. *Proceedings of the American Philosophical Society*, **23**, 271–287.

Crawford AJ, Smith EN (2005) Cenozoic biogeography and evolution in direct-developing frogs of Central America (Leptodactylidae: *Eleutherodactylus*) as inferred from a phylogenetic analysis of nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution*, **35**, 536–555.

Crawford AJ, Bermingham E, Polanía C (2007) The role of tropical dry forest as a long-term barrier to dispersal: a comparative phylogeographical analysis of dry forest tolerant and intolerant frogs. *Molecular Ecology*, **16**, 4789–4807.

Crawford AJ, Lips KR, Bermingham E (2010a) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 13777–13782.

Crawford AJ, Ryan MJ, Jaramillo CA (2010b) A new species of *Pristimantis* (Anura: Strabomantidae) from the Pacific coast of the Darien Province, Panama, with a molecular analysis of its phylogenetic position. *Herpetologica*, **66**, 192–206.

Daugherty CH, Cree A, Hay JM, Thompson MB (1990) Neglected taxonomy and continuing extinctions of tuatara (*Sphenodon*). *Nature*, **347**, 177–179.

Dayrat B (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society*, **85**, 407–415.

De Queiroz K (2007) Species concepts and species delineation. *Systematic Biology*, **56**, 879–886.

Dubois A (2003) The relationships between taxonomy and conservation biology in the century of extinctions. *Comptes Rendus Biologies*, **326**, 9–21.

Dunn ER (1933) Amphibians and reptiles from El Valle de Anton, Panama. *Occasional Papers of the Boston Society of Natural History*, **8**, 65–79.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, **17**, 368–376.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Felsenstein J (2005) *PHYLIP (Phylogeny Inference Package) Version 3.6*. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Felsenstein J, Churchill GA (1996) A Hidden Markov Model approach to variation among sites in rate of evolution. *Molecular Biology and Evolution*, **13**, 93–104.

Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. *Molecular Ecology*, **11**, 839–850.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Fouquet A, Gilles A, Vences M *et al.* (2007) Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE*, **2**, e1109.

Fujita MK, Leaché AD, Burbrink FT, McGuire JA, Moritz C (2012) Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution*, **27**, 480–488.

Funk WC, Caminer M, Ron SR (2011) High levels of cryptic species diversity uncovered in Amazonian frogs. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 1806–1814.

Gagliardo R, Crump P, Griffith E *et al.* (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. *International Zoo Yearbook*, **42**, 125–135.

Glaw F, Köhler J (1998) Amphibian species diversity exceeds that of mammals. *Herpetological Review*, **29**, 11–12.

Goldstein PZ, DeSalle R (2011) Integrating DNA barcode data and taxonomic practice: determination, discovery, and description. *BioEssays*, **33**, 135–147.

Hailer F, Kutschera VE, Hallström BM *et al.* (2012) Nuclear genomic sequences reveal that polar bears are an old and distinct bear lineage. *Science*, **336**, 344–347.

Hanken J (1999) Why are there so many new amphibian species when amphibians are declining? *Trends in Ecology & Evolution*, **14**, 7–8.

Hasegawa M, Kishino H, Yano T-A (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160–174.

Hebert PDN, Ratnasingham S, de Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **270**, S96–S99.

Hof C, Araújo MB, Jetz W, Rahbek C (2011) Additive threats from pathogens, climate and land-use change for global amphibian diversity. *Nature*, **480**, 516–519.

Hoffmann M, Hilton-Taylor C, Angulo A *et al.* (2010) The impact of conservation on the status of the World's vertebrates. *Science*, **330**, 1503–1509.

Howard JG, Raphael BL, Brown JL *et al.* (1989) Male sterility associated with karyotypic hybridization in Kirk's dik-dik (*Madoqua kirkii*). Proceedings American Association of Zoo Veterinarians Annual Meeting, pp. 58–60.

Hudson RR (1990) Gene genealogies and the coalescent process. In: *Oxford Surveys in Evolutionary Biology* (eds. Futuyma D, Antonovics J), vol. **7**, pp. 1–44. Oxford University Press, New York.

