Species Status Assessment Report for the Eastern Population of the Boreal Toad, *Anaxyrus boreas boreas*

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EXECUTIVE SUMMARY

This species status assessment (SSA) reports the results of the comprehensive biological status review by the U.S. Fish and Wildlife Service (Service) for the Eastern Population of the boreal toad (*Anaxyrus boreas boreas*) and provides a thorough account of the species' overall viability and, therefore, extinction risk. The boreal toad is a subspecies of the western toad (*Anaxyrus boreas*, formerly *Bufo boreas*). The Eastern Population of the boreal toad occurs in southeastern Idaho, Wyoming, Colorado, northern New Mexico, and most of Utah. This SSA Report is intended to provide the best available biological information to inform a 12-month finding and decision on whether or not the Eastern Population of boreal toad is warranted for listing under the Endangered Species Act (Act), and if so, whether and where to propose designating critical habitat.

To evaluate the biological status of the boreal toad both currently and into the future, we assessed a range of conditions to allow us to consider the species' resiliency, redundancy, and representation (together, the 3Rs). The boreal toad needs multiple resilient populations widely distributed across its range to maintain its persistence into the future and to avoid extinction. A number of factors influence whether boreal toad populations are considered resilient to stochastic events. These factors include (1) sufficient population size (abundance), (2) recruitment of toads into the population, as evidenced by the presence of all life stages at some point during the year, and (3) connectivity between breeding populations. We evaluated a number of potential stressors that could influence the health and resilience of boreal toad populations. We found that the main factor influencing the status of populations is the presence of Chytrid Fungus, *Batrachochytrium dendrobatidis* (Bd); however, the response of boreal toads to Bd varies across the species' range. If boreal toads display some resistance to Bd, and recruitment continues within breeding sites despite exposure to Bd, boreal toads should continue to persist with some level of resiliency across their range.

We have assessed the Eastern Population's levels of resiliency, redundancy, and representation currently and into the future by evaluating known occupied hydrologic unit code-12 (HUC-12s) as a proxy for populations. The HUC-12 is the finest-grained sub-watershed delineated in the National Watershed Boundary Database (USGS 2009), and is used to represent historical or active breeding sites, also referred to as populations. As we consider the future viability of the species, we believe that a greater number of self-sustaining populations that are distributed across the known range of the species would be associated with a higher overall viability of the species. We consider occupied sites where Bd infection is absent to be the most resilient; some populations exist where Bd is present, but are highly resistant to Bd infection, and we also consider these populations highly resilient. Other areas display moderate resistance to Bd and are therefore moderately resilient; low resiliency populations are those that have little or no resistance to Bd, and suffer severe population declines or extirpation. Therefore, we believe that if suitable habitat parameters continue to be maintained, coupled with some level of resistance to Bd, we anticipate the Eastern Population of the boreal toad would continue to survive into the future.

The historical range of the Eastern Population of boreal toad includes 439 known HUC-12s across the range of this subspecies. Currently, approximately 194 HUC-12s are considered occupied. Of these, approximately 83 HUC-12s are positive for Bd infection. Occupancy within the remaining approximately 245 HUC-12s is currently unknown due primarily to the lack of recent survey effort. However, this number includes approximately 62 HUC-12s within in the Southern Rocky Mountains subpopulation area that are considered unoccupied, and may have been extirpated by Bd. We recognize that the 439 known HUC-12s within the range of the species likely represents a minimum number of possible breeding sites, since every area that could possibly support boreal toads has not been surveyed.

Future Scenarios

The most significant stressor to boreal toads is Bd. Chytrid Fungus is distributed across the entire range of the species. However, toads within different parts of the range respond differently to Bd infection, highlighting the broad representation across their range. The variability in the toads' response to Bd infection informs our understanding of the future of the boreal toad. Toads in the Southern Rocky Mountains subpopulation area appear to respond the most negatively when exposed to Bd, resulting in drastic declines in toad numbers at breeding sites, or the extirpation of toads at some sites. Toads in Utah do not appear to be significantly affected by Bd, and toads in western Wyoming display slow population declines through time. As part of the Southern Rocky Mountains Recovery Team's update of its conservation plan, a population persistence model was developed which is believed to provide a statistically rigorous assessment of viability of toads in the southern Rocky Mountains. This model predicts a greater than 95 percent probability of persistence of toads within the southern Rocky Mountains over the next 50 years, but with lower population levels, fewer breeding sites, and reduced geographic distribution. Given that boreal toads in other geographic areas display higher levels of resistance to Bd infection, we believe this model represents a worst-case scenario when considering the future condition of the Eastern Population as a whole. If we anticipate this high level of persistence to occur within an area most susceptible to Bd infection, toads in other population areas are likely to maintain robust breeding populations into the future, although there is uncertainty regarding how climate change may factor into the future condition of the Eastern Population.

Summary: Boreal toad populations are currently experiencing variability in their response to Bd infection, which we considered to be the primary stressor on boreal toad population resilience for the purposes of our analysis. The most susceptible population to Bd infection experiences high population losses and localized extirpations, but some breeding sites continue to persist despite significant population declines. Some populations within the range show little or no evidence of impacts caused by Bd infection, and remain robust despite the presence of Bd. Other areas show some population decline, but at much lower severity than observed in the southern Rocky Mountains. Conservation efforts, conducted primarily by Colorado Parks and Wildlife in the southern Rocky Mountains are anticipated to increase the likelihood of persistence to approximately 100% during the next 50 years. Therefore, we anticipate that the Eastern Population of boreal toad will likely continue to maintain high levels of resiliency, redundancy, and representation across its range over the next 50 years.

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INTRODUCTION

The boreal toad (*Anaxyrus boreas boreas*) is one of two subspecies of the Western toad (*Anaxyrus boreas,* formerly *Bufo boreas*), which is found throughout much of western North America (Stebbins 2003, p. 475, map 32). The subject of this Species Status Assessment (SSA) Report is the Eastern Population of the boreal toad (boreal toad) found in Colorado, southeastern Idaho, northern New Mexico, Utah, and Wyoming (Figure 1). The U.S. Fish and Wildlife Service (Service) is reviewing the status of the Eastern Population of the boreal toad in response to a May 25, 2011, petition to list a distinct population segment of the boreal toad (*Anaxyrus boreas boreas*) under the Endangered Species Act of 1973 (Act), as amended (16 U.S.C. 1531 *et seq.*).

