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Morphological Change during Rapid Population Expansion Confounds Leopard Frog Identifications in the Southwestern United States

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Lowland Leopard Frogs (*Rana yavapaiensis*) have experienced extensive population declines over the last century. In California, this species was historically known to occur in scattered localities in the extreme southeastern portion of the state, but it has not been positively documented since 1965. Subsequent to this decline in California, nonnative Rio Grande Leopard Frogs (*R. berlandieri*) have expanded into localities previously occupied by *R. yavapaiensis*. The lack of extensive formal surveys and the difficulty distinguishing between these species using morphological characters have caused uncertainty about whether Lowland Leopard Frogs persist within their historical range in California. Recently, leopard frogs that could not be confidently identified to species have been observed at historical localities of *R. yavapaiensis*. Thus, we undertook a formal study of these populations to characterize their morphological and genetic variation, and conclusively determine to which species they belong. Our genetic analyses demonstrate that these frogs are *R. berlandieri*, but the morphological characters typically used to diagnose these species are largely overlapping. Further complicating field identifications, for some morphological characters, the California *R. berlandieri* are more similar to *R. yavapaiensis* than to native-range *R. berlandieri*. Additionally, invasive *R. berlandieri* show greater variation in a key character—the condition of the inset dorsolateral folds—than that found across much of the species' native range. These results demonstrate the potential for morphological change during rapid population expansions to confound species identifications. Our findings have implications for future efforts to resolve the status of *R. yavapaiensis* in California and to identify other native leopard frogs found within the expanding range of *R. berlandieri*. Our results also highlight the utility of genetic approaches for reliably identifying morphologically similar leopard frogs.

OVER the past century, ranid frog populations in the American Southwest have experienced tremendous flux. The native leopard frog species have declined dramatically, with the extirpation of many populations and the near extinction of at least one species, the Relict Leopard Frog, *Rana onca* (Clarkson and Rorabaugh, 1989; Jennings and Hayes, 1994a; Jaeger et al., 2001; Bradford et al., 2005; Savage et al., 2011). Simultaneously, nonnative American Bullfrogs (*Rana catesbeiana*) and Rio Grande Leopard Frogs (*Rana berlandieri*) have been introduced and have rapidly expanded their range (Rorabaugh et al., 2002; Stebbins, 2003; Goodward and Wilcox, 2019). Tracking these rapid range shifts has been especially challenging because the native and nonnative leopard frog species (*Rana pipiens* complex) are so morphologically similar that positive field identifications are often not possible. This presents a serious challenge for survey and monitoring efforts, especially in areas where native species have declined and documenting their persistence could affect conservation management efforts. As a result, researchers have increasingly turned to genetic analyses to identify species and track distributional changes (Platz et al., 1990; Benedict and Quinn, 1999; Rorabaugh et al., 2002).

The Lowland Leopard Frog (*Rana yavapaiensis*) presents one such challenge. This species is native to the southwestern United States and northern Mexico, ranging from southwestern New Mexico through central and southern Arizona, northern Sonora, southwestern Utah, southern Nevada, and southeastern California. It has declined severely throughout its range, notably in California where it has not been documented since 1965 and may be extirpated (Clarkson and Rorabaugh, 1989; Jennings and Hayes, 1994a, 1994b; Sredl, 2005). As a result of these declines, *R. yavapaiensis* is designated as a Species of Special Concern in California (Thomson et al., 2016). Because this species may persist at low densities, and because much of its historical range is remote and difficult to survey completely, it remains possible, though extremely unlikely, that the species persists in areas where it has not been documented for long periods. If extant populations of *R. yavapaiensis* were to be detected in California, this would pose both a dramatic herpetological rediscovery, as well as a critical conservation opportunity. A needed step following any such claim would be a convincing demonstration that the newly found leopard frogs were in fact *R. yavapaiensis*.

Rana yavapaiensis is difficult to distinguish morphologically from *R. berlandieri*, which has invaded the historical range of *R. yavapaiensis*. This poses a serious difficulty in developing a convincing positive species identification if remaining populations of *R. yavapaiensis* are found. While the two species differ slightly for some morphological characteristics (e.g., *R. berlandieri* has a slightly larger body size and a

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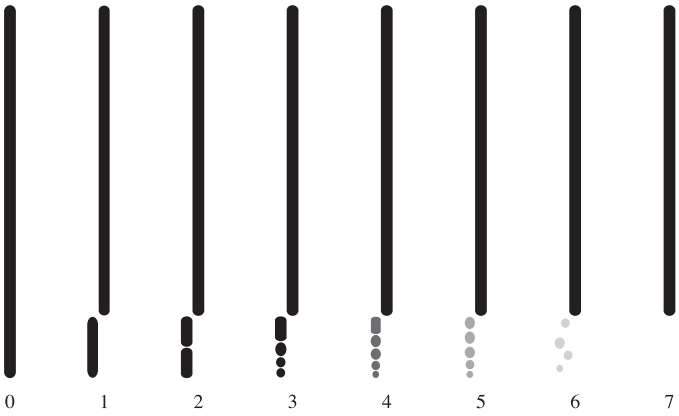