Ibáñez R, Jaramillo CA, Arosemena F (1994) A new species of *Eleutherodactylus* (Anura: Leptodactylidae) from Panamá. *Amphibia-Reptilia*, **15**, 337–341.

Ibáñez R, Jaramillo CA, Solís FA (1995) Una especies nueva de *Atelopus* (Amphibia: Bufonidae) de Panamá. *Caribbean Journal of Science*, **31**, 57–64.

Jansen M, Bloch R, Schulze A, Pfenninger M (2011) Integrative inventory of Bolivia's lowland anurans reveals hidden diversity. *Zoologica Scripta*, **40**, 567–583.

Jiménez de la Espada M (1872) Nuevos batrácios Americanos. *Anales de la Sociedad Española de Historia Natural. Madrid*, **1**, 84–88.

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: *Mammalian Protein Metabolism* (ed. Munro HN), pp. 21–132. Academic Press, New York.

Kessing B, Croom H, Martin A *et al.* (2004) *The Simple Fool's Guide to PCR*, version 1.0. In: Special Publication, Dept. of Zoology, University of Hawaii, Honolulu, USA.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 6196–6200.

Köhler J, Vieites DR, Bonett RM *et al.* (2005) New amphibians and global conservation: A boost in species discoveries in a highly endangered vertebrate group. *BioScience*, **55**, 693–696.

Lind MI, Ingvarsson PK, Johansson H, Hall D, Johansson F (2011) Gene flow and selection on phenotypic plasticity in an island system of *Rana temporaria*. *Evolution*, **65**, 684–697.

Lips KR (1999) Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology*, **13**, 117–125.

Lips KR, Brem F, Brenes R *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 3165–3170.

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, **91**, 219–227.