For simplicity in this SSA report, we use the terms "species" and "boreal toad" interchangeably to refer to the Eastern Population of boreal toad, although this population is not a taxonomic species.

Using the SSA analytical framework (U.S. Fish and Wildlife Service 2016, entire), this SSA report provides an in-depth review of the species' biology and stressors, an evaluation of its biological status, and an assessment of the resources and conditions needed to maintain long- term viability. The intent is for the SSA Report to be easily updated as new information becomes available and to support all functions of the Endangered Species Program from Candidate Assessment to Listing to Consultations to Recovery. As such, the SSA Report will be a living document upon which other documents, such as listing rules, critical habitat designation, recovery plan, and 5-year review, would be based if the species warrants listing under the Act.

This SSA Report for the Eastern Population of boreal toad is intended to provide the biological support for the decision on whether to propose to list the species as threatened or endangered and, if so, whether to and where to propose designating critical habitat. Importantly, the SSA Report does not result in a decision by the Service on whether this taxon should be proposed for listing as a threatened or endangered species under the Act. Instead, this SSA Report provides a review of the available information strictly related to the biological status of the boreal toad. The listing decision will be made by the Service after reviewing this report and all relevant laws, regulations, and policies, and the results of a proposed decision will be announced in the Federal Register, with appropriate opportunities for public input should public input be necessary.

For the purpose of this assessment, we define viability as the ability of the species to naturally sustain populations in suitable habitat (i.e. wetland areas, natural spring systems, ponds, lakes, etc.) beyond a biologically meaningful timeframe, in this case, 50 years. We chose 50 years because it is within the range of the available hydrological and climate change model forecasts, encompasses approximately 5 to15 generations, and represents a biologically meaningful time frame in which we could expect to observe any plausible changes in the status of the Eastern Population of boreal toad.

Using the SSA framework, we consider what is needed by the species to maintain viability by characterizing the status of the species in terms of its resiliency, redundancy, and representation (U.S. Fish and Wildlife Service 2016, entire).

- **Resiliency** is having sufficiently large populations for the species to withstand stochastic events (arising from random factors). We can measure resiliency based on metrics of population health; for example, birth versus death rates and population size. Resilient populations are better able to withstand disturbances such as random fluctuations in birth rates (demographic stochasticity), variations in rainfall (environmental stochasticity), or the effects of anthropogenic activities.

- **Redundancy** is having a sufficient number of populations for the species to withstand catastrophic events (such as a rare destructive natural event or episode involving many populations). Redundancy is about spreading the risk, and can be measured through the duplication and distribution of populations across the range of the species. The greater the number of populations a species has distributed over a larger landscape, the better it can withstand catastrophic events.

- **Representation** is having the breadth of genetic makeup of the species to adapt to changing environmental conditions. Representation can be measured through the genetic diversity within and among populations and the ecological diversity (also called environmental variation or diversity) of populations across the species' range. The more representation, or diversity, a species has, the more it is capable of adapting to changes (natural or human caused) in its environment. In the absence of species- specific genetic and ecological diversity information, we evaluate representation based on the extent and variability of habitat characteristics within the geographical range.

We assessed a range of conditions that allow us to consider the species' resiliency, redundancy, and representation (together, the 3 Rs) now, and to project those conditions into the future. This SSA Report provides an assessment of biology and natural history, and assesses demographic risks, stressors, and limiting factors in the context of determining the viability and risks of extinction for the species.

The format for this SSA Report includes: (1) an overview of background information on taxonomy and life history of the species (Chapter 1); (2) resource needs at the individual, population, and species level; (Chapter 2); (3) an assessment of the current condition of the species, including a review of the likely causes of the current and future status of the species and determining which of these risk factors affect the species' viability and to what degree (Chapter 3); and (4) an assessment of the likely future condition of the species in terms of resiliency, redundancy, and representation (Chapter 4). This document is a compilation of the best available scientific and commercial information and a description of past, present, and likely future risk factors to the boreal toad.

CHAPTER 1 – TAXONOMY AND LIFE HISTORY

Taxonomy and Genetics

The boreal toad (*Anaxyrus boreas boreas*) is a subspecies of the Western toad (*Anaxyrus boreas*, formerly *Bufo boreas*), which occurs throughout much of the western United States. Our assessment of taxonomy in our 2012 90-day finding (77 FR 21922, April 12, 2012) relied on two studies analyzing mitochondrial DNA (mtDNA) from boreal toads and other closely related species and subspecies. We concluded that boreal toads within southeastern and western Wyoming, Colorado, northern New Mexico, southeastern Idaho, northeastern Nevada, and Utah comprised a population of genetically similar toads identified as the Eastern Major Clade by Goebel et al. (2009, p. 210, fig. 1), and Clade 3–1 by Switzer et al. (2009, p. 8). The combination of Goebel et al.'s Eastern Major Clade and Switzer et al.'s Clade 3–1 formed the Eastern Population of the boreal toad used in that 90-day finding (77 FR 21922, April 12, 2012).

Currently, analyses of mtDNA alone are no longer accepted as definitive due to the unique pattern of maternal inheritance of mtDNA, which can result in conflicts with nuclear data (Goebel 2017a, pers. comm). Gene introgression, or the introduction of mtDNA into unrelated populations from hybridization, is common because mtDNA is inherited maternally with each female passing on her mtDNA genome to all her offspring. In addition, mtDNA does not recombine, as do nuclear genes, so introgression can be rapid. When results of analyses of mtDNA and multiple nuclear genes conflict, the nuclear DNA analyses are accepted and the pattern of mtDNA is interpreted as lineage sorting or gene introgression (Goebel 2017a pers. comm.).

