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A diamond in the rough desert shrublands of the Great Basin in the Western United States: A new cryptic toad species (Amphibia: Bufonidae: *Bufo* (*Anaxyrus*)) discovered in Northern Nevada

MICHELLE R. GORDON[1], ERIC T. SIMANDLE[2] & C. RICHARD TRACY[1]

[1] *Department of Biology, University of Nevada, Reno, NV 89557.*
[2] *Department of Natural Sciences, Paul Smith's College, Paul Smiths, NY, 12970*
[*] *Correspondence: C. Richard Tracy (Email: dtracy@unr.edu)*

Abstract

We describe a new species of toad from the Great Basin region of northern Nevada belonging to the *Bufo* (*Anaxyrus*) *boreas* species complex. This cryptic species was detected through genetic analyses of toad populations sampled throughout the Great Basin and the morphological evidence was quantified through extensive sampling of live toads within the region. The new species has the smallest body size in the species complex, and can be further diagnosed from other species in the complex by its large tibial glands and unique coloration. The known distribution of the new species is restricted to an area less than 6 km^2 in Dixie Valley, Churchill Co., Nevada. The Great Basin is an arid region where aquatic resources are both rare and widely scattered, making habitat suitable for anuran populations highly vulnerable to anthropogenic change. The habitat occupied by this newly described species is threatened by the incipient installation of geothermal and solar power development projects that require the water that defines its habitat.

Keywords: *Bufo* (*Anaxyrus*) *williamsi* **sp. nov.,** Dixie Valley Toad, Western Toad, *Bufo(Anaxyrus) boreas* species complex, cryptic species, morphology, new species, conservation, geothermal

Introduction

The Great Basin, which was covered by large marshes and giant inland lakes during the Pleistocene Epoch, is among the most arid regions in the United States (Sada & Vinyard 2002). Only one percent of the landscape contains an aquatic resource, often in the form of widely dispersed springs, seeps, and small streams. These rare habitats provide an enormous value to flora and fauna that are dependent on these aquatic resources (Shepard 1993; Bogan *et al*. 2014) and represent regional biodiversity hotspots (Shepard 1993). Aside from supporting widespread taxa, Great Basin springs and wetland habitats also harbor high levels of endemic species, including aquatic organisms such as desert fishes (Hubbs & Miller 1948; Hewitt 1996, 2000; Smith *et al*. 2002), springsnails (Hershler & Sada 2002; Sada & Vinyard 2002), and insects (Shepard 1992). Despite the relatively recent recognition of numerous new species of plants and animals associated with these rare habitats, undetected diversity is still suspected given the rarity and isolation of aquatic sites within the region (Sada & Vinyard 2002).

The *Bufo* (*Anaxyrus*) *boreas* species complex occurs within the western United States (Blair 1972; Stebbins, 2003) and includes subspecies *B. b. boreas* (Baird & Girard 1852), *B. b. halophilus* (Baird & Girard 1853), and three narrow endemics known only to occur within the hydrological Great Basin: *B. canorus* (Camp 1916), *B. exsul* (Myers 1942) and *B. nelsoni* (Stejneger 1893). Studies of evolutionary divergence within this species complex have suggested that localized species are relics of more continuous toad populations that diversified allopatrically from *B. boreas* during the Pleistocene (Myers 1942; Karlstrom 1962; Feder 1973; Graybeal 1993; Goebel *et al*. 2009). Analyses of diversity across the large geographic range of *B. boreas* suggest that diversification and speciation within the complex has been underrepresented (Stephens 2001; Goebel 2005; Goebel *et al*. 2009). While prior studies have included samples of populations across much of the geographic distribution of the *B. boreas* species complex, samples were limited or absent from the interior of the Great Basin. Our recent morphological and

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genetic analyses of *B.* cf. *boreas* populations within the Great Basin (Tracy *et al*. unpubl. data) has uncovered significant divergences among toads in the isolated basin of Dixie Valley, Nevada, indicating that this population should be recognized as a new species. Here, we use morphological and genetic evidence to describe and diagnose this new endemic member of the *B. boreas* species complex.

Materials and Methods

Morphological data collection. Measurements were collected from live adult toads (n = 380) from 17 distinct populations throughout the hydrological Great Basin (Fig. 1a) including *B. boreas* (n = 289), *B. nelsoni* (n = 31), *B. exsul* (n = 30), and *B.* **sp. nov** (n = 76). Fourteen morphological characters were recorded: snout–vent length (SVL; tip of snout to posterior end of urostyle), head length (HL; tip of snout to occiput), head width (HW; at widest part of the head), snout length (SL; tip of snout to anterior corner of eye), internarial distance (IND; distance between nares), eye diameter (ED; at widest part of eye), interorbital space (IOD; shortest distance between medial margin of upper eyelids), tympanum diameter (TYM; at maximum width of tympanum), parotoid length (PTL; horizontal length of parotoid gland) and width (PTW; maximum width of parotoid), interparotoid distance (IPD; shortest distance between medial margin of parotoid glands), femur length (FML; distance between vent and knee), tibia length (TBL; distance between knee and heel), and hind foot length (FTL; distance from anterior margin of heel to distal end of the third toe). All morphological characters were measured using Mitutoyo digital calipers to a precision of 0.01mm. ETS measured all individuals with the exception of 46 individuals collected from Dixie Valley, Nevada, which were measured by MRG, including the holotype and paratypic series. Sex was determined in the field near breeding sites where adults congregate, noting behavior and secondary sex characteristics such as the presence of nuptial pads on males as identifiers.

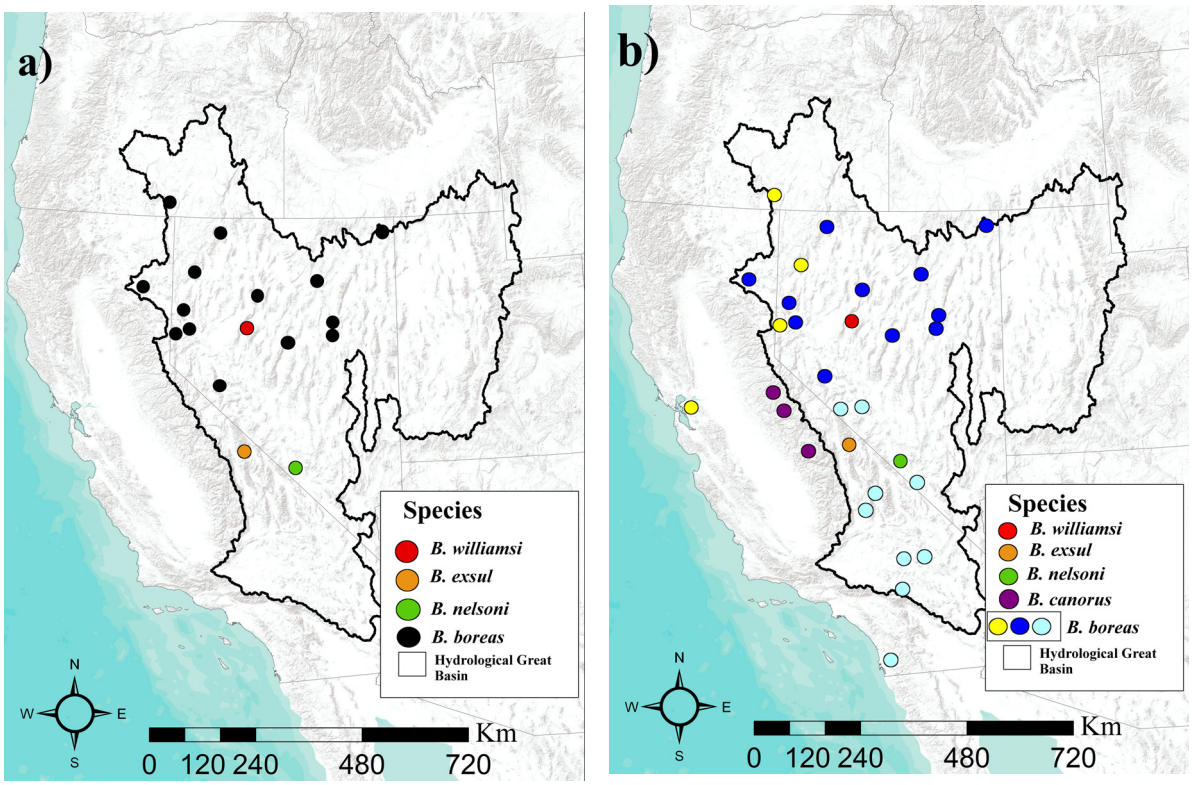

FIGURE 1. Sampling localities of populations included for morphological (a) and DNA (b) collections within the hydrological Great Basin and surrounding states. a) Colors indicate species-specific populations measured for morphological analysis. b) Colors correspond with localized species and *B. boreas* colors correspond with major mtDNA haplotype clades (ONV- Oregon-NW Nevada (yellow), HL-Humboldt-Lahontan (blue), M-Mojave (aqua)) identified in Tracy *et al*. (*in progress*) molecular study of *B. boreas* diversity. Maps created using ArcGIS software by ESRI (2011: Release 10).