Fig. 1. Types of dorsolateral folds observed in *Rana berlandieri* and *Rana yavapaiensis*. These representations depict the right dorsolateral folds. The color of the medially inset, posterior folds (states 1–6) indicate similarity of the tissue to the primary dorsolateral folds (see text for more information). Black depicts posterior folds that are similar in appearance to the primary fold, and lighter shades of gray indicate skin features that are less pronounced than the raised, glandular tissue of the primary folds. State 1 is typical of *R. berlandieri* across its native range, and state 5 is typical of *R. yavapaiensis*. Modeled after Pace (1974: fig. 23).

different configuration of the dorsolateral folds), their identifying characteristics broadly overlap (Stebbins, 2003). Any populations of *R. yavapaiensis* that remain in California, or other areas where the species is declining, therefore run the risk of being overlooked due to the close morphological similarities of these two species. Similarly, populations of *R. berlandieri,* especially if found in locations previously occupied by *R. yavapaienis,* may be misidentified and incorrectly assumed to be a declining native species of conservation concern. Positive identification of an individual as *R. yavapaiensis* is therefore dependent on genetic data.

Three populations of leopard frogs that appear to be morphologically intermediate between *R. yavapaiensis* and *R. berlandieri* were recently reported in California. In November 2014, B. J. Stacey and J. Keller photographed three leopard frogs southeast of the Salton Sea near Calipatria, Imperial County, California and uploaded these observations to the Reptiles and Amphibians of Southern California (RASCals) project on the iNaturalist citizen science platform (iNaturalist 1066114 and 1066936). These observations were made in a highly-modified agricultural area but within the historical range of *R. yavapaiensis* and in close proximity (ca. 11.5 km) to the collection locality of one of the last specimens of *R. yavapaiensis* collected in the state (LACM 91310, collected March 1956). One photograph showed a frog's entire left dorsolateral fold (DLF). Dorsolateral folds are lines of raised glandular skin, and in many ranid frogs, the length and configuration of these folds is a useful character for distinguishing among species (e.g., McAlister, 1962; Pace, 1974). *Rana berlandieri* and *R. yavapaiensis* have folds that are discontinuous, with the posterior section inset medially. This short, inset DLF is usually a single fold (i.e., a single, long segment) in *R. berlandieri* but a series of small dots of raised tissue in *R. yavapaiensis* (Fig. 1; Pace, 1974; Miera and Sredl, 2000; Stebbins, 2003). Surprisingly, in iNaturalist 1066936, the inset DLF is a series of small dots, consistent with this frog being *R. yavapaiensis,* which, if confirmed, would make it the first Lowland Leopard Frog documented in the state in over 50 years. Later, in February 2016, one of us (SS) observed leopard frogs in the San Felipe Creek watershed, southwest of the Salton Sea, at some of the last localities where *R. yavapaiensis* were observed in California (KU 194236–194241 from 1956 and 1959; see also Ruibal, 1959). Because San Felipe Wash is the only historical locality of *R. yavapaiensis* in California that has not been heavily altered by agricultural development, reservoir construction, and/or irrigation activities, and because this site is isolated and receives only infrequent surveys, there was optimism that these morphologically ambiguous frogs might be *R. yavapaiensis*. Later, in April 2016, leopard frogs of questionable identity were also observed in Indio, Riverside County, California, north of the Salton Sea, and more than 70 km from the two aforementioned localities. The discovery of leopard frogs that could not be confidently identified to species by multiple biologists highlights the need for a better understanding of the morphological variation in these frogs and a genetically confirmed species identification.

Given the extreme conservation importance of any remaining populations of *R. yavapaiensis* in California, we conducted a morphological and genetic study to both positively identify the morphologically intermediate frogs and to more fully characterize the extent of morphological and genetic variation present within these two species. We positively identified these morphologically intermediate populations as nonnative *R. berlandieri*. Our morphological and genetic datasets provide a baseline to which future leopard frog populations in California can be compared, thus easing positive identification for any future leopard frog discoveries.

MATERIALS AND METHODS

Field surveys.—In May 2015, nocturnal searches were conducted along agricultural ditches in the vicinity (<3.5 km) of the iNaturalist observations. We also conducted diurnal surveys of a lower reach of San Felipe Creek near Highway 78. San Felipe Creek was targeted because it was home to one of the last documented populations of *R. yavapaiensis* in California, although the surveys were conducted downstream of the historical breeding localities (Ruibal, 1959). Frogs encountered during these surveys showed a great deal of variation in the inset DLFs, including several individuals with folds more consistent with *R. yavapaiensis* than *R. berlandieri,* as was also seen in iNaturalist 1066936. However, the presence of prominent external vocal sacs in adult males and the occurrence of individuals with inset DLFs typical of *R. berlandieri* convinced us these were not *R. yavapaiensis*, and two to nine individuals were collected opportunistically from each of four localities (lower San Felipe Creek and three localities near the iNaturalist observations). Additional surveys were conducted in March 2016 more broadly around Calipatria and the iNaturalist observations. For the 2016 surveys, collections were made at five localities in an area approximately 14 km N-S and 13 km E-W around Calipatria. Surveys in 2016 also included the upper reaches of the San Felipe Creek watershed including localities with historical records of *R. yavapaiensis* along San Felipe Creek, Fish Creek, and Carrizo Wash. Using the same criteria as in the 2015 surveys, one to 14 individuals were collected opportunistically from each locality. Captured frogs were euthanized via anesthetic overdose (5% benzocaine), and then liver samples