Three types of genetic analyses were completed or are in progress, all focusing on the eastern portion of the boreal toad's distribution. Each used different genetic methods and different numbers of samples, resulting in different strengths and weaknesses, as described above. In one type of analysis, the phylogeography of mtDNA were analyzed by Goebel (e.g., 1996, 2000; Goebel et al. (2009)); these studies examined both a large and a small fragment of a control region from over 200 samples, as well as several additional mtDNA genes in a smaller number of samples (N=21). Similarly, Switzer et al. (2009, entire) examined a smaller fragment of the control region from a larger number of specimens (N=540, 243 samples were from the Southern Rocky Mountain subpopulation). In a second type of analysis, three analyses of different nuclear DNA markers were completed or are in progress. Switzer et al. (2009 et al., entire) investigated microsatellite data from 12 loci from 856 specimens to investigate the genetic distinctness of the Southern Rocky Mountain subpopulation. In addition, Goebel (Appendix B and in progress) is investigating sequence data from seven nuclear genes (seven loci) from 99 samples, to identify the genetic distinctness and geographic distribution of the Eastern Population. Finally, in a third type of analysis, Sara Oyler-McCance, PhD, USGS, (Appendix 3 and in progress) is examining whole-genome single nuclear polymorphisms data using data from 64 individuals, to identify the

genetic distinctness of the Eastern Population group. From these analyses, several conclusions are evident:

1. A genetically distinct group of boreal toads exists in the eastern portion of the range and is found within all of Colorado, northern New Mexico, most of Utah, Wyoming, and southeastern Idaho. Part of this group also may occur in southwestern Montana. This is supported by all nuclear DNA analyses and all mtDNA analyses (Switzer et al, 2009, Appendix 2, Appendix 3). In some areas the boundaries of the group are uncertain based on mtDNA analyses, as described below.

2. The geographic boundaries of the eastern group are unclear in several regions based on mtDNA analyses, but nuclear DNA analyses have helped clarify some of these boundaries.
   - First, boreal toads in northwestern Utah and northeastern Nevada (Box Elder Co. UT and Elko Co. NV) have a mix of distinct eastern and western alleles. However, a combined analysis of multiple nuclear genes suggests that populations of boreal toads in this region have differentiated from the eastern group (Switzer et al. 2009, Figure 6; Appendix 2, Appendix 3).
   - Second, boreal toad populations in middle-eastern Nevada (Hot Creek and Railroad Valley, NV) have unique mtDNA haplotypes within the Eastern mtDNA clade; however analyses of nuclear genes show that these toads have differentiated from the Eastern Clade and are more closely related to boreal toads in Nevada (Appendix 2, Appendix 3).
   - The third region where boundaries are unclear is in southwestern Montana, where sampling was sparse, but individuals with alleles from both the eastern and western middle-Rocky Mountain groups occur. It is unclear whether there is a clear break, or whether this will be a zone of intergradation between the two groups. Switzer et al. (2009, figures 4 and 5) also found a mix of mtDNA haplotypes from the eastern and northwest mtDNA groups in southeast Idaho.

3. Results of mtDNA analyses are slightly different than the combined results of seven- nuclear genes analyzed. We note these differences, but accept the nuclear DNA analyses as more informative of the evolution of the group, for the reasons described above. We describe these differences here because our previous understanding of the groups was based solely on mtDNA, and we outline how our understanding has changed due to new data.
   - While mtDNA suggested that Wyoming consisted of a large zone of intergradation of the northwestern (Middle Rocky Mountain) and eastern mtDNA haplotypes, the analysis of multiple nuclear genes suggests that Wyoming is comprised of toads most genetically similar to the eastern group.
   - The nuclear DNA analyses presented here (Appendix 2, Appendix 3) do not identify as much genetic differentiation within the Eastern Clade as the mtDNA analyses.

4. Results of genomic analysis (Appendix 3) are largely congruent with the analyses of sequence data by Goebel (Appendix 2). Most of the samples used in the genomic analysis (Appendix 3) were also included in the analyses of sequence data.

In summary, current and ongoing genetic analyses suggest the occurrence of an eastern group of boreal toads that are genetically distinct from the rest of the subspecies. Genetic studies have helped clarify the boundaries of this group, which we now understand to include boreal toads in southeastern Idaho, western and south-central Wyoming, most of Utah (except western Box Elder County), Colorado, and north-central New Mexico. For the purposes of this report, we refer to this group as the Eastern Population of boreal toads.

Range and Distribution

Based on the most up to date genetic information and analyses as described above and presented in Appendix B, the historical range of the Eastern Population of boreal toad considered in this SSA Report includes southeastern Idaho, western and south-central Wyoming, most of Utah (except western Box Elder County), Colorado, and north-central New Mexico (Figure 1).

We lack detailed survey data across the range of the species to precisely map all active discrete breeding sites of boreal toads, because of the variation in survey protocols used and the data collected across the range of the species. Some recent location data collected within last 10 years result from targeted and repetitive site visits to known, or suspected, boreal toad breeding sites. Other data are limited to incidental sightings of individual toads. We recognize that an incidental sighting of an individual adult toad does not necessarily indicate a breeding population, where the presence of eggs, tadpoles, or metamorphs indicates breeding activity. Furthermore, we cannot automatically assume that breeding is not occurring if earlier life stages are not detected where the incidental siting of an adult toad occurred. Our data suggest there are sites where breeding activity or incidental siting was recorded in the past, but have not been revisited within the last ten years. In these situations, the occupancy and/ or breeding status of these sites is unknown, but we have no reason to assume extirpation of these sites.

Crocket (2017a, p.3), provided occupancy data at the 12-digit hydrologic unit code (HUC-12) level. The 12-digit HUC is the finest- grained sub- watershed delineated in the National Watershed Boundary Dataset, representing areas of 10,000- 40,000 acres (USGS 2009). Toad encounters at a specific location represent a larger range occupied by the toad and others in the population to which it belongs Crockett 2017a, p. 3). The Service agrees with Crockett (2017 a, p. 3) that the use of HUC-12 to up-scale point encounter data is a reasonable approach to identify possible breeding populations. For consistency, we converted all location data submitted to us to the HUC-12 level. This approach allowed us to use consistent units for analysis across the range of the boreal toad.