Individuals selected to represent the type series were euthanized and preserved following the guidelines under the Institutional Animal Care and Use Committee (IACUC) from University of Nevada (UNR IACUC #00066). Tissue samples were extracted and preserved in 70% ethanol and specimens were fixed in 10% buffered formalin and transferred to 70% ethanol.

Morphological analyses. We used multivariate analysis of covariance (MANCOVA) to quantify morphological differences among species and populations. We used SVL as a covariate to account for the effect of body-size variability in regressions against each morphological variable (Dahl & Peckarsky 2002; McCoy *et al*. 2006). This analysis results in least squares means from each regression for each size corrected morphological character which identify subtle, but significant differences in the fine features of these toads. Additionally, we log transformed the morphological measurements as an additional way to account for allometric differences among measured toads. Likewise, we analyzed these scaled data using MANCOVA to quantify morphological differences by population and by species (Lleonart *et al*. 2000). Tukey HSD post-hoc tests were used to identify significant differences among the morphological characters in pairwise comparisons by species resulting from the MANCOVA analyses for both size-corrected data sets. We used a cross-validated discriminant function analysis (DFA) to evaluate the variation in multivariate space to identify variables that best discriminated among the species. Hayek *et al*. (2001) cautioned that multiple measurers, despite care, result in interobserver error, particularly on fine features of amphibian anatomy and that these biases could result in different biological interpretations of morphometric analyses. To avoid interobserver biases, only measurements by ETS were used in the multivariate analyses, whereas the means table (Table 1), including the holotype are raw, unadjusted measures from both ETS and MRG. Our Table 2 provides the least squares means generated from the regression analyses, plus results from post hoc tests where significant differences were detected. All statistics were conducted using JMP Pro v. 10 (SAS Institute Inc. Cary, N.C.).

TABLE 1. Morphological variation of four species of the *Bufo* (*Anaxyrus*) *boreas* species complex within the Great Basin. Fourteen morphological measurements (in mm) are as follows: snout–vent length (SVL), head length (HL), head width (HW), snout length (SL), internarial distance (IND), eye diameter (ED), interorbital distance (IOD), tympanum diameter (TYM), parotoid width (PTW), parotoid length (PTL), interparotoid distance (IPD), femur length (FML), tibial length (TBL), and foot length (FTL). Data include sample size (n), character mean ± standard deviation, and range. The values reflect unadjusted raw data, which includes additional individuals of *B. williamsi* from sampling efforts in 2014 and 2015 (n = 46), plus the holotype.

	Holotype	*B. williamsi* (n=76)	*B. exsul* (n=30)	*B. nelsoni* (n=31)	*B. boreas* (n=289)
SVL	52.92	54.6±4.73 44.01–69.97	64.0±8.43 53.00–79.00	80.8±13.0 57–122	82.3±12.2 53–113
HL	17.45	16.02±1.62 11.63–19.98	18.64±2.42 15.04–22.90	23.99±3.34 17.2–31.8	23.96±2.98 16.9–30.9
HW	18.35	18.23±1.50 14.81–24.32	20.6±3.00 16.55–25.02	28.1 ±4.35 19.0–37.7	27.6±3.89 18.5–36.21
SL	6.61	5.43±0.90 3.92–7.41	4.42±0.65 3.30–6.21	5.41±0.79 4.46–7.56	5.57±0.77 3.67–8.44
IND	2.13	3.19±0.86 1.50–4.61	4.31±0.51 3.08–5.56	5.23±0.72 3.99–7.16	4.97±0.62 3.1–6.70
ED	4.94	5.76±0.92 3.17–7.70	6.04±0.70 4.68–7.10	7.59±1.09 5.39–10.91	7.47±0.96 4.65–10.31
IOD	3.83	5.37±3.22 1.69–10.55	9.73±1.04 7.79–11.85	12.2±2.03 9.3–18.66	12.9±1.68 9.09–17.79
TYM	2.89	2.81±0.50 1.84–3.88	3.23±0.34 2.74–4.16	4.17±0.70 3.19–5.54	4.28±0.83 2.43–6.62

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TABLE 1. (Continued)

	Holotype	*B. williamsi* (n=76)	*B. exsul* (n=30)	*B. nelsoni* (n=31)	*B. boreas* (n=289)
PTW	3.90	5.18±0.79	5.38±0.82	6.89±1.01	7.1±1.14
		3.36–7.40	3.76–6.52	5.12–9.47	4.49–10.59
PTL	5.56	6.50±0.95	6.82±0.92	8.83±1.33	9.99±1.58
		4.20–9.49	5.53–8.75	5.69–11.52	6.61–14.58
IPD	9.18	10.35±1.11	11.9±1.53	15.6±2.66	15.8±2.39
		8.23–14.00	10.00–15.00	11.50–23.0	11.00–23.0
FML	19.54	19.60±2.65	24.5±3.30	31.13±4.59	32.8±4.38
		14.22–27.00	19.0–30.0	20.0–41.0	22.0–44.0
TBL	18.75	18.20±2.68	24.2±3.36	30.3±4.57	32.2±4.43
		13.21–24.0	18.0–29.0	19.0–38.0	22.0–43.0
FTL	33.10	26.52±4.18	26.2±3.84	32.6±5.02	33.8±4.44
		19.0–38.68	19.0–31.0	23.0–45.0	21.0–44.0

Genetic data. Tissue samples were collected from measured individuals of *B. boreas* populations (Fig.1b: n = 308), *B. nelsoni* (n = 32), *B. exsul* (n = 30), and *B. canorus* (n = 32) for a broader study of *B. boreas* phylogeography and species diversity within the hydrological Great Basin (Tracy *et al*. unpubl. data). Whole genomic DNA was extracted from dried toes or liver tissues stored in 95% ethanol and extracted using DNeasy Blood and Tissue Kit (Qiagen). Primers were designed based on the sequenced whole mitochondrial genome of *B. boreas* (not published). We used the mitochondrial control region (CR) as our genetic marker. This is a rapidly evolving region of mtDNA that is ideal for evaluating intraspecific polymorphism (Avise *et al*. 1987) and has been used in previous phylogenetic studies of *B. boreas* (Stephens 2001; Goebel *et al*. 2009). A 1.6 kb fragment of the CR was amplified via PCR with primers Bmt14844F and Bmt14200R. The PCR reagent included 1-5ng/uL template DNA, 1X PCR buffer, 4 mM dNTP mix, 0.5 uM of each primer, and TaqPlus Long PCR enzyme (Stratagene) in water to achieve the desired reaction volume. PCR conditions for the target genetic marker consisted of 30 cycles for 30 s at 95˚C, 45 s at 55˚C, and 1 min at 68˚C, followed by 5 min for final elongation at 68˚C. Fragments were purified by either gel purification (fragment sizes over 1 kb) or by column filtration. DNA concentration was determined by fluorescence and then sequenced with the same primers used for PCR and with primers internal from the PCR primers: Bmt14844F, Bmt14999R, Bmt 14223F, Bmt 15273F, Bmt 15400R, Bmt 15612R, Bmt 15777F, Bmt 15930R, Bmt 16207F, Bmt 16237R, Bmt 14200R. In previous studies of the *B. boreas* complex (Stephens 2001; Goebel *et al*. 2009), distantly related bufonids within the genus were adequate outgroups to evaluate genetic variation of the CR, and *Bufo punctatus* was selected for our study. For *B. punctatus*, primers were designed first based on the *B. boreas* sequence, then later based on D-loop sequences for *B. punctatus* that are within the CR. We amplified 300 to 500 bp fragments of D-loop and then sequenced these fragments using the same PCR primers. DNA was sequenced by an ABI 3730 Sequencer and data were analyzed with Sequencher software (Gene Codes, Ann Arbor, Michigan).