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were taken prior to preserving the specimen in 10% formalin. Tissue samples and the specimens were deposited at the Natural History Museum of Los Angeles County (LACM). Fieldwork was conducted under a scientific collecting permit issued to GBP by the California Department of Fish and Wildlife (SCP-4307).

Morphological analyses.—We examined seven external morphological characters that have previously been suggested to be useful in differentiating *R. berlandieri* and *R. yavapaiensis*. These included the presence of prominent external vocal sacs in males, five morphometric characters, and condition of the posterior dorsolateral folds. The five morphometric characters were (i) snout–urostyle length (SUL), distance from the tip of the snout to the base of the urostyle; (ii) tibiofibula length; (iii) head width at the posterior margin of the tympanum, measured below the labial fold in cases where a pronounced fold was present (this follows Platz et al., 1990; but see Platz, 1976 for a similar measurement taken at the jaw articulation); (iv) head length, distance from the jaw articulation to the tip of the snout; and (v) tympanum diameter. We selected these characters because they were examined in previous studies of *R. yavapaiensis* (Platz, 1976; Platz and Frost, 1984; Platz et al., 1990), with characters iii–v being the most informative characters for differentiating *R. yavapaiensis* and *R. berlandieri* in a previous statistical analysis (Platz et al., 1990: fig. 5, table 2). Characters ii, iv, and v were measured from the right side of the specimen except in cases of injury or damage to the specimen, in which case the character was measured from the left side. All measurements were taken to the nearest 0.01 mm with a digital caliper, and all measurements were taken by one person (GBP).

We also examined the dorsolateral folds. *Rana berlandieri* is typically described as having a discontinuous dorsolateral fold in which the posterior section is interrupted and inset medially near the groin (Fig. 1; Pace, 1974; Powell et al., 1998; Miera and Sredl, 2000; Stebbins, 2003). *Rana yavapaiensis* has a similar configuration except that the small inset fold near the groin tends to be broken into a series of short segments or small dots of raised glandular tissue (Fig. 1; Pace, 1974; Miera and Sredl, 2000; Stebbins, 2003). We scored the inset DLFs as one of the following character states, all of which are depicted in Figure 1:

0. DLF is continuous.
1. DLF is discontinuous, with the posterior section inset medially. Inset DLF is a single fold (i.e., a single, long segment) similar in appearance to the primary DLF. This is the typical condition in *R. berlandieri* (see also Pace, 1974: fig. 23d).
2. DLF is discontinuous, with the posterior section inset medially. Inset DLF has 2 longer segments, each segment consisting of raised tissue similar in appearance to the primary fold.
3. DLF is discontinuous, with the posterior section inset medially. Inset DLF has 1 or 2 segments and $\geq$1 raised dots of tissue similar in appearance to the primary fold.
4. DLF is discontinuous, with the posterior section inset medially. Inset DLF has a single short segment or a series of short segments and dots with the tissue showing some similarity to the raised, glandular tissue in the primary fold.
5. DLF is discontinuous, with the posterior section inset medially. Inset DLF has a series of dots with minimal or no similarity to the raised, glandular tissue in the primary fold. This is the typical condition in *R. yavapaiensis* (see also Pace, 1974: fig. 23e).
6. DLF is discontinuous, with the posterior section inset medially. Inset DLF has some dots but not in a distinct row.
7. DLF is absent in the groin region (i.e., no posterior DLF).

The left and right inset DLFs were found to often vary in the same individual, so both sides were scored. Although this could introduce pseudoreplication into statistical analyses of the DLFs, our goal was to examine the extent to which characters frequently used for identification were diagnostic; thus, understanding the overall levels of variability was a more central concern. The character states were treated as unordered, though states 1 through 5 can be considered points along a continuum of variation from the typical condition in *R. berlandieri* through the typical condition in *R. yavapaiensis*. However, how these states are related developmentally and how they relate to states 0, 6, and 7 are unknown.

Sex was determined by examination of the size and shape of the first digit of the hand especially presence/absence of nuptial pads, examination of the gonads in those specimens previously cut open or for which permission to examine the gonads was secured, and presence/absence of a prominent external vocal sac. This third character is only reliable for differentiating sexes in *R. berlandieri* because *R. yavapaiensis* does not have a prominent external vocal sac, although a less-conspicuous vocal sac can be observed in some adult males during the breeding season (e.g., LACM 13846).