Since we lack consistent survey data across the range of the boreal toad to indicate breeding activity, we assumed that any observation of an adult toad occurring within the last 10 years, represented an active breeding population, as indicated at the HUC-12 level. We acknowledge that using HUC-12s as a proxy for breeding populations is a coarse-scale approach and that this approach may either overestimate or underestimate the actual number of breeding populations. We believe that at the scale of our range-wide analysis, any over- or under- estimate will average out. We believe this is a reasonable approach because, some occupied HUC-12s likely contain multiple discrete breeding sites, may contain breeding adult toads that are persisting at low numbers, or are limited to incidental observations of dispersing individuals.

As stated above, an individual HUC-12 may include one or more currently active or historical breeding (status unknown) breeding sites. In some cases, an individual breeding site within a HUC-12 may have recently become extirpated, but another breeding site within the HUC may still be active. In such cases, any active breeding site within a HUC-12 results in an occupied status for the entire HUC-12. In some cases the occupancy status is unknown, which represent population areas that have not been surveyed recently.

Population declines have been documented within the southern Rocky Mountains (Carey et al., p. 236; Corn et al. 1989, p. 10, Crockett 2017a, p.7), likely referring to known breeding sites. However, we have no indication that boreal toads within entire HUC-12s have become extirpated within the southern Rocky Mountains. Although Pilliod et al. (2010, p. 1265) documented reduced survival of adult toads (individuals), we have no data or information documenting extirpation of entire breeding sites elsewhere within the range of the species. Therefore, we believe it is reasonable to assume that HUC-12s outside of the southern Rocky Mountains likely contain breeding populations of boreal toads. Based on the information described herein, we have little reason to believe that the current range has changed substantially from the historical range (Figure 1). The historical range is represented by all of the HUC-12 displayed in Figure 1, including HUC-12s of known occupancy (yellow) and those of unknown occupancy (pink). It is also likely that the range of the species may include additional HUC-12s that contain the appropriate habitat for boreal toads, but have not been surveyed.

Figure 1. Boreal Toad Current and Historical Range.

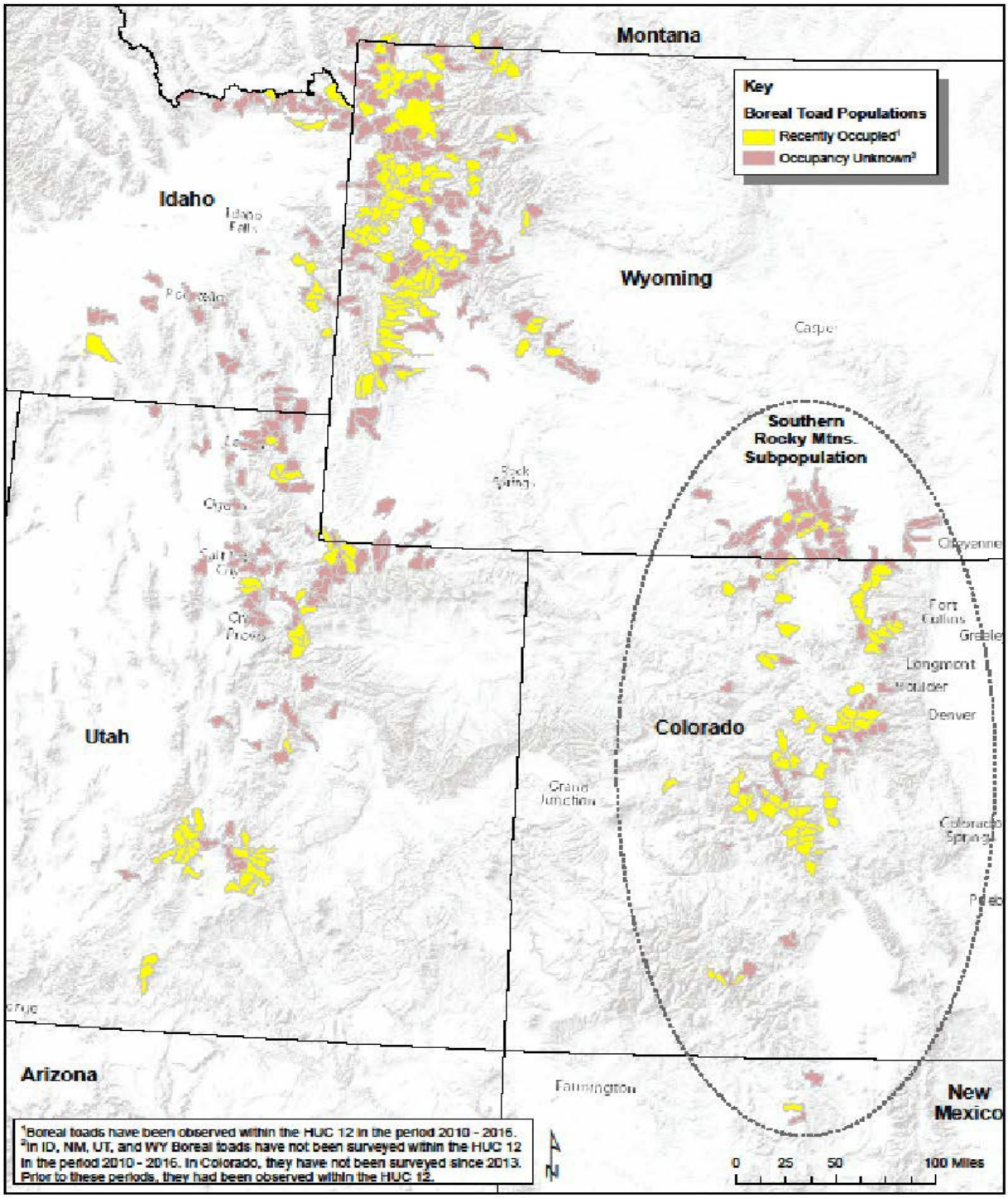


Table 1. Land ownership estimate (percent of total) of occupied boreal toad habitat within the HUC-12. Acreage estimates likely overestimate the actual amount of boreal toad habitat, because not all areas within the HUC-12 are occupied or provide suitable habitats.