Mace GM (2004) The role of taxonomy in species conservation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 711–719.
Maddison DR, Maddison WP (2005) *MacClade 4*. Sinauer Associates, Inc., Sunderland, Massachusetts.
May RM (1990) Taxonomy as destiny. *Nature*, **347**, 129–130.
Meegaskumbura M, Bossuyt F, Pethiyagoda R *et al.* (2002) Sri Lanka: an amphibian hot spot. *Science*, **298**, 379.
Mendelson JR, III, Lips KR, Gagliardo RW *et al.* (2006) Confronting amphibian declines and extinctions. *Science*, **313**, 48.
Mendelson JR, Savage JM, Griffith E *et al.* (2008) Spectacular new gliding species of *Ecnomiohyla* (Anura: Hylidae) from Central Panama. *Journal of Herpetology*, **42**, 750–759.
Mendoza ÁM, García-Ramirez JC, Cárdenas-Henao H (2012) Epithelial mucosa as an alternative tissue for DNA extraction in amphibians. *Conservation Genetics Resources*, **4**, 1097–1099.
Meyer CP, Geller JB, Paulay G (2005) Fine scale endemism on coral reefs: Archipelagic differentiation in turbinid gastropods. *Evolution*, **59**, 113–125.
Miyamoto MM (1983) Frogs of the *Eleutherodactylus rugulosus* group: a cladistic study of allozymes, morphological, and karyological data. *Systematic Zoology*, **32**, 109–124.
Miyamoto MM (1984) Central American frogs allied to *Eleutherodactylus cruentus*: allozyme and morphological data. *Journal of Herpetology*, **18**, 256–263.
Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.
Nabholz B, Mauffrey J-F, Bazin E, Galtier N, Glemin S (2008) Determination of mitochondrial genetic diversity in mammals. *Genetics*, **178**, 351–361.
Padial JM, de la Riva I (2007) Integrative taxonomists should use and produce DNA barcodes. *Zootaxa*, **1586**, 67–68.
Padial JM, Castroviejo-Fisher S, Köhler J *et al.* (2009) Deciphering the products of evolution at the species level: the need for an integrative taxonomy. *Zoologica Scripta*, **38**, 431–447.
Padial J, Miralles A, de la Riva I, Vences M (2010) The integrative future of taxonomy. *Frontiers in Zoology*, **7**, 16.
Paz A, Crawford AJ (2012) Molecular-based rapid inventories of sympatric diversity: a comparison of DNA barcode clustering methods applied to geography-based vs. clade-based sampling of amphibians. *Journal of Biosciences*, **37**, 887–896.
Peters WCH (1862) Über die batrachier-gattung *Hemiphractus*. *Monatsberichte der Königlichen Preussische Akademie des Wissenschaften zu Berlin*, **1862**, 144–152.
Peters WCH (1863) Fernere mittheilungen über neue batrachier. *Monatsberichte der Königlichen Preussische Akademie des Wissenschaften zu Berlin*, **1863**, 445–470.
Peters WCH (1873) Über eine neue schildrötenart, *Cinosternon Effeldtii* und einige andere neue oder weniger bekannte amphibien. *Monatsberichte der Königlichen Preussische Akademie des Wissenschaften zu Berlin*, **1873**, 603–618.
Phillimore AB, Hadfield JD, Jones OR, Smithers RJ (2010) Differences in spawning date between populations of common frog reveal local adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 8292–8297.
Piganeau G, Eyre-Walker A (2009) Evidence for variation in the effective population size of animal mitochondrial DNA. *PLoS ONE*, **4**, e4396.
Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–1256.
Prunier J, Kaufmann B, Grolet O *et al.* (2012) Skin swabbing as a new efficient DNA sampling technique in amphibians, and 14 new microsatellite markers in the alpine newt (*Ichthyosaura alpestris*). *Molecular Ecology Resources*, **12**, 524–531.
Puillandre N, Lambert A, Brouillet S, Achaz G (2011) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, **21**, 1864–1877.
Ratnasingham S, Hebert PDN (2007) BoLD: the barcode of life data system. *Molecular Ecology Notes*, **7**, 355–364. Available from http://www.barcodinglife.org.
Rissler LJ, Apodaca JJ (2007) Adding more ecology into species delimitation: ecological niche models and phylogeography help define cryptic species in the Black Salamander (*Aneides flavipunctatus*). *Systematic Biology*, **56**, 924–942.
Rocha L, Craig M, Bowen B (2007) Phylogeography and the conservation of coral reef fishes. *Coral Reefs*, **26**, 501–512.
Ryan MJ, Savage JM, Lips KR, Giermakowski JT (2010) A new species of the *Craugastor rugulosus* series (Anura: Craugastoridae) from West-Central Panama. *Copeia*, **2010**, 405–409.
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.
Samadi S, Barberousse A (2006) The tree, the network, and the species. *Biological Journal of the Linnean Society*, **89**, 509–521.
Sarkar IN, Planet PJ, DeSalle R (2008) CAOS software for use in character-based DNA barcoding. *Molecular Ecology Resources*, **8**, 1256–1259.
Savage JM (1981) The systematic status of Central American frogs confused with *Eleutherodactylus cruentus*. *Proceedings of the Biological Society of Washington*, **94**, 413–420.
Savage JM, Hollingsworth BD, Lips KR, Jaslow AP (2004) A new species of rainfrog (genus *Eleutherodactylus*) from the Serranía de Tabasará, west-central Panama and reanalysis of the *fitzingeri* species group. *Herpetologica*, **60**, 519–529.
Sites JW, Marshall JC (2003) Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology & Evolution*, **18**, 462–470.
Slatkin M (1991) Inbreeding coefficients and coalescence times. *Genetical Research*, **58**, 167–175.
Srivathsan A, Meier R (2012) On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics*, **28**, 190–194.
Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.
Tamura K (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, **9**, 678–687.
Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28**, 2731–2739.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876–4882.
Turmelle AS, Kunz TH, Sorenson MD (2011) A tale of two genomes: contrasting patterns of phylogeographic structure in a widely distributed bat. *Molecular Ecology*, **20**, 357–375.
Vences M, Thomas M, Bonett RM, Vieites DR (2005a) Deciphering amphibian diversity through DNA barcoding: Chances and challenges. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **360**, 1859–1868.
Vences M, Thomas M, Van der Meijden A, Chiari Y, Vieites DR (2005b) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, **2**, 5.
Vieites DR, Wollenberg KC, Andreone F *et al.* (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 8267–8272.
Voyles J, Young S, Berger L *et al.* (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, **326**, 582–585.
Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11466–11473.
Wang IJ, Crawford AJ, Bermingham E (2008) Phylogeography of the Pygmy Rain Frog (*Pristimantis ridens*) across the lowland wet forests of isthmian Central America. *Molecular Phylogenetics and Evolution*, **47**, 992–1004.