The morphological analyses were largely restricted to adult individuals. Platz (1988) lists minimum sizes at sexual maturity for *R. yavapaiensis* as 46 mm for males and 53 mm for females, but these cut-offs are for SVL, not SUL, which was measured here. Depending on measurement technique, SVL is either the same or slightly larger than SUL. Similar cut-off data do not exist for *R. berlandieri* so specimens between 45 and 55 mm SUL were examined for development of secondary sexual characteristics in males (vocal sac and nuptial pads) and ovaries in females. To add to the existing knowledge of minimum size at sexual maturity for *R. yavapaiensis,* we similarly examined males and females between 46 and 53 mm SUL.

Sex and all morphological characters were examined for 84 museum specimens of *R. berlandieri* from their native range in Texas, New Mexico, and Mexico; 41 museum specimens of *R. yavapaiensis*; and 38 recently collected leopard frogs of unknown identity from southeastern California. One *R. yavapaiensis* was not scored for inset DLFs because of inadequate preservation. After finding high levels of variation in the inset DLFs of some South and West Texas populations of *R. berlandieri*, we scored 6–14 additional individuals from each of four localities (total of 50 additional individuals). We also scored inset DLFs for an additional four *R. berlandieri,* nine unknown individuals from southeastern California, and four *R. yavapaiensis*. In total, we scored inset DLFs from 229 specimens. Locality information, sex, morphological measurements, and DLF condition are provided in the supplemental materials (see Data Accessibility).

To assess whether morphometric trait values differed among *R. berlandieri*, *R. yavapaiensis*, and the frogs of uncertain identity, we performed analyses of variance

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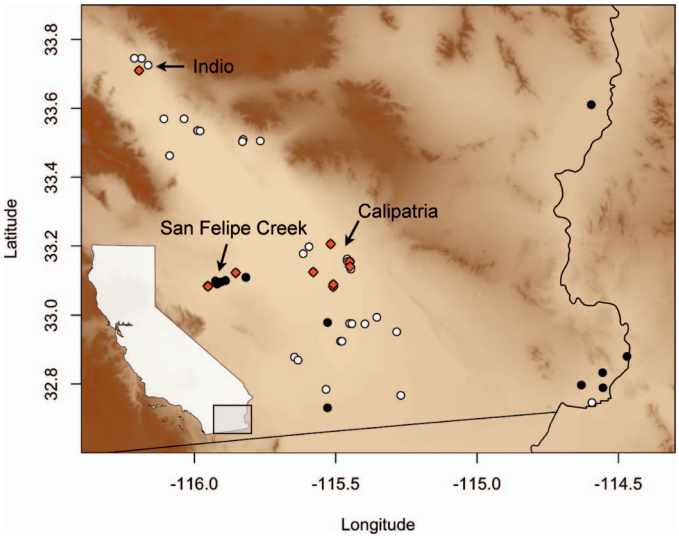


Fig. 2. Map of southeastern California, USA depicting historical localities of *Rana yavapaiensis* (black dots), contemporary localities of *Rana berlandieri* (white dots), and the uncertain specimens that we examine here (red dots).

(ANOVA). To do so, we log transformed each trait and then divided the transformed tibiofibula length, head width, head length, and tympanum diameter by transformed SUL. We treated the two species and the uncertain individuals as separate groups and performed a one-way ANOVA on SUL and each of the four morphometric ratios to ask whether trait means differed among the three groups. When significant differences were found, we identified which comparisons were different using a Tukey multiple pairwise comparison. We then tested for normality of residuals for each ANOVA using a Shapiro-Wilk test and by visualizing the residuals directly using q-q plots. These analyses were carried out in the R programming environment (R Core Team, 2017) and made use of the dplyr v0.7.4 (Wickham et al., 2018) and car v2.1-5 packages (Fox and Weisberg, 2019).

Molecular identification.—We used molecular tools to determine the identity of the unknown individuals. To do so, we assembled a comparative panel from individuals that spanned much of the native range of *R. berlandieri* in the US ($n = 5$), two localities in the nonnative range of *R. berlandieri* in Arizona ($n = 10$), and two localities of *R. yavapaiensis* in Arizona ($n = 4$). We included only individuals for which morphological identifications were unequivocal. We also included samples of the unidentified California frogs, including two from Indio, four from the San Felipe Creek watershed (three from the upper watershed at Fish Creek, and one from the lower watershed), and 11 collected across seven sampling sites in the vicinity of Calipatria (Fig. 2). Many of the individuals from San Felipe Creek and the vicinity of Calipatria were specifically selected because they had inset DLFs more representative of *R. yavapaiensis* than *R. berlandieri* (i.e., inset DLF states 4, 5, and 7). Locality information and GenBank numbers are provided in the supplemental materials (see Data Accessibility).