The final alignment of the *B. boreas* group (CR 1622bp) was completed in ClustalW (Larkin *et al*. 2007) within Mega 7.0 (Kumar *et al*. 2015) resulting in 72 unique haplotypes, which were included in subsequent analyses. To examine pairwise genetic distances among sequences relative to the haplotypes identified, a Jukes-Cantor model (Jukes & Cantor 1969) was applied in Mega 7.0 (Kumar *et al*. 2015).

Genetic analyses. Previous molecular studies have shown that taxa within the *B. boreas* species complex are close relatives that have diverged recently (Graybeal 1993; Shaffer *et al*. 2000; Stephens 2001; Pauly *et al*. 2004; Goebel *et al*. 2009). To examine population level genealogy, a TCS haplotype network was constructed in PopART (Clement *et al*. 2002; Leigh & Bryant 2015). Phylogenetic hypotheses were tested using both Bayesian inference (BI) and maximum likelihood (ML) methods to compare tree reconstructions highlighting relationships between taxa of this species complex.

Bayesian inference analyses were conducted using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003). The BI analysis included 2 x 10^7 generation of Markov chains sampled every 1000 generations. A standard 25 % burn-in was performed. In Mega 7.0 (Kumar *et al*. 2015), a ML phylogeny was constructed with the model GTR + G+ I. The program Tracer v. 1.6 (Rambaut et al. 2014) confirmed that stationarity was obtained and trees were constructed using FigTree v. 1.4.2 (Rambaut 2014). A condensed tree was constructed in Mega 7.0 (Kumar *et al*. 2015) for simplicity as the broader phylogeographic analyses for the *B. boreas* group is in progress.

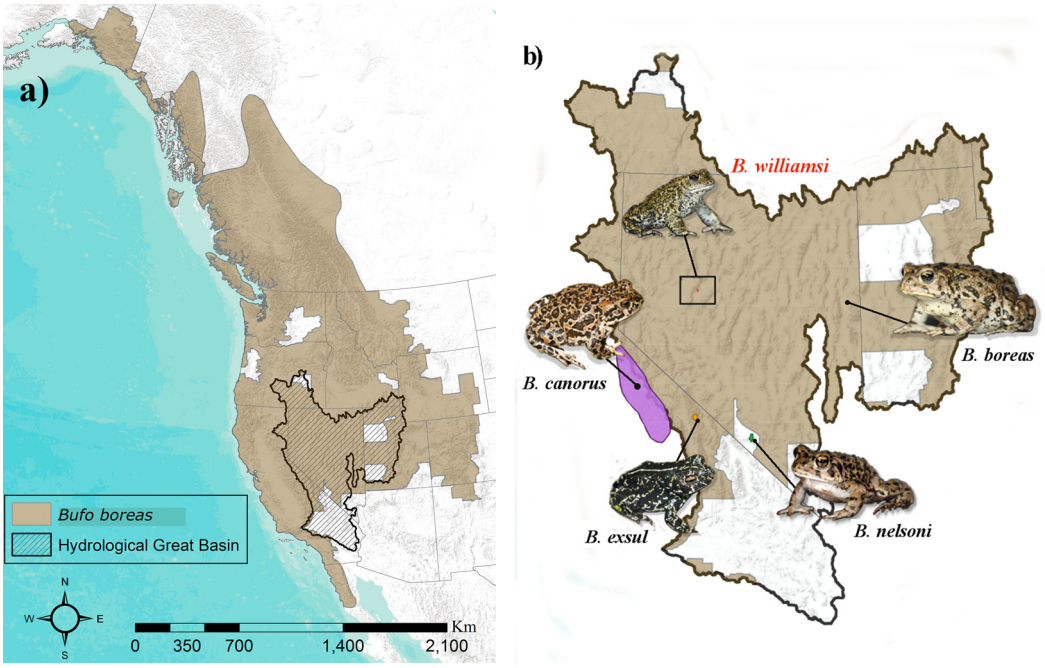

FIGURE 2. *Bufo* (*Anaxyrus*) *boreas* species complex distribution**.** a) *Bufo* (*Anaxyrus*) *boreas* distribution (shown in brown) across the Western United States with hydrological Great Basin shown with black outline and hash mark interior; b) *Bufo* (*Anaxyrus*) *boreas* species complex and ranges for toads including new species, illustrating the narrow distribution of localized endemics. Spatial data for all toads except *B. williamsi* provided by IUCN (2015). Images taken by M.R.Gordon except *B. canorus* with photo credit to G. Nafis.

***Bufo* (*Anaxyrus*) *williamsi.* sp. nov.**
Dixie Valley Toad
(Fig. 2b, Fig.4)

Holotype. CAS 259271 (California Academy of Science Herpetology Collection), adult male (Fig. 4, Table 1), Dixie Valley, Churchill County, Nevada, United States (39°47'39.02"N, 118° 3'32.08"W), on 3 June 2015 by M. R. Gordon, K. Nicholson, C. Mo and C. Gibson.

Paratypes. UNR 7918, adult male; UNR 7919, adult female; UNR 7920, subadult; UNR 7921, adult male; UNR 7922, adult female; UNR 7923, subadult; UNR 7924, adult male. Same locality, collection date, and collectors as holotype.

Diagnosis. *Bufo* (*Anaxyrus*) *williamsi* is distinguishable from *B. boreas* by a combination of diagnostic morphological characters (Fig. 4; Table 1, Table 2), genetic evidence (Fig.3, Fig. 6), and localized distribution (Fig. 2b). *Bufo* (*Anaxyrus*) *williamsi* is distinct from *B. boreas* by: a small adult body size (SVL is more than 2.5 cm smaller than *B. boreas*; Table 1); significantly, but modestly, larger, closely-set eyes, and smaller head (Table 2); statistically and perceptibly larger tympanum, and shorter hind limbs; conspicuously large and elevated tibial glands; and distinctive color pattern (Fig. 4a, Fig. 4b).