Land Owner	Percent of Total Area
Federal	97%
Private	<1%
State	1.5%
Tribal	<1%
Other	<1%
Total	1,894,038 hectares (4,680,270 acres)

Species Description

Boreal toads are dark, brown-black, with warty skin and a white to cream-colored dorsal stripe on the back that may sometimes be broken or incomplete (Loeffler 2001, p. 7). Dark spots blotch the light underside over the chest and abdominal area. Adult toads lack cranial crests and have oval parotoid glands. Adults are large in size, with males being 60 to 80 millimeters (mm) (2.4 to 3.2 inches (in)) long and females being 75 to 100 mm (3 to 4 in) long from snout to vent. Eggs are laid in shallow waters. Round black eggs are linearly spaced in two rows within long strings of two-layered jelly. Tadpoles are jet-black in color, though sometimes turning brown, and generally aggregate in shallow water. Juvenile toads are similar in appearance to adults, but have yellow coloration on the toes and often lack the mid-dorsal stripe, especially when small (Stebbins 2003, pp. 208-209).

Habitat and Life History

The boreal toad occurs between 2000 meters (m) (6,550 feet (ft.)) (Hogrefe et al. 2005, p.7) and 3,670 m (12,232 ft.) (Loeffler 2001, p.7; Lambert and Schneider 2013, pp. 10-11; Crockett 2017a, p.5). The distribution of toads is restricted to areas with suitable breeding habitat (described below) within a landscape containing a variety of vegetation types, including pinon-juniper, lodgepole pine, spruce-fir forests, mountain shrubs, and alpine meadows. Movement and dispersal of toads is likely limited by water availability on the landscape.

Boreal toads occupy three different types of habitat during the course of the year: breeding ponds, summer range, and overwinter refugia. Breeding takes place in shallow, quiet water in lakes, marshes, bogs, ponds, and wet meadows, often with egg placement optimizing thermal effects of the summer sun. A study of egg placement in Colorado found that eggs were laid at an average depth of 6.1 cm and pond slopes averaged 0.07 percent (Holland 2002, p. 13; Holland 2006, p. 158). Breeding has been recorded from large permanent lakes, glacial kettle ponds, man-made ponds, beaver ponds, marshes, and roadside ditches (Loeffler 2001, p. 8; Carey et al. 2005, p. 227; Holland 2006, p. 158; Hossack et al. 2015, entire).

The life cycle and stages of the boreal toad are displayed in Figure 2. The term “subadult” represents animals that are 1 to 3 years old for males and 1 to 5 years old for females. Subadults do not breed. Males are capable of breeding in 4-5 years and females in 5-7 years (Carey 1976, p. 162; Carey et al. 2005, pp. 230, 235). Females are obligatory non-breeders the year following a breeding event and may skip multiple years between breeding attempts (Muths et al. 2010, p. 128).

Figure 2. Boreal Toad Life Cycle

Life Stage (*age*)	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Eggs (from laying up to hatching at 7-14 Days)												
Tadpoles (from hatching, 45-92 Days until metamorphosis)												
Metamorph (from 50-120 Days, up to 1 year of age)											First-Fall Hibernation	
Subadult (Males: 1-4 years-old, Females: 1-5 years-old)	Hibernation										Hibernation	
Adult (Males: 4-16+ years-old, Females: 6-20+ years old)	Hibernation										Hibernation	

The availability of moist habitat restricts the movement and distribution of young toads (Livo 1998, p. 130; Loeffler 2001, p. 8). Adults are less dependent on moist habitats than metamorphs, but must rehydrate daily (Campbell 1970a, p. 29-34), and may move several miles and reside in marshes, wet meadows, or forested areas (Goates, et al. 2007, pp. 478-481; Loeffler 2001, p. 8; Muths 2003, p. 163). Toads may use large boulders, small mammal burrows, beaver lodges and dams, as hibernacula (Campbell 1970a, p. 90, Jones and Goettl 1998, pp. 29-30; Loeffler 2001, p. 8).

At higher elevations, adult boreal toads emerge from winter refugia when snowmelt has cleared an opening and daily temperatures remain above freezing (Campbell 1970a, pp. 22, 99; Campbell 1970b, p. 281). Breeding of Western toads can occur from late January to July, depending on latitude, elevation, and local conditions (Stebbins 2003, p. 209). However, in the Eastern Population of boreal toad, breeding in Utah likely starts in April (Hogrefe et al. 2005, p. 8). In the southern Rocky Mountains, breeding occurs during a two to four week period from mid-May to mid-June at lower elevations, and as late as mid-July at higher elevations (Hammerson 1999, p. 96). Boreal toads have been observed to lay up to 16,500 eggs (Campbell 1970a, p. 24), and in Colorado they have been observed laying up to 10,900 eggs (Hammerson 1999, p. 96), with an overall mean clutch size of 6,661 eggs (Carey et al. 2005, p. 224). Eggs hatch one to two weeks

after being laid. Egg and tadpole development is temperature-dependent, and reproductive efforts may fail if tadpoles do not have sufficient time to metamorphose before the onset of winter. Persistent, shallow bodies of water are vital to breeding success, and if the breeding site dries before metamorphosis is complete, desiccation of the tadpoles or eggs will occur. Tadpoles typically metamorphose by late July to late August, but at higher elevations metamorphosis may not be complete until late September (Loeffler 2001, p. 7).

Recently metamorphosed juveniles may aggregate within a few meters of the water to prevent desiccation, heat retention, or to avoid predation (Black and Black 1969, p. 156; Livo 1998, pp. 128, 130, 134). However, these young toads move into nearby moist habitats later in summer. After mating, adults often disperse to upland, terrestrial habitats, where they are mostly active during the day in early and late summer (Mullally 1958, entire; Campbell 1970a, pp. 84–86; Carey 1978, pp. 203, 206, 211), foraging primarily on ants, beetles, spiders, and other invertebrates (Schonberger 1945, p. 121; Campbell 1970a, p. 69–71). Late in the summer, adult toads will expand their movements, generally in the direction of wintering habitats, which include cavities among streamside boulders, small mammal burrows, and beaver lodges and dams (Campbell 1970a, pp. 50, 87; Hammerson 1999, p. 94; Jones and Goettl 1998, p. 29).