We extracted genomic DNA from toe or liver tissue using a salt extraction protocol (Sambrook and Russell, 2001), then amplified and sequenced two mitochondrial markers (NADH subunit two and 12S) and four nuclear markers (NTF3, RAG1, Rhodopsin, and Tyrosinase) for all individuals (Table 1). We amplified each marker using PCR in either a 11 µl MangoTaq (Bioline) or 25 µl GoTaqGreen (Promega) mediated reaction. Each reaction was carried out with an initial denaturation of 1 min at 95°C, 38 subsequent cycles with denaturation for 30 s at 94°C, locus-specific annealing temperatures (see Table 1) for 45 s, and extension at 72°C for 1 min, followed by a final extension of 10 min at 72°C. Products were then purified with ExoSAP-IT (Affymetrix) and sequenced in both directions on an Applied Biosystems 3730XL DNA Analyzer at the University of Hawai'i.

We edited, assembled, and aligned sequences using MUSCLE in Geneious v7.1.5 (Kearse et al., 2012). We checked each nuclear locus for heterozygous positions and coded these using IUPAC ambiguity codes. For each alignment, we selected a model of molecular evolution using Akaike Information Criterion (AICc) implemented in jModelTest2 (Darriba et al., 2012). We then estimated phylogeny using MrBayes v3.2.5 (Ronquist et al., 2012) for each individual marker and the concatenated dataset as a whole. These analyses used four independent runs that each used one cold and three incrementally heated Markov chains (temperature parameter = 0.05). We ran each analysis for 10 million generations, recording the state of the cold chain every 10,000th generation. We examined the Markov chain Monte Carlo (MCMC) output in Tracer v1.6 to verify that all chains mixed well, appeared to converge to the same target distribution, and had obtained an adequate number of effectively independent samples from the posterior (ESS > 1000 for all parameters). Finally, we discarded the first 25% of samples as 'burnin,' combined the remaining samples from the four runs, and summarized the results.

RESULTS

Morphological analyses.—We found a large amount of variation in most morphological traits that we measured. No single measurement was adequate to identify the two species or the unknown individuals with certainty. We did

Table 1. Primers used for amplification and sequencing and the associated model of sequence evolution selected via jModelTest2 and used in the Bayesian analyses.

Gene	Primer name (forward; reverse)	Source	Annealing temp (°C)	Model
ND2	ND2_Frog-F; ND2_Frog-R	Newman et al., 2012	56	GTR
12S	12SJ-L; 12SK-H	Zaldívar-Riverón et al., 2004	50	GTR
NTF3	NTF3-F; NTF3-R	Newman et al., 2012	65	F81
Tyrosinase	Tyr1b*Rana*; Tyr1gBufo	Newman et al., 2012	65	K80
Rag1	MartFL1; AMPR1*Rana*	Newman et al., 2012	64.5	HKY
Rhodopsin	Rhod1A; Rhod1D	Bossuyt and Milinkovitch, 2000	56	F81

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Table 2. Mean (and standard deviation) for morphometric traits measured in this study. SUL: snout–urostyle length; TFL: tibiofibula length; HL: head length; HW: head width; TYMP: diameter of tympanum.

Identification	SUL (mm)	TFL/SUL	HL/SUL	HW/SUL	TYMP/SUL	Fraction with vocal sac[a]
R. berlandieri	70.35 (10.89)	0.56 (0.03)	0.37 (0.01)	0.34 (0.02)	0.08 (0.01)	1.0
Uncertain	65.74 (9.18)	0.56 (0.02)	0.38 (0.02)	0.35 (0.02)	0.08 (0.00)	1.0
R. yavapaiensis	62.19 (11.57)	0.55 (0.02)	0.38 (0.01)	0.37 (0.01)	0.08 (0.01)	0[b]

[a] Fraction of adult males with prominent external vocal sacs.
[b] 30% of male *R. yavapaiensis* showed visible outpocketings of skin associated with vocal sac development, but these are not considered prominent external vocal sacs.

find differences in means for several traits, although their distributions often overlapped widely (Table 2, Fig. 3).

The most conspicuous morphological trait that differentiates *R. berlandieri* and *R. yavapaiensis* is the relatively larger size (measured by SUL) of adult *R. berlandieri* (Table 2). In addition, adult male *R. berlandieri* have well developed vocal sacs, and these are absent or only weakly developed in *R. yavapaiensis* (Platz and Frost, 1984). Adult males from the Southern California populations had well developed vocal sacs, suggesting that they are *R. berlandieri*. For large and male frogs, these two traits may suffice to reliably differentiate the two species and identify uncertain individuals.

The remaining morphometric traits were far less useful for differentiating the two species, or for identification of the uncertain specimens, owing to a large amount of overlap in trait values (Table 2, Fig. 3). One-way analysis of variance confirms that *R. berlandieri* has a larger mean SUL and a relatively smaller head width and length than *R. yavapaiensis*. We found no significant difference between the two species in relative length of the tibiofibula or size of the tympanum. Finally, we observed a large amount of variation in the condition of the dorsolateral folds within both *R. berlandieri* and *R. yavapaiensis*, as well as between the left and right dorsolateral fold of individual frogs (Fig. 4). The most common DLF character state differed between the two species, although both showed a large and overlapping amount of variation, such that it is not possible to identify an individual frog with this trait alone. We also saw an effect of locality in the DLF condition for *R. berlandieri* (Table 3). Twenty of the 35 *R. berlandieri* with dorsolateral folds in states 4 through 7 came from a single locality (Miller Ranch, Presidio County, TX). Thus, across most of the native US range of this species, states 4 through 7 are uncommon. Yet, these same character states make up 27% of the observations for the specimens with uncertain identification in California.