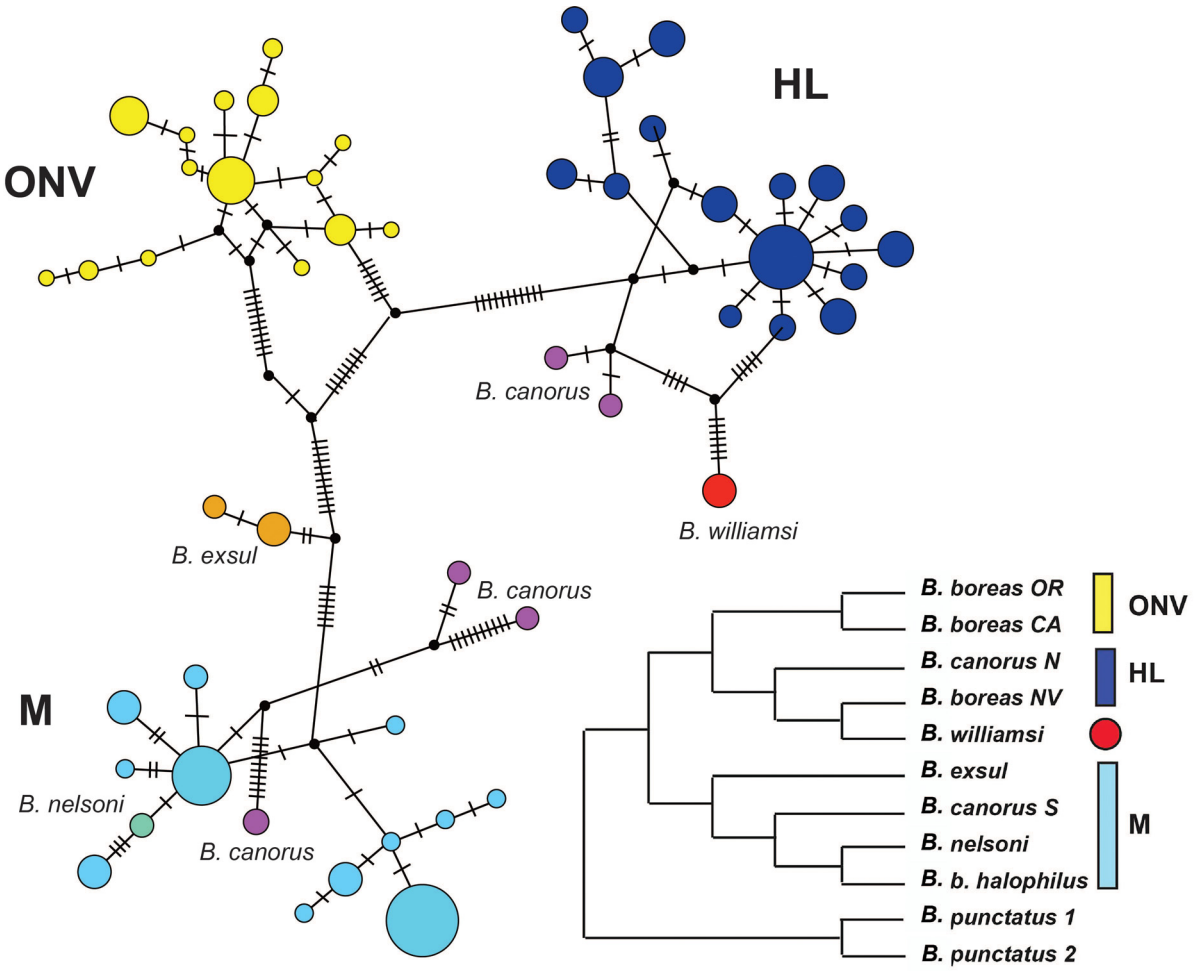

FIGURE 3. Molecular examination of *Bufo* (*Anaxyrus*) *boreas* species complex.The TCS haplotype network was constructed using 246 sequences (1622 aligned sites) obtained from toad sampling (Fig.1b) resulting in 72 unique haplotypes, with circle sizes corresponding with the number of individuals of a particular haplotype. Haplotype colors correspond geographically (Fig. 1b) and to localized species (*B. canorus* (purple), *B. exsul* (green) and *B. nelsoni* (orange)) and highlight the genetic divergence of *B. williamsi* (red). The condensed phylogeny identifying Great Basin *Bufo* (*Anaxyrus*) *boreas* species complex major haplotype clades: maximum likelihood of 10 samples (1436 aligned sites) using GTR +G+I evolutionary model. The terminals are identified by taxon name and followed by locality of collection for *B. boreas*. *Bufo williamsi*, noted with a red circle, is sister to *boreas* of the HL clade. Heavy bars correspond with major haplotype clades.

TABLE 2. Least squares means, confidence intervals and Tukey HSD post-hoc test results for four species of the *Bufo* (*Anaxyrus*) *boreas* species complex within the Great Basin. The least square values were generated from the MANCOVA analysis of 14 size-corrected morphometric characters described in Table 1 with corresponding lower and upper 95% confidence intervals (CI) and sample size for each species (n). Tukey HSD post-hoc tests results identify significant smaller (↓) or larger (↑) states exhibited by congeneric species when compared to *B. williamsi*.

	B. williamsi (n=30)	*B. exsul* (n=30)	*B. nelsoni* (n=31)	*B. boreas* (n=289)
SVL	56.3	64.0 ↑	80.8 ↑	82.3 ↑
CI	52.1, 60.4	59.8, 68.1	76.8, 84.9	81.0, 83.7
HL	22.2	22.0	23.4 ↑	23.1 ↑
CI	21.7, 22.6	21.5, 22.4	23.0, 23.8	23.0, 23.3
HW	25.2	25.0	27.4 ↑	26.5 ↑

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TABLE 2. (Continued)

	B. williamsi (n=30)	*B. exsul* (n=30)	*B. nelsoni* (n=31)	*B. boreas* (n=289)
CI	24.6, 25.8	24.5, 25.6	27.0, 27.9	26.4, 26.7
SL	5.62	5.10↓	5.31	5.40
CI	5.43, 5.83	4.91, 5.30	5.14, 5.50	5.34, 5.46
IND	4.87	4.81	5.15	4.84
CI	4.68, 5.05	4.64, 4.99	4.99, 5.31	4.79, 4.90
ED	7.83	6.98 ↓	7.45	7.24 ↓
CI	7.58, 8.01	6.77, 7.19	7.26, 7.65	7.18, 7.31
IOD	11.8	11.4	11.9	12.5 ↑
CI	11.5, 12.2	11.07, 11.77	11.58, 12.22	12.40, 12.62
TYM	4.46	4.06 ↓	4.06 ↓	4.10 ↓
CI	4.28, 4.65	3.88, 4.23	3.89, 4.22	4.04, 4.15
PTW	6.65	6.32	6.75	6.87
CI	6.33, 6.97	6.02, 6.62	6.47, 7.02	6.78, 6.96
PTL	8.57	8.04	8.65	9.69 ↑
CI	8.12, 9.02	7.62, 8.46	8.26, 9.05	9.55, 9.82
IPD	14.9	14.4	15.2	15.2
CI	14.5, 15.4	14.0, 14.9	14.8, 15.6	15.1, 15.3
FML	29.5	29.3	30.4	31.6 ↑
CI	28.8, 30.2	28.6, 30.0	29.8, 31.0	31.4, 31.8
TBL	28.3	29.1	29.6 ↑	31.0 ↑
CI	27.6, 29.0	28.5, 29.8	29.0, 30.2	30.8, 31.2
FTL	29.9	30.8	31.9 ↑	32.7 ↑
CI	28.9, 30.8	30.0, 31.7	31.1, 32.7	32.4, 32.9

Bufo **(***Anaxyrus***)** *williamsi* is the smallest bufonid within the *B. boreas* species complex (Table 1, Table 2). This new species has a statistically, but modestly short, narrow head similar to the small sized *B. exsul*, but *B. williamsi* can be distinguished from *B. exsul* by a significantly, but modestly, longer relative snout length comparable to that *B. boreas* and *B. nelsoni* (Table 2). *Bufo* **(***Anaxyrus***)** *williamsi* has relatively large, closely set eyes and perceptively large tympanum, which distinguishes this toad from all taxa within the *B. boreas* species complex. The parotoid glands are slightly longer than wide, but are comparatively shorter overall than parotoids of *B. boreas* (Table 2*). Bufo* (*Anaxyrus*) *williamsi* has hind legs that are similar in relative size to *B. exsul*, but significantly and perceptibly shorter than those of *B. boreas* and *B. nelsoni.* The tibial glands exhibited in *B. williamsi* are conspicuous and approximately the width of the parotoid glands, regular in shape and rust colored with little variation among individuals of this species. In addition to morphological shape differences, *B. williamsi* exhibits unique coloration different from taxa of the *B. boreas* species complex. The dorsal ground color consists of olive shades that contain minute black flecks, rust colored warts are bordered by fine, black halos, and prominent parotoid glands are pale tan and black specked. The venter of *B. williamsi* is similar to *B. exsul*, exhibiting sharply contrasted black marbling against a white background color on the anterior sides of the limbs and belly. The presence of a dorsal stripe is variable among individuals of *B. williamsi*, as is similar to the other members of the *B. boreas* complex, with the exception of *B. exsul*.

Distinct nuptial pads develop on the dorsal side of the thumb in males of *B. williamsi*, a typical secondary sexual characteristic exhibited among most bufonids. This species lacks an advertisement call, but retains a release call that sounds like the weeping of a chick (Stebbins 2003). The call is emitted when males come into contact with one another, similar to congeners of the *B. boreas* complex.