Weigt LA, Crawford AJ, Rand AS, Ryan MJ (2005) Biogeography of the túngara frog, *Physalaemus pustulosus*: a molecular perspective. *Molecular Ecology*, **14**, 3857–3876.

Werle E, Schneider C, Renner M, Volker M, Fiehn W (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Research*, **22**, 4354–4355.

Wiens JJ, Penkrot TA (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology*, **51**, 69–91.

Will KW, Mishler BD, Wheeler QD (2005) The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, **54**, 844–851.

Woodhams D, Kilburn V, Reinert L *et al.* (2008) Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. *EcoHealth*, **5**, 268–274.

Wynn A, Heyer WR (2001) Do geographically widespread species of tropical amphibians exist? An estimate of genetic relatedness within the neotropical frog Leptodactylus fuscus (Schneider 1799) (Anura Leptodactylidae). *Tropical Zoology*, **14**, 255–285.

Xia YUN, Gu H-F, Peng RUI *et al.* (2012) COI is better than 16S rRNA for DNA barcoding Asiatic salamanders (Amphibia: Caudata: Hynobiidae). *Molecular Ecology Resources*, **12**, 48–56.

Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *Journal of Molecular Evolution*, **39**, 306–324.

Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. *Molecular Ecology*, **17**, 2107–2121.

Zippel KC, Ibáñez R, Lindquist ED *et al.* (2006) Implicaciones en la conservación de las ranas doradas de Panamá, asociadas con su revision taxonómica. *Herpetotropicos*, **3**, 29–39.

Zippel K, Johnson K, Gagliardo R *et al.* (2011) The Amphibian Ark: a global community for *ex situ* conservation of amphibians. *Herpetological Conservation and Biology*, **6**, 340–352.

Zwickl DJ (2006) *Genetic Algorithm Approaches for the Phylogenetic Analysis of Large Biological Sequence Datasets Under the Maximum Likelihood Criterion*. The University of Texas, Austin.

P.C., E.G., H.R., E.B. and A.J.C. designed research; A.J.C., E.G., H.R., C.C., A.C.D., R.I. and K.R.L. performed research; A.J.C., C.C. and A.C.D. analysed data; and A.J. C., P.C., K.R.L., E.G., A.C.D. and R.I. wrote the manuscript

Data Accessibility

DNA sequences: GenBank accession numbers are provided in Supplementary Table S1 and DNA sequence data, chromatograms and specimen data are available in two public projects, 'EVACC' and 'EVACW', in the Barcode of Life Database (BoLD).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Maximum likelihood phylogeny based on a partitioned analysis of COI and 16S mitochondrial DNA gene fragments from *Ecnomiohyla* samples, inferred using the software *GARLI* 2.0 and rooted at mid-point. Double asterisks (**) indicate support $\geq 95\%$. Scale bar indicates inferred patristic distance.

Table S1 Taxonomy, sample number, museum voucher numbers (when available), collecting locality and GenBank numbers for all samples used in this study. All localities are in the Republic of Panama. See Fig. 1 in main text for map. AJC = Andrew J. Crawford field number; CH = Círculo Herpetológico de Panamá, Panama City, Republic of Panama; EVACC = El Valle Amphibian Conservation Center sample number, Republic of Panama; FB = Federico Bolaños field number; KRL = Karen R. Lips field number; MVUP = Museo de Vertebrados de la Universidad de Panamá, Republic of Panama; 'swab' = non-vouchered genetic sample from live specimen; UCR = Universidad de Costa Rica, Museo de Zoología, San Pedro, Costa Rica; USNM = Smithsonian Institution's National Museum of Natural History, Division of Amphibians and Reptiles, Washington, D.C., USA.

Table S2 Model of nucleotide substitution best supported by the Bayesian Information Criterion as implemented in *jModeltest* and applied to each mitochondrial DNA data set (EVACC and wild-caught samples combined).