The specimens with uncertain identification did not show clear affinities with either *R. berlandieri* or *R. yavapaiensis*. They were intermediate between the two in size and head width, and significantly more similar to *R. yavapaiensis* for head length (Fig. 3). The dorsolateral folds were more similar to those of *R. berlandieri*, although again a large amount of variation within and across individuals was present, and some specimens had character states identical to *R. yavapaiensis*. Taken together, their larger size, head width, and presence of well-developed vocal sacs suggests that they are *R. berlandieri*, although the widely overlapping morphometric data and dorsolateral folds suggest that caution is warranted when making identifications based on morphology, especially when vocal sac condition cannot be assessed.

Molecular identification.—We obtained a total of 4066 bp (1706 bp mitochondrial, 2360 bp nuclear) of sequence data for the 36 individuals. All sequences have been deposited in GenBank (MT114437–MT114472 for 12S; MT112715–MT112858 for NTF3, Rhodopsin, Tyrosinase, and ND2; and MT124627–MT124661 for RAG1; see Data Accessibility). The aligned matrix was nearly complete and contained 2.2% missing data that arose from a small amount of low quality sequence data that we trimmed, and a small number of insertion–deletion events. The phylogenetic analyses mixed well and appeared to converge within the first 25% of MCMC samples.

The phylogenetic analysis of the concatenated alignment recovers a well-supported (Posterior Probability ~1.0) bipartition separating *R. berlandieri* from *R. yavapaiensis* (Fig. 5). All frogs of questionable identity are included within the *R. berlandieri* clade with high posterior probability, indicating that they belong to this species. The independent gene tree estimates were less well resolved but were compatible with the concatenated tree. None of the gene trees gave any indication that the uncertain specimens could potentially be *R. yavapaiensis*, or be hybrids of *R. yavapaiensis* and *R. berlandieri*. The tree contained no strong geographic signal within either the *R. berlandieri* or *R. yavapaiensis* clades.

DISCUSSION

Morphological variation.—Our results have several implications for ongoing efforts to document, conserve, and manage declining leopard frog populations in the southwestern United States. The dataset reported here provides a quantitative assessment of the extent of morphological variation within wild populations of an invasive species with an expanding range. Morphological traits that have previously been suggested as being useful for differentiating the two species do vary between *R. yavapaiensis* and *R. berlandieri*, but there is a great deal of overlap. Many individual frogs cannot be positively identified on the basis of morphology alone, using any known morphological character. Thus, these traits offer little utility for field identifications.

Most importantly, our morphological analyses confirm that the invasive populations of *R. berlandieri* are morphologically intermediate, and for some characters, are actually more similar to *R. yavapaiensis* than they are to native-range *R. berlandieri*. The condition of the DLFs is an especially useful character for rapid identification of many leopard frog species (McAlister, 1962; Pace, 1974; Stebbins, 2003), but in southeastern California, some nonnative *R. berlandieri* have DLFs similar to those observed in *R. yavapaiensis* and rarely observed in native-range populations of *R. berlandieri* (Table 3; Fig. 4; supplemental material; see Data Accessibility). Further, two of five morphometric traits are statistically

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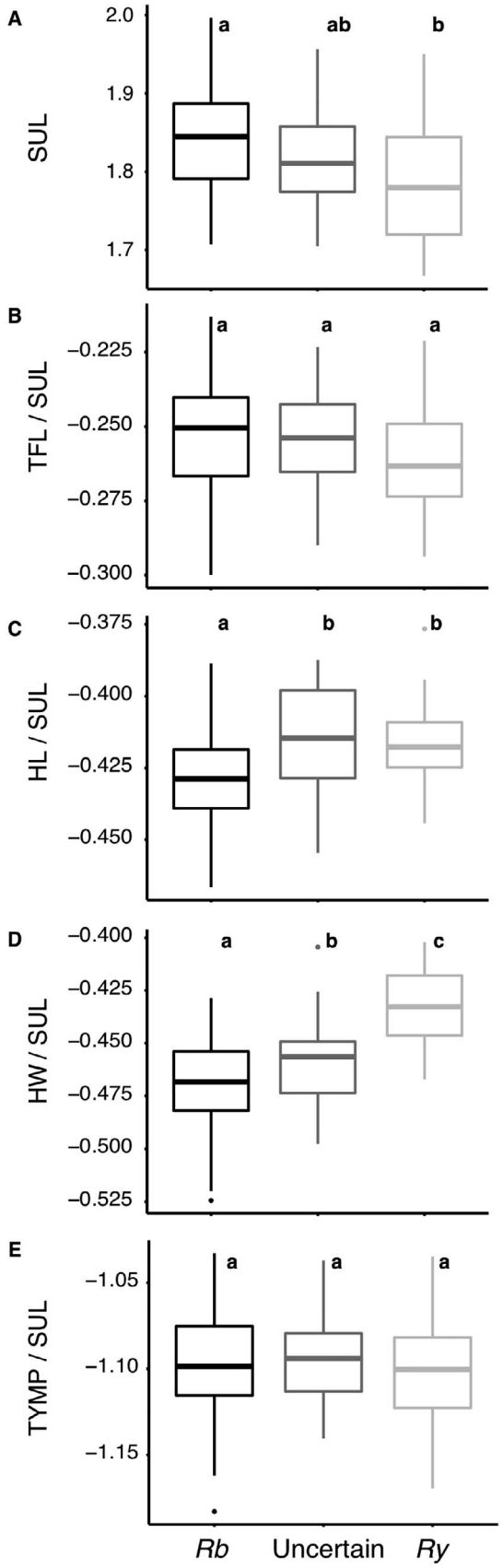