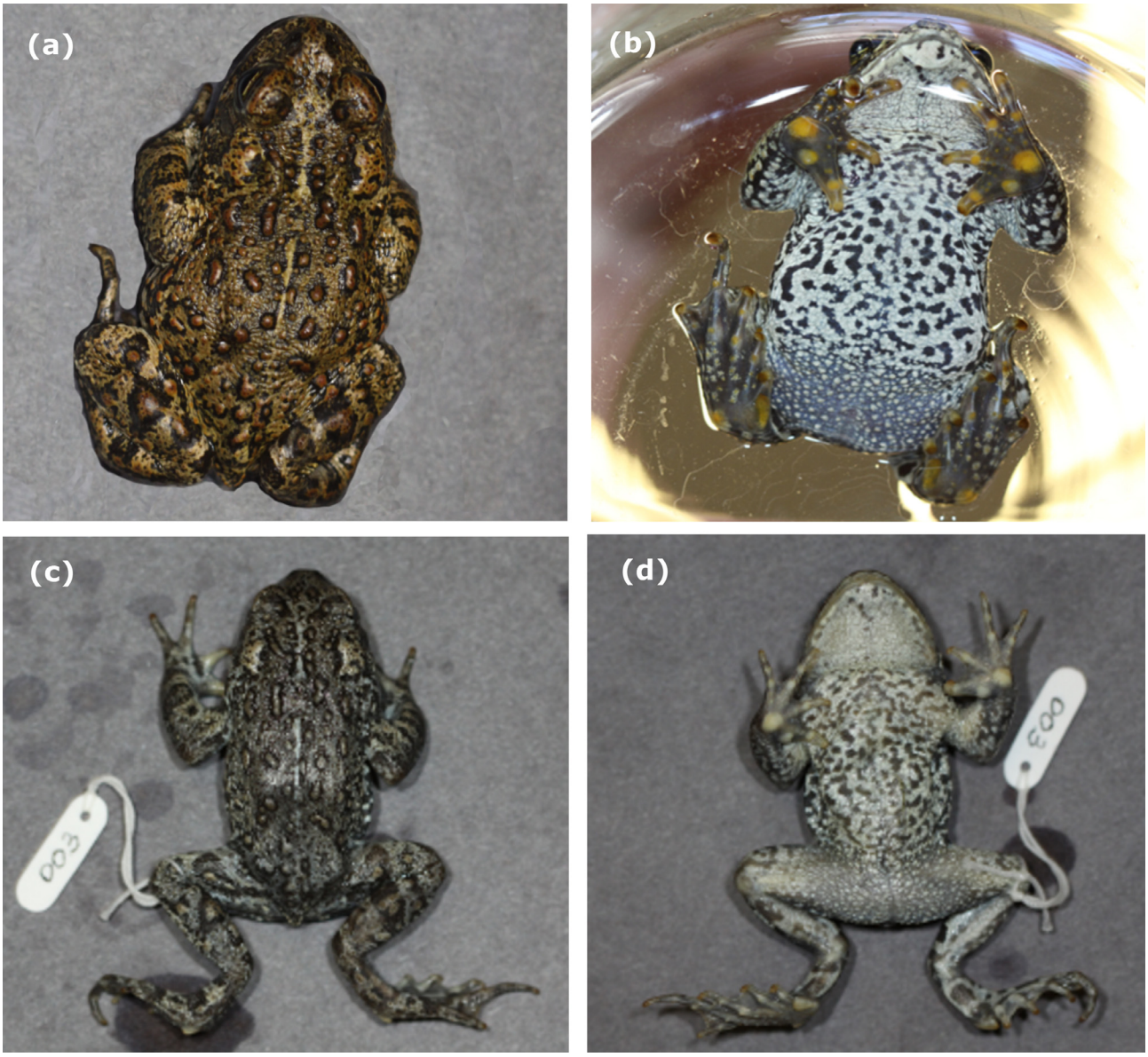

FIGURE 4. Photographs of *Bufo* (*Anaxyrus*) *williamsi* **sp. nov.** holotype (CAS 259271). Adult male toad presented live: (a) dorsal view and (b) ventral view; and preserved: (c) dorsal view and (d) ventral view. Photographs taken by M.R.Gordon.

Description of holotype. Body small (SVL = 52.92 mm), robust; head nearly long (17.45 mm) as wide (18.35 mm; 95 % head length to head width). Dorsal outline of snout is moderately truncate; snout long in lateral view (6.61 mm; 1.3 times longer than eye diameter). Canthus rostralis distinct, slightly concave and abrupt at nares, sloping up to anterior margin of orbits. Loreal region moderately concave. Nostrils protuberant, directed dorsolaterally and closer to anterior corner of eye than end of snout. Internarial distance 75% of eye-to-naris distance. Eyes large (4.94 mm), close spaced (3.83 mm); interorbital distance 75% of eye diameter. Eyes prominent, breaching snout profile in dorsal view. Tympanum distinct, ovoid, relatively large (2.89 mm; 58% of eye diameter). Supratympanic fold present. Parotoid glands sub-elliptical, tapered at posterior margin of eye, longer (5.56 mm) than wide (3.90 mm; 77%). Parotoids elevated dorsally, slightly divergent and separated (9.18 mm); gland width smaller than eye diameter (75%). Forearms robust. Fingers unwebbed; relative lengths III > VI > I > II; nuptial pads present, raised on dorsal side of digit I; tips rounded, subarticular tubercles moderate, round; accessory palmar tubercles small and round. Thenar tubercle raised, prominent, and round. Palmar tubercle is distinct, large, subovoid, separated from medial margin of lesser thenar tubercle. Hind limbs short (FML =19.54 mm; TBL =18.75 mm; FTL =33.10 mm), robust; femur slightly longer than tibia. Tarsal fold present. Hind feet webbed proximally. Relative toe lengths IV > III > V > II > I; tips rounded. Subarticular tubercles moderate, small,

round; plantar tubercles small, numerous. Inner metatarsal tubercle pronounced, elevated, and elliptical. Outer metatarsal tubercle smaller than inner metatarsal tubercle, conspicuous, ovoid.

Longitudinally along dorsum, dorsal stripe broken, weakly present, originating posterior to interorbital space and terminating at sacral hump; irregular, elevated, scattered tubercles present, increasing in size from interorbital space to posterior margin of urostyle. Skin between tubercles nearly smooth; forearms smooth; hind leg tubercles vary in size. Tibial glands present on dorsal surface of legs, prominent, equivalent to the width of parotoid gland. Small densely concentrated tubercles present, originating posterior to labial commissure, inferior to tympanum, terminating in axillary region. Small, densely concentrated tubercles present longitudinally along mid axillary line, terminating at articulation of femur. Venter granular; seat patch dark, conspicuous.

Color in life. Dorsal ground color of the holotype is complex, with chromatic hues of olive with small, diverse and irregular black flecks, (Fig. 4a). Face heavily specked. Upper eyelids flecked black against olive background color. Pupil black, horizontal, with gold-streaked iris. Parotoid glands tan; minor black spotting on crown of gland with black streaks along margins. Small, dense tubercles occur between labial commissure and axillary region and are rust colored. Rust colored tubercles irregularly distributed across dorsum, small but variable in size, with black margins. Tubercles between mid-axillary line and articulation with femur rust colored, bordered by fine black halos. Dorsal stripe cream, originating at interorbital space, broken just posterior to terminal margin of parotoid glands, resumes along vertebral region, and terminates at sacral hump. Forearms with black flecks dorsally and medium to dark brown overlying olive background. Hind legs with rusty tubercles arranged atop dark brown banding overlying ground olive color with black flecking. Inferior to midaxillary line, tubercles diminish in size until absent. Inferior mid-axillary line with heavy black mottling against white. Small black spots along inferior lower labial margin. Anterior forearms and hind legs heavily marbled black against the white background color. Throat white, immaculate. White venter heavily mottled in black; seat patch conspicuous and dark brown, with round, white spotting (Fig. 4b). Undersides of hands and feet dark gray. Tubercles of hands and feet, fingers, and toes bright orange.

Color in preservative. Color is notably different and muted (Fig. 4c, Fig. 4d) relative to life (Fig. 4a, Fig. 4b). Distinctive differences include nearly monotone ground color which is dark greenish gray, warts to dark brown, dorsal stripe faint. Parotoid glands pale brown and conspicuous, streaked, and spotted a muted black color. In preservative, the bright coloration of the spinose tubercles inferior to tympanum and tubercles of hands and feet fade to white. Black mottle on the venter and limbs appears duller than in life. Tubercles on feet and hands are white with brown tips.