Survival of eggs is variable, with high mortality events observed due to desiccation, predation, disease, and unknown causes (Blaustein and Olson 1991, entire; Holland 2002, p. 14; Carey et al. 2005, p. 234). Survival of tadpoles is also variable with frequently high mortality primarily caused by predation, desiccation, or other adverse environmental conditions (Campbell 1970a, p. 61; Carey et al. 2005, p. 234; Muths and Nanjappa 2005, pp. 394-395; Lanier 2015, p. iv). Approximately 95 to 99 percent of juveniles die before reaching their second year of life (Samollow 1980, p. 33). However, low egg, tadpole, and juvenile survival is not unusual among anurans (frogs and toads); most anuran populations have highly variable recruitment that is compensated by more stable adult survival (e.g., Alford and Richards 1999, p. 133), such as in boreal toads where males have been recorded to live at least 16 years (Lambert and Schneider 2013, p. 104) and females have been recorded to live more than 21 years (Lambert and Schneider 2016, p. 41).

Habitat use by adult toads in Colorado indicates considerable use of rocky areas and wet areas in aspen/conifer and other montane forests (Campbell 1970a, p. 33; Jones and Goettl 1998, p. 27; Lambert and Schneider 2013, p. 4). Patterns of habitat use by adult toads after breeding may vary according to sex of the toad and composition of the terrestrial landscape (Campbell 1970a, 22, 29-34, 67). Available evidence indicates that female toads disperse farther and into drier habitats than do males (Loeffler 2001, p. 8; Muths 2003, p. 162; Bartelt et al. 2004, p. 460; Carey et al. 2005, pp. 230, 235; Goates et al. 2007, p. 479). Seasonal movements of up to 6 kilometers (km) (3.7 miles (mi)) have been observed (Bartelt et al. 2004, p. 460; Bartelt et al. 2010, p. 2683, fig. 7A, 7B). In Utah, boreal toads were documented having traveled up to 5.3 km (3.3 mi) in two years (Seegert 2017, p. 5) and they have been observed having traveled 8 km (5 mi) in 3 years in Colorado (Lambert 2003, p. 88). Adult boreal toads tend to display high breeding site fidelity, similar to

other amphibians (Oldham 1966, 1 p.; Breden 1987, p. 386; Berven and Grudzien 1990, p. 2051; Carey et al. 2005, pp. 233, 235; Muths et al. 2006, p. 1054).

Feeding

Boreal toads are predominately insectivorous after metamorphosis. Prey items found in boreal toads primarily include ground dwelling arthropods, including ants, beetles, spiders mosquitoes, grasshoppers, crane flies, stink bugs, damsel bugs, deer flies, wasps, bees, water striders, alder flies, backswimmers, muscid flies, mites, and snails (*Formicidae, Carabidae, and Araneae* (Campbell 1970a, pp. 69-72; McGee and Keinath 2004, p. 21).

CHAPTER 2 – RESOURCE NEEDS

Individual Needs

Each life stage of the boreal toad requires biological and physical resources that support feeding, breeding, sheltering, and dispersal of the species. Table 2 provides a detailed account of the individual needs for each life stage we described above. In addition, the table indicates the function that the resource provides for feeding, breeding, sheltering, and dispersal.

Table 2. Individual Life Stage Needs

Life Stage	**Resources and/or circumstances needed for INDIVIDUALS to complete each life stage**	**Resource Function***
Eggs		
-	Ponded water (wetland, pond, tarn, beaver pond, lake, man-made ponds, roadside ditches) (Carey et al. 2005, pg. 223; Loeffler 2001, p. 7) at elevations of 2000 m (6,550 ft) (Hogrefe et al. 2005, p.7) to 3,670 m (12,232 ft) (Lambert and Schneider 2013, p.10, 11; Crockett 2017a, p. 5) though typically 2,440 m to 3,350 m (8,000-11,000 ft)(Loeffler 2001, p.6)	S
-	Stable water levels to prevent desiccation or deepening water that prevents development (Corn 1998, p.21; Carey et al. 2005, pg. 227)	S
	Shallow, gently sloping bottom, generally < 10 centimeters (cm) (4 in) deep for egg thermoregulation (Holland 2002, p. 13; Carey et al. 2005, pg. 227; Holland 2006, p. 158), but up to or over 1 m (3 ft) deep (Olson 1989, p. 392)	S
-	Water temperature within a range of 10-30˚C (50-86˚F) (Carey et al. 2005, pg. 227, 234)	S
-	Minimum pH 4.9 (Corn et al. 1989, p. 28; Corn and Vertucci 1992, p. 365)	S
Tadpoles		
-	Ponded water, daily fluctuating temperature of 10-30˚C (50-86˚F) (Carey et al. 2005, pp. 227, 234)	S
-	Stable water level through metamorphosis (Carey et al. 2005, pp. 227- 228, 234)	S