Fig. 3. Distribution of morphometric characters for *Rana berlandieri* (*Rb*), uncertain individuals, and *Rana yavapaiensis* (*Ry*). Trait values are log transformed. Different letters indicate significant differences between groups based on Tukey multiple comparison tests (ANOVA, $P < 0.05$). Abbreviations as in Table 2.

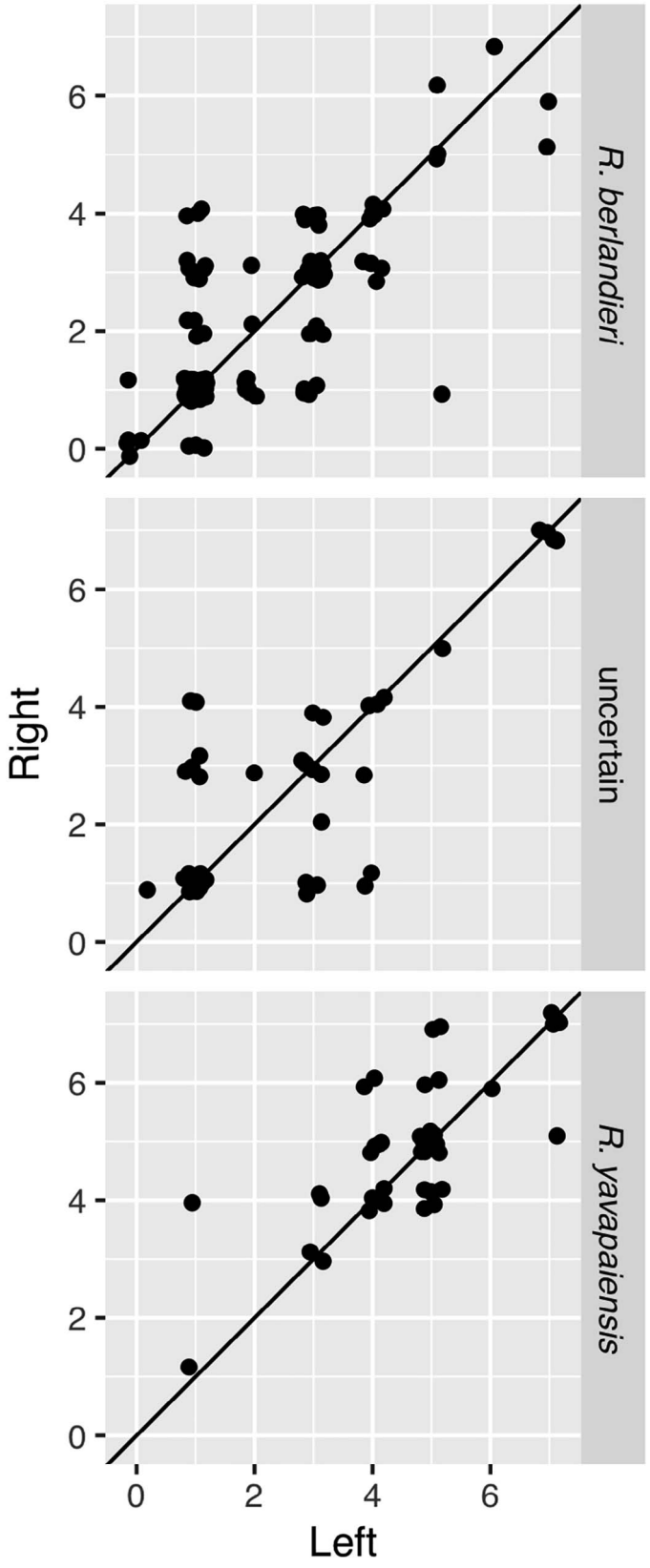


Fig. 4. Character states for the left and right dorsolateral folds for *R. berlandieri*, uncertain individuals, and *R. yavapaiensis*. Points along the dark line indicate individuals with the same character state for the left and right dorsolateral folds. See text for description of character states. We have applied a small amount of random noise to the points to enhance visibility.

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Table 3. Variation in dorsolateral folds. Numbers given are counts of dorsolateral fold character states for the given species or locality. We scored left and right dorsolateral folds for each individual. Thus, the sample size (*n*) corresponds to twice the number of specimens examined.