Morphological results. Results of statistical analyses were consistent for both log-transformed data, and for using regression of SVL against the morphological variables. Both analyses detected significant differences for all 14 morphological characters evaluated at the species level among *B. boreas*, *B. nelsoni*, *B. exsul*, and *B. williamsi* (Table 1, Table 2*)*. *Bufo* **(***Anaxyrus***)** *williamsi* is the smallest of this group ($F_{3, 376}$= 77.9, $p < 0.0001$; $F_{3, 376}$= 63.4, $p < 0.0001$) with a relatively short ($F_{4, 379}$ = 903.8, $p < 0.0001$; $F_{4, 379}$= 830.8, $p < 0.0001$; Table 2) and narrow head ($F_{4, 379}$ =1219.0, $p < 0.0001$; $F_{4, 379}$ = 1080.1, $p < 0.0001$; Table 2). There were significant differences in snout length among species ($F_{4, 379}$ = 164.9, $p < 0.0001$; $F_{4, 379}$ =160.9, $p < 0.0001$), and in pairwise comparisons of Tukey HSD post-hoc tests, *B. williamsi* differed significantly from *B. exsul* by having a relatively longer snout more like larger species *B. boreas* and *B. nelsoni,* a similarity detected in the least squares means generated from the MANCOVA analyses and corresponding linear regression that normalize SVL against this character (Table 2). *Bufo* **(***Anaxyrus***)** *williamsi* has relatively large eyes ($F_{4, 379}$ = 259.9, $p < 0.0001$; $F_{4, 379}$ = 240.0, $p < 0.001$) that are close together ($F_{4, 379}$ = 422.5, $p < 0.0001$; $F_{4, 379}$ = 371.1, $p < 0.0001$), which is distinct from *B. boreas*, and a larger tympanum compared to all three other species examined ($F_{4, 379}$= 231.4, $p < 0.0001$). While the width of the parotoid gland in *B. williamsi* is similar to *B. boreas,* the comparative length of the parotoid is shorter (Table 2). Additionally, the characters that define the length of the leg of *B. williamsi* are short (FL: $F_{4, 379}$= 910.4, $p < 0.0001$; $F_{4, 379}$= 801.0, $p < 0.0001$; TL: $F_{4, 379}$= 1063.8, $p < 0.0001$; $F_{4, 379}$ = 909.0, $p < 0.0001$; FTL: $F_{4, 379}$= 571.8, $p < 0.0001$; $F_{4, 379}$= 470.7, $p < 0.0001$), and differ significantly from *B. boreas* (Tukey HSD post-hoc pairwise comparisons, $p < 0.0001$). MANCOVA results evaluating log-transformed data by population yielded similar results with significant differences detected among localities sampled (Fig. 1a), and this analysis confirmed that *B. williamsi* is the smallest toad among all populations examined ($F_{16, 379}$= 34.7, $p < 0.0001$), with a comparatively short, narrow head (HL: $F_{17,379}$= 285.1, $p < 0.0001$; HW: $F_{17,379}$= 353.3, $p < 0.0001$), long snout ($F_{17,379}$= 55.1, $p < 0.0001$) and relatively the largest eyes among

regional *B. boreas* and congeneric taxa examined ($F_{17,379}$ = 86.07, p < 0.0001). This additional analysis confirmed that the parotoid glands of *B. williamsi* are relatively shorter in length ($F_{17, 379}$ = 81.1, p < 0.0001) compared to *B. boreas*. However, the size of the parotoids and internarial distance (IND) were among those traits that were similar in relative sizes to *B. boreas, B exsul, and B. nelsoni*. On the other hand, the tympanic diameter is relatively large ($F_{17, 379}$ = 82.4, p < 0.0001) in *B. williamsi*, and its legs are the shortest among all populations sampled (FL: $F_{17, 379}$ = 248.9, p < 0.0001; TB: $F_{17, 379}$ = 312.6, p < 0.0001; FTL: $F_{17, 379}$ = 139.1, p < 0.0001), similar to leg sizes among *B. exsul*.

Discriminant function analysis (DFA) illustrates significant morphological differences among species ($F_{42, 173}$ = 2.80, p < 0.0001; Fig. 5). The DFA correctly classified 77.3 % of predicted species, with some morphological overlap detected among *B. boreas*, *B. nelsoni* and *B. exsul* (Fig. 5). The morphological characters were accurate predictors of *B. williamsi* in all thirty predictions. The first canonical axis explained 60% of the variation in the DFA with tibial length loading most heavily, while the second canonical axis accounted for 24 % of the variation with head width loading more heavily than other characters.

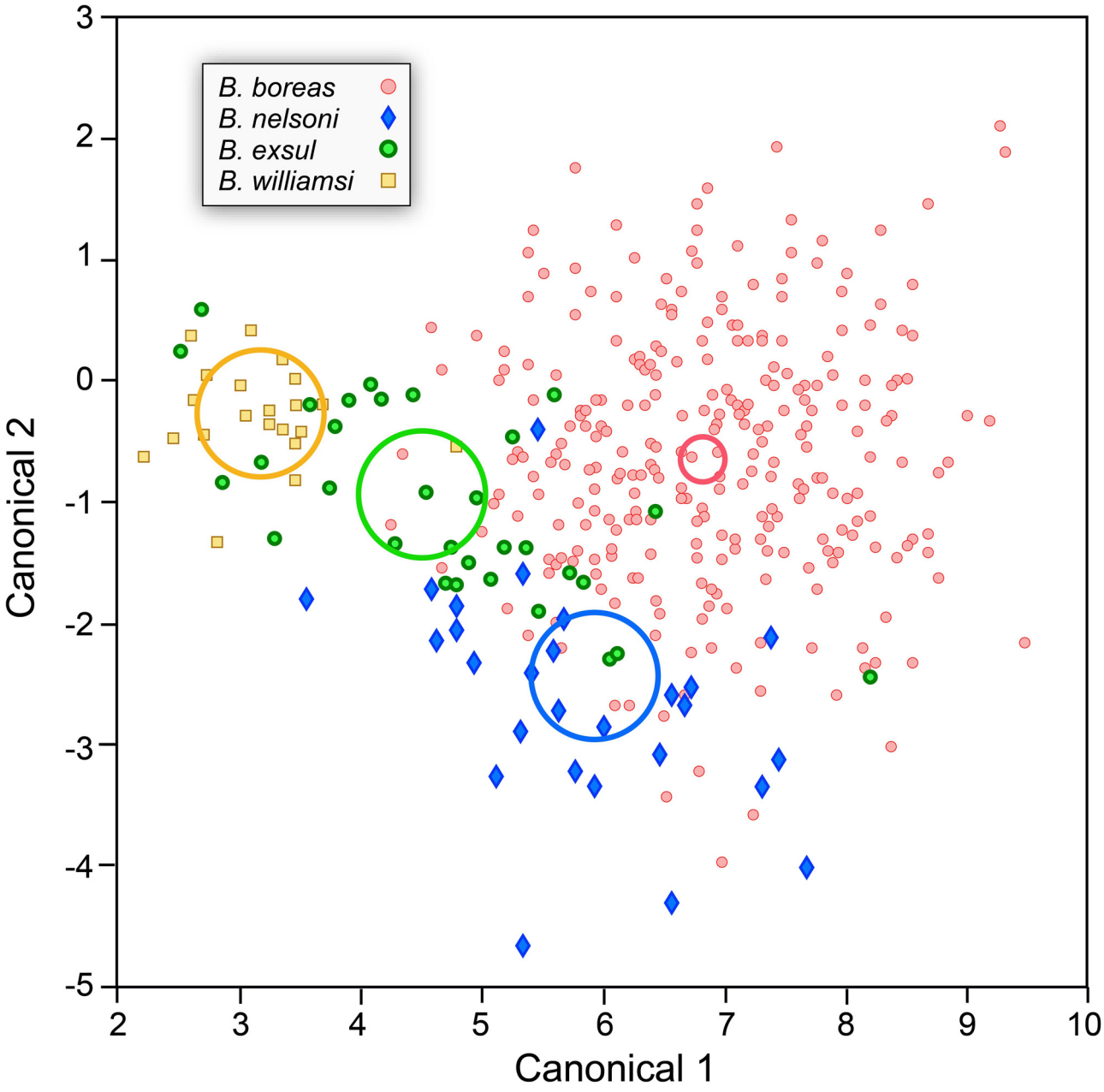

FIGURE 5. Discriminant function analysis (DFA). Cross validated DFA using 14 size corrected morphological characters measured from 380 live adult toads (Fig. 1a) examined within the hydrological Great Basin *Bufo* (*Anaxyrus*) *boreas* species complex. Species identified as *B. boreas* (red circle), *B. nelsoni* (blue diamond), *B. exsul* (green circle), and *B. williamsi* (yellow square).