-	Shallow, gently sloping bottom < 10 cm (4 in) deep for thermoregulation (Carey et al. 2005, p. 227;Holland 2006, p. 158)	S
-	South-facing exposure to sun; typically northeast to northwest shores (Carey et al. 2005, pg. 227; Loeffler 2001, p. 6)	S
-	Algae, detritus, carrion (Muths and Nanjappa 2005, p. 394)	F
Juveniles (metamorphosis to 1 year old)		
-	Moist soil, water, for hydration (Livo 1998, p. 130)	S
-	Wide variety of arthropods (Hammerson 1999, p. 97; McGee and Keinath 2004, p. 21)	F
-	Vegetation for shelter and to prevent desiccation (Bartelt et al. 2004 (p. 464; Bartelt et al. 2010, p. 2685)	S
-	Bare ground, exposure to sun for thermoregulation. Open areas to bask in sun for thermoregulation (Carey et al. 2005, p. 223)	S
-	Movement or dispersal corridors with water availability (Livo 1998, p. 130)	D
-	Hibernacula (ground squirrel holes, chambers under rocks, beaver lodges or dams that are maintained above freezing by groundwater, or are below frost line) (Campbell 1970a, p. 87; Loeffler 2001, p.7; Jones and Goettl 1998, p. 29)	S
Subadults/Juveniles (1- 6 years old, non- breeding)		
-	Upland habitat (generally lodgepole pine, spruce-fir, and alpine meadows (Loeffler 2001, p. 6); also aspen, ponderosa, and blue spruce (Lambert and Schneider 2013, p. 4)	S, F
-	Bogs, wet meadows with varying amounts of emergent vegetation such as sedges, willows and mossy hummocks (Campbell 1970a, p. 33; Lambert and Schneider 2013, p. 4)	S, F
-	Moist soil, water, precipitation, and humid conditions for hydration (Livo 1998, p. 130; Bartelt et al. 2010, p. 2679-2685).	S, F, D

-	Vegetation for thermoregulation (Bartelt et al. 2010, p. 2685)	S, F, D
-	Wide variety of ground dwelling arthropod prey (e.g. ants, beetles, spiders) (Campbell 1970a, p. 69-72; McGee and Keinath 2004, p. 21)	F
-	Bare ground, open areas to bask in sun for thermoregulation (Carey et al. 2005, p. 223)	S
-	Movement or dispersal corridors with water availability (Livo 1998, p. 130)	D
-	Hibernacula up to or beyond 2,720 m (8,924 ft) away from natal ponds (Campbell 1970b, p. 281)	S
Adults (males 3-16+ years; females 5-20+ years)		
-	Ponded water for breeding (wetland, pond, tarn, beaver pond, lake, man-made ponds, roadside ditches) (Carey et al. 2005, pg. 223; Loeffler 2001, p. 7; Holland et al. 2006, p. 157) at elevations of 2000 m (6,550 ft) (Hogrefe et al. 2005, p. 7) to 3,670 m (12,232 ft) (Lambert and Schneider 2013, pp. 10-11; Crockett 2017a, p.5) though typically 2,440 m to 3,350 m (8, 000 - 11,000 ft) (Loeffler 2001, p. 6)	B
-	Generally occur in lodgepole pine, spruce-fir, and alpine meadows (Loeffler 2001, p. 6). Also aspen, ponderosa, and blue spruce (Lambert and Schneider 2013, p. 4). Bogs, wet meadows with varying amounts of emergent vegetation such as sedges, willows and mossy hummocks (Campbell 1970a, p. 33; Lambert and Schneider 2013, p. 4). Usually close to water (Campbell 1970a, p. 33)	S, F, D
-	Wide variety of arthropod prey (ants, beetles, spiders primarily) (Campbell 1970a, pp. 69-72; McGee and Keinath 2004, p. 21)	F
-	Bare ground, open areas to bask in sun for thermoregulation (Carey et al. 2005, p. 223)	S
-	Movement or dispersal corridors to and from breeding ponds (Bartelt et al. 2004, p. 464)	D
-	Hibernacula (small mammal burrows, chambers under rocks, beaver lodges or dams that are maintained above freezing by groundwater or are below frost line) (Campbell 1970a, p. 87; Loeffler 2001, p.7; Jones and Goettl 1998, p. 29; Hammerson 1999, p. 94)	S

*Breeding – B, Feeding – F, Sheltering – S, Dispersal – D

Population Needs

At the population level, the SSA describes the resources, circumstances, and demographics that most influence resiliency of a population. These may vary if populations inhabit different ecological settings. Species viability corresponds to the resiliency of its populations, and therefore, it is necessary to understand and determine for the analysis how populations should be defined for the species. Resiliency describes the ability of a species to withstand stochastic disturbance, and is positively related to population size and growth rate and may be influenced by connectivity among populations. Generally speaking, populations need abundant individuals within habitat patches of adequate area and quality to maintain survival and reproduction in spite of disturbance.

Populations have been defined in various ways for boreal toads. For example, populations have been defined as toads associated with one or more breeding sites that are located within a common second or third order drainage, and separated by no more than five (5) miles (approx. 8 km) (Loeffler et al. 2001, p. 19; Hogrefe et al. 2005, p. vii). However, there is no standard definition of a population throughout the range, nor is there data available that will allow us to identify discrete populations consistently across the range, so we used the HUC-12's as a proxy for this SSA, as explained above.

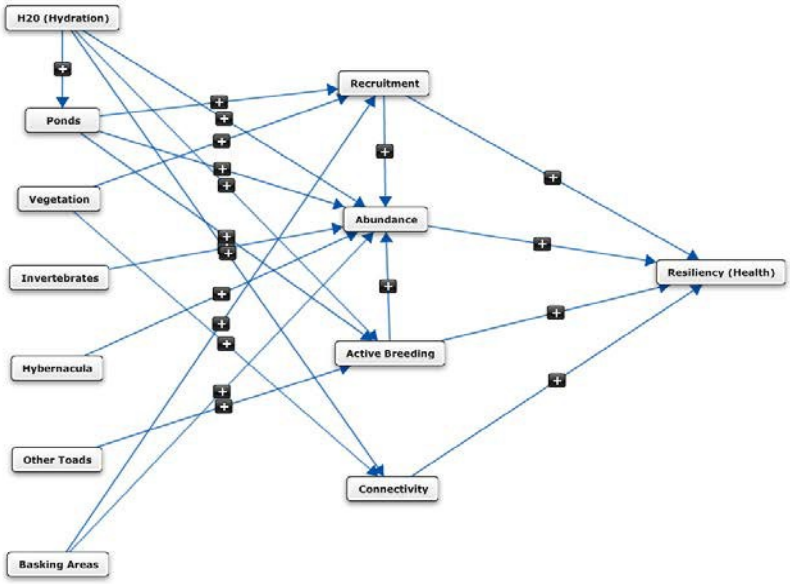
We reviewed two conservation plans developed for portions of the Eastern Population. These plans provided us with examples of site-specific criteria for describing population viability or sustainability within specific ecological settings. Since the ecological settings occupied by boreal toads vary across its range, we developed the following general attributes of boreal toad populations we believe are necessary for a population to be considered resilient:

Consist of a sufficient number of breeding adults (abundance) to withstand stochastic events;

1) Consist of individuals of different ages and sexes;
2) Recruits new breeders into the population;
3) Has successful breeding and recruitment in most years; and
4) Has connectivity with other populations for genetic interchange to maintain genetic diversity; Conversely, it may be beneficial for a few populations to have limited connectivity with other populations so that the species may be less susceptible to stochastic events such as the spread of a novel pathogen among populations.