	Character states							
	0	1	2	3	4	5	6	7
Rana berlandieri ($n = 276$)	12	162	17	50	22	7	3	3
Presidio Co., TX ($n = 38$)	0	5	3	10	13	5	1	1
Hidalgo Co., TX ($n = 38$)	8	23	3	4	0	0	0	0
Uncertain specimens ($n = 94$)	1	46	2	20	13	2	0	10
Rana yavapaiensis ($n = 88$)	0	3	0	6	22	40	6	11

distinguishable between introduced and native populations of *R. berlandieri* (HL and HW; Fig. 3), with nonnative *R. berlandieri* being more similar to *R. yavapaiensis* than would be expected based on analyses of native-range *R. berlandieri* (see also SUL; Fig. 3A).

These results highlight the potential for morphometric trait means to vary in different parts of a species' range, particularly when parts of the range are the result of recent expansions. Thus, we advise caution when using morphological traits to identify leopard frogs, particularly when these traits are being applied to populations outside of those in which the traits were originally assessed.

The unexpected morphological variation observed in nonnative *R. berlandieri* could, in principle, simply result from differences in life stage or growth rates in different parts of the species range, plastic responses to varying environmental cues across the range, or due to evolution of the trait itself. Rapid evolution of morphometric traits has been documented in other invasive populations. This is especially common on invasion fronts, where dispersal-adapted morphological traits are favored, potentially resulting in range-edge phenotypes that are not observed in the range-core area of introduced populations nor in the native range (Chuang and Peterson, 2016). Such patterns have been especially well studied in invasive anuran populations (Phillips et al., 2006; Shine et al., 2011; Perkins et al., 2013) and are also found in other taxa (Laparie et al., 2013). Adaptation to novel food sources, habitats, and climates can also result in rapid morphological shifts in introduced populations (Losos et al., 1997; Herrel et al., 2008). While speculative, it is possible that such a process could be occurring here as well. The available habitat for leopard frogs in Southern California has changed dramatically over the last half century, and it is possible that this change in habitat has led to plastic and/or evolutionary changes in the morphometric characters that we use to recognize species. The fact that these traits are useful for species identification (at least for other leopard frog species pairs) means that they are variable and therefore relatively labile. A better understanding of this variation would be most easily generated by assembling larger morphometric datasets across species ranges and directly assessing variation (and co-variation) that is present. Such data are conceptually simple to collect and would greatly help our understanding of the morphological diversity present in this threatened group of frogs.

Molecular identification and the need for more surveys.—Our phylogenetic analysis establishes the identity of morphologically intermediate leopard frog populations in Southern California as *Rana berlandieri*. We find no evidence for hybrid ancestry or other potential explanations for the morphological intermediacy of these populations, although it is possible that this could exist and go undetected in an analysis of a small number of loci, as we have done here. These molecular tools provide a simple means of species identification for future surveys if additional morphologically intermediate populations are discovered.

It deserves emphasis that these results do not change our understanding of whether *R. yavapaiensis* is extirpated in California. Rather, they should encourage additional survey efforts and the collection of genetic samples. Leopard frogs are known to persist at low densities and to undergo rather marked fluctuations in population size in other areas of the desert southwest (Rorabaugh, 2005). If the species persists in California, it probably does so at low densities. In addition, surveys of tadpoles in suitable waterways could be undertaken to increase detectability. Non-lethal tissue samples are simple to collect for leopard frog tadpoles, and this may serve as our best chance to rapidly inventory and identify leopard frog populations within the state.

Native populations of *Rana berlandieri* encompass an extensive geographic range, occurring from Texas and New Mexico south through Mexico and into Central America. More recent introductions of this species into Arizona, California, and Baja California have led to the establishment of populations that appear to be expanding into additional suitable habitat in these regions (Rorabaugh et al., 2002; Goodward and Wilcox, 2019). This may partially be facilitated by the fact that *R. berlandieri* is successful in a wide range of habitats, and in particular, human modified habitats such as agricultural landscapes. By contrast, *R. yavapaiensis* has experienced extensive declines throughout its range over the past century, and appears to be less tolerant of anthropogenic disturbances (Clarkson and Rorabaugh, 1989). Thus, there is general concern about the potential displacement of any existing native leopard frog populations by introduced *R. berlandieri*, especially in the highly modified remaining habitats in California. This should also motivate surveys of additional localities to more definitively determine if any remaining populations of *R. yavapaiensis* might persist in California as well as to monitor changing distributions of these two species and potential threats posed by *R. berlandieri* in Arizona.

DATA ACCESSIBILITY

Supplemental material is available at https://www.copeiajournal.org/ch-19-222.

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Fig. 5. Majority rule consensus tree of the posterior distribution of trees from the Bayesian analysis of the concatenated alignment (two mitochrondrial and four nuclear markers, with the model of evolution for each marker specified in Table 1). Individuals with uncertain species identification are shown in bold. Numbers below nodes are estimated posterior probabilities. See Data Accessibility for tree file.

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