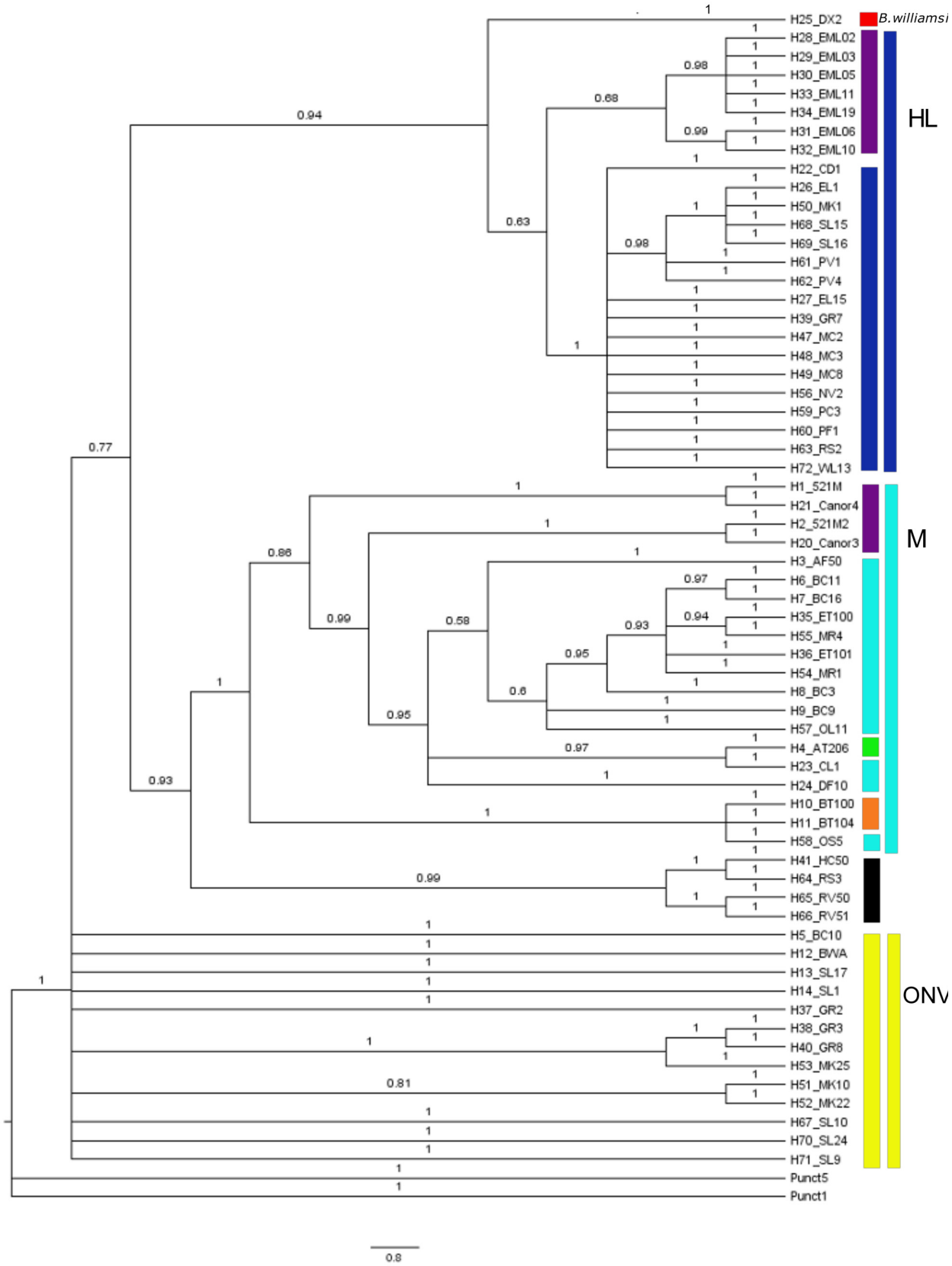

FIGURE 6. Major and minor groups identified: Bayesian inference phylogenetic tree constructed from analyses from unique haplotype sequences of 1622bp fragment of the control region of the mitochondrial genome (Fig. 1b; n = 308). Posterior probabilities are shown. Haplotype number (n = 72) and sampling locality comprise terminal ends of tree and two haplotypes of the root are shown. Minor groups include localized species: *Bufo* (*Anaxyrus*) *nelsoni* (green), *B. exsul* (orange), *B. canorus* (purple), *B. williamsi* (red) and undescribed divergent species (black). Large bars identify major groups, which include populations of *B. boreas*, sampled within the hydrological Great Basin (Fig. 1b).

There was no sexual dimorphism detected in SVL of *B. williamsi* ($F_{1, 28} = 0.09$, $p > 0.05$). However, males exhibited 3 % larger eyes ($F_{2, 29} = 4.9$, $p < 0.014$) and longer feet than females ($F_{2, 29} = 12.2$, $p < 0.0002$ for 5.2% longer FTL). The unadjusted raw data collected for the four species examined for all fourteen characters are presented in Table 1.

Genetic Results. The combined DNA analyses of the control region support the differentiation of the new species, *B. williamsi* (Fig.3, Fig. 6). Three major geographic clades were identified as Oregon-NW Nevada (ONV), Humboldt-Lahontan (HL) and Mojave (M) and genetic relationships are illustrated in the *B. boreas* TCS haplotype network (Fig. 1b, Fig. 3*)*, confirming the differentiation of *B. williamsi* from neighboring populations of *B. boreas* identified under the HL clade and northern populations of *B. canorus*. The TCS network highlights the fact that *B. boreas* from northern Nevada are less divergent from each other, a result common within the clades identified here, but illustrates that each are disconnected from each other, supporting the strong geographic signal within the Great Basin (Fig. 1b, Fig. 3, Fig. 6). Of the three, the southern clade (M) is more diverse, which includes haplotypes of *B. boreas*, the localized endemic, *B. nelsoni,* and southern populations of *B. canorus* (Fig. 1b, Fig. 3). This pattern is a consistent result of previous studies (Stephens 2001; Goebel *et al*. 2009). There are minor differences in the topologies of our phylogenetic analyses, yet the differentiation of *B. williamsi* as a unique and sister lineage to both the HL group of *B. boreas* and northern *B. canorus* is a consistent result (Fig. 6). Pairwise comparisons of nucleotide diversity revealed that *B. williamsi* is comparably differentiated as are other species in the *boreas* species complex with an average genetic distance of 2.1 %, indicating recent divergence from *B. boreas*, a consistent result from previous studies examining *boreas* diversity within the species complex (Graybeal 1993; Shaffer *et al*. 2000; Stephens 2001; Pauly *et al*. 2004; Goebel *et al*. 2009; Table 3).

TABLE 3. Estimates of evolutionary divergence among sequences from the *Bufo* **(***Anaxyrus*) *boreas* species complex within the Great Basin. The number of base substitutions per site from between sequences are shown. The analysis involved 11 nucleotide sequences conducted using the Jukes-Cantor model (Jukes & Cantor 1969) in Mega7 (Kumar *et al*. 2015). All positions containing gaps or missing data were deleted. The final dataset included a total of 1436 positions. Pairwise comparisons for *B. williamsi* against congeners within the species complex are indicated in bold print.

	1	2	3	4	5	6	7	8	9	10
1—*B. canorus* (S)										
2—*B. nelsoni*	0.011									
3—*B. exsul*	0.011	0.010								
4—*B. boreas* (CA)	0.030	0.031	0.027							
5—*B. williamsi*	**0.031**	**0.030**	**0.027**	**0.018**						
6—*B. canorus* (N)	0.030	0.029	0.026	0.018	**0.009**					
7—*B. b. halophilus*	0.013	0.006	0.011	0.031	**0.031**	0.031				
8—*B. boreas* (OR)	0.028	0.028	0.024	0.004	**0.015**	0.015	0.028			
9—*B. boreas* (NV)	0.031	0.030	0.027	0.018	**0.010**	0.009	0.031	0.015		
10—*B. punctatus*	0.183	0.184	0.183	0.184	0.189	0.184	0.183	0.182	0.187	
11—*B. punctatus*	0.187	0.188	0.187	0.188	0.194	0.188	0.187	0.186	0.190	0.012

Etymology. The specific epithet is in tribute to Robert Williams, former Field Supervisor of the U.S. Fish and Wildlife, whose Herculean efforts on behalf of the fauna of Nevada and California were critically important in discovering additional biodiversity of anurans in the Great Basin, and in focusing on the needs to provide protection to the rare and imperiled fauna, and the ecosystems upon which they depend, in Nevada and California. The Dixie Valley toad would not have been discovered without the efforts of this courageous public servant.

Distribution. *Bufo* **(***Anaxyrus*) *williamsi* is found only within wetlands of limited extent fed from artesian springs on the western edge of the Dixie Valley Playa, east of the Stillwater Range in Dixie Valley, NV (Fig. 2b). Very isolated and restricted in size, the entire estimated geographic range is approximately 6 km^2, with no usable corridors to other toad habitat outside Dixie Valley. Four spring discharge sites and the marsh habitat downstream of the springs are separated from each other and interrupted by sagebrush steppe dominated by big sagebrush

(*Artemisia tridentata* ssp. *tridentata*), greasewood (*Sarcobatus vermiculatus*), rubber rabbitbrush (*Ericameria nauseosa*) and saltbush (*Atriplex* spp.) (BLM 2011). The spring-fed wetlands support marsh vegetation such as spikerush (*Eleocharis* spp.), knotweed (*Polygonum* spp.), canarygrass (*Phalaris* spp.), duckweed (*Lemna* sp.), various species of rush (*Juncus* sp.), common reed (*Phragmites australis*), and cattail (*Typha* spp.) (BLM 2011), and toads are typically found in shallow water or associated with moist soils within the immediate perimeter of the riparian areas that border sagebrush habitat.