While we lack data on the precise quantity of these factors that a population needs (e.g. what is the minimum viable population size, what rates of breeding or recruitment are needed for population persistence, what level of connectivity, etc.), we recognize that these factors each contribute to maintaining resilient populations of boreal toads. These population-level needs and how they contribute to resiliency are displayed in the core conceptual model below (Figure 3).

Figure 3. Conceptual Model of Needs Leading to Resiliency



Species Needs

We lack specific quantitative data regarding species-level needs of the Eastern Population of the boreal toad across its range. However, to maintain viability, the populations, or some portion of its populations, must be resilient (e.g. healthy). The species also needs representation, or diversity, which allows adaptability to changing conditions over time. In addition, multiple resilient populations distributed throughout the toad's range provide redundancy. Redundancy spreads risk, which reduces the risk that a large portion of populations will be affected negatively by a single catastrophic event at a given point in time. Species that are well distributed across their range are less susceptible to extinction and more likely to be viable than species confined to a small portion of their range (Carroll et al. 2010, entire; Redford et al. 2011, entire).

Summary of Species Needs:

Boreal toad individuals need a variety of resources to support each stage of their life cycle, such as water, vegetation, invertebrate prey, and hibernacula. At the population level, breeding and recruitment are necessary, an abundance of toads and other individuals for breeding are needed, and connectivity to other sites or populations is needed to maintain resiliency, although we lack specific information to determine quantitative values for these requirements. At the species level, boreal toads need multiple resilient populations, distributed throughout their range (redundancy), and diversity in ecological settings and genetics (representation).

CHAPTER 3 – CURRENT CONDITIONS

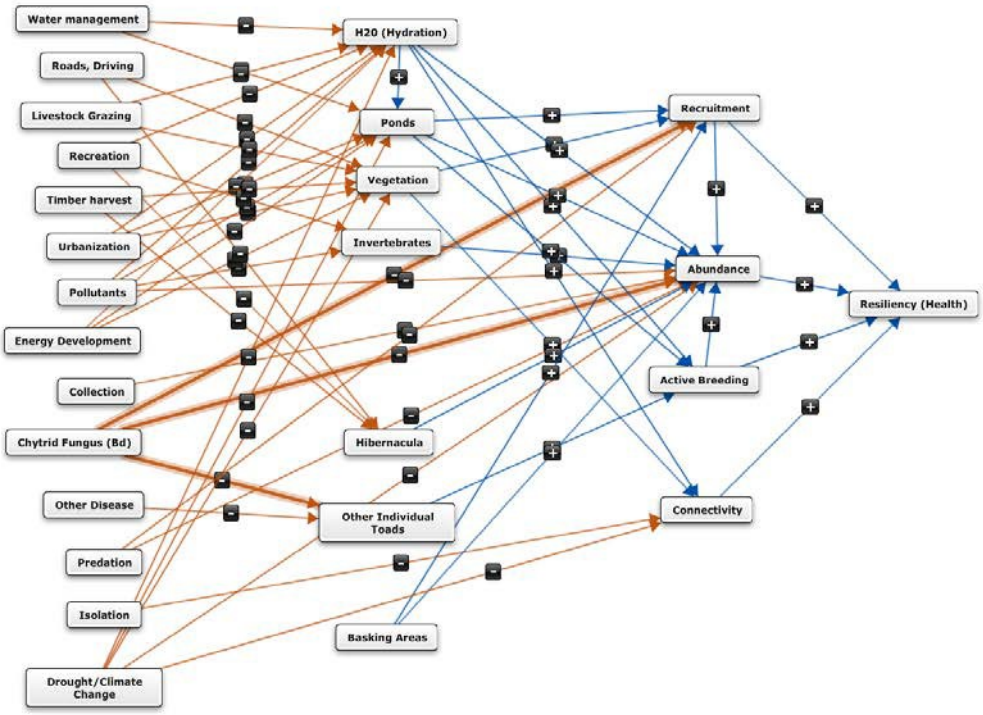
In this chapter, we describe the current condition of the species' habitat and demographics, and the probable explanations for past and ongoing changes in abundance and distribution within areas representing important geographic, genetic, or life history variation (i.e., the species' ecological settings). The species' ecological needs, as assessed above, provide the basis to explain changes in abundance and distribution. The species' current condition is an empirical assessment based on available data and knowledge.

Chapter 2 describes the boreal toad's specific individual needs, and general, over-arching population and species-level needs. Here, we assess the species' current condition by examining how stressors and other influences impact the toads' ecological setting, and its lifecycle. We assess the current condition of the species in terms of resiliency, redundancy, and representation.

Potential Stressors and Influences

In our 90-Day finding, we identified potential threats (stressors) that could possibly affect boreal toads, including water management; roads (including use); livestock grazing; recreation; timber harvest; urbanization; pollutants; energy development; collection; predation; isolation; drought/climate change; ultraviolet radiation; and the presence of Bd and other diseases. As we noted in our 90-Day finding, the 2011 petition also suggested that invasive species could affect boreal toads through disease transmission or predation. Therefore, as we did in our 90-day finding, we incorporate into our evaluation diseases and predation related to invasive species into this SSA Report. The severity to which these stressors act on the species varies. Appendix A provides a detailed analysis of each of these potential stressors and their effects on the species. Appendix A also describes our level of confidence in the relationship between a stressor and it's affect to boreal toads. The