Natural history. *Bufo* **(***Anaxyrus***)** *williamsi* is restricted to the spring fed-wetland habitat along the western edge of the Dixie Valley playa. Similar to other toads in the *B. boreas* complex (except perhaps *B. exsul*, which is more aquatic), the terrestrial *B. williamsi* is typically nocturnal, emerging at dusk, and can be found in moist vegetation or in very still, shallow water with very little vegetation canopy. Dixie Valley experiences extreme temperature fluctuations between day and night temperatures, as well as season-to-season extremes, characteristic of cold desert ecosystems.

In autumn, it is likely that *B. williamsi* retreats to burrows to hibernate, emerging in spring to breed. Breeding occurs from March to June (Forrest *et al*. 2013). Sexually mature males congregate in shallow water around the perimeter of wetland vegetation. *Bufo* **(***Anaxyrus***)** *williamsi* does not have an advertisement vocalization, but retains a release call used by males when in contact with other males. Egg masses and tadpoles develop in still, shallow water within the margins of the marsh habitat, where there are adequate temperatures for development as is seen within *B. boreas* (Karlstrom 1962; Carey *et al*. 2005). Toadlets are generally fully metamorphosed in approximately 10 weeks (Forrest *et al*. 2013).

While *B. williamsi* is reportedly active throughout the summer (Kris Urquhart, pers. comm.), little is known regarding dispersal and non-breeding behavior of this toad. The overall population numbers of this toad are unknown; however, the current range is severely restricted, suggesting that this species' population is likely very small.

The coloration of this toad is striking, but within the wetland vegetation, the disrupted olive and flecking of *B. williamsi* is very cryptic causing their detection to be difficult. The main stores of bufotoxin are in the parotoid glands, which are conspicuous in shape and tan color, which contrasts with the olive background color of the body, and may trigger a warning to potential predators, such as common ravens (*Corvus corax*) and coyotes (*Canis latrans*). Large and conspicuous tibial glands (not typical of congeners within the *B. boreas* complex) are also present in *B. williamsi* and are additional stores of bufotoxins.

Remarks. The Dixie Valley Toad is the newest addition to the *B. boreas* species complex, increasing the regional diversity in the complex to five species (Frost 2015). The taxonomy within the genus *Bufo* remains unstable and controversial, and to provide continuity for the nomenclature under *B. boreas* and for the delimitation of the Dixie Valley toad, we recommend that *B. williamsi* retains *Bufo*, increasing the Nearctic bufonids in the subgenus *Anaxyrus* to 23 species (Pauly *et al*. 2009; Frost 2015). New anuran discoveries have been rare within the United States, with only three newly described frogs (that were not simply elevated from subspecies status) since 1985 (Moler 1985; Lemmon *et al*. 2008; Feinberg *et al*. 2014). *Bufo* **(***Anaxyrus***)** *williamsi* represents the first newly described bufonid species to occur north of Mexico since 1968 (Frost 2015) and demonstrates that our knowledge of Nearctic anuran diversity remains incomplete and that novel discoveries continue to occur, even in unlikely settings. The most recently named new anuran species, *Rana kauffeldi* (Feinberg *et al*. 2014) and *Pseudacris fouquettei* (Lemmon *et al*. 2008) remained undetected despite occurring in a highly populated region (*R. kauffeldi*) or having a broad distribution (*P. fouquettei*). *Rana* and *Pseudacris* contain multiple species complexes, where the taxonomy remains unstable and controversial due to cryptic diversity, (Platz & Forester 1988; Moriarty & Cannatella 2004; Vredenburg *et al*. 2007; Lemmon *et al*. 2008; Feinberg *et al*. 2014), an issue highlighted by numerous subspecies reclassifications of frogs within these groups (Green *et al*. 1996; Lemmon *et al*. 2008), and evidenced by the newest species described that were both themselves cryptic.

The arid Great Basin has few aquatic resources, with high endemism associated with widely dispersed springs, small streams and seeps within the region (Hubbs & Miller 1948; Shepard 1992, Hershler & Sada 2002; Sada & Vinyard 2002; Smith *et al*. 2002). Due to isolation and rarity, springs and resulting wetlands may harbor cryptic species (Shepard 1993). Recent molecular studies investigating the fine scale relationships within the *B. boreas* species complex have suggested that the western toad diversity across its broad geographic range is not accurately reflected under the current taxonomy with results indicating that even additional hidden diversity is likely (Goebel, 2005; Goebel *et al*., 2009), particularly around the edges of the Great Basin (Goebel *et al*. 2009). However,

sampling within the Great Basin had been very limited or absent until the recent localized study (Tracy *et al*. unpubl. data) examining *B. boreas* diversity within the region. That study also indicates that *B. williamsi* is genetically distinct from *B. boreas*. Evidence suggests aquatic isolation of Dixie Valley is estimated to have occurred approximately 650KYA when the climate shifted (Reheis *et al*. 2002; Noles 2010) which could have stranded aquatic organisms to this endorheic basin. The undetected diversity identified within toads of Dixie Valley yielded genetic distances (Table 3) similar to those of the closely related species within the *B. boreas* species complex when compared to *B. boreas*. The novel discovery of *B. williamsi*, whose concealment was due to its occurrence within the range of the widely distributed *B. boreas*, is an example of remarkable results through the coupled use of both systematics and taxonomy, leading to the identification of hidden diversity.

Bufo **(***Anaxyrus*) *williamsi* has the smallest range of all congeners of the *B. boreas* species complex, and these results highlight the importance of accurate taxonomy having profound implications for the management and conservation initiatives for taxa (Bickford *et al*. 2006; Trontelj & Fišer 2009), particularly rare species occurring within the range of widely nominal species.

Conservation Concerns. Amphibians are the most imperiled clade of vertebrates with declines and extinctions occurring globally (IUCN 2015). In the United States, 31.7 % of amphibian species are in decline (Adams *et al*. 2013) and 26% of rare endemics are listed as threatened (IUCN 2015). In the western United States, populations of *B. boreas* have experienced declines across their large geographic range (Blaustein & Wake 1990; Blaustein *et al*. 1994; Bull & Carey 2008; Pilliod *et al*. 2010). Within the Great Basin, *B. boreas* occupancy is declining because of habitat loss (Wente *et al*. 2005), and all three endemics are threatened (IUCN 2015).

The Dixie Valley Toad faces a staggering number of threats to its persistence which are compounded by its remarkably small geographic range. The most urgent concern is the expansion of geothermal energy production, which could imperil the fragile marsh habitat upon which this rare toad relies. Dixie Valley is the hottest and most geothermally active system in the Basin and Range Province, and it is home to the largest geothermal energy plant in Nevada, which has been in operation for over 20 years (Blackwell *et al*. 2007). However, new proposals for geothermal, or solar, energy development could reduce the rare water resources within this valley, devastating critical breeding habitat for the species.

While *B. williamsi* is not sympatric with *B. boreas*, introduced North American bullfrogs (*Rana catesbeiana*), are present at the southern edge of the Dixie Valley toad range. Bullfrogs are much larger than *B. williamsi* (as are metamorphs of these bullfrogs which are larger than adult *B. williamsi*) and bullfrogs are known to prey upon other amphibians. In addition, bullfrogs are a known vector for diseases (Kats & Ferrer 2003; Daszak *et al*. 2004) such as chytridiomycosis, a potentially lethal pathogen thought to cause amphibian declines and extirpations, and implicated in some declines noted among populations of *B. boreas* (Muths 2003; Muths *et al*. 2008; Bull 2009). In 2012, a survey did not detect chytridiomycosis among *B. williamsi*, however, there was an increase in the infection among bullfrogs from 18 % in 2011 to 75 % in 2012 (Forrest *et al*. 2013), which poses a serious threat to the endemic Dixie Valley toad.

The US Fish and Wildlife Service and Nevada Department of Wildlife have been monitoring *B. williamsi* since 2008. Although the species range has been defined, the population size remains unknown. This limited distribution is strong indicator that the current population is exceedingly small, similar to related toads, *B. exsul* and *B. nelsoni*, and will similarly warrant strong conservation initiatives to protect and monitor this new species. At smaller population sizes, habitat loss, nonnative species, and disease may act synergistically, negatively impacting this indigenous toad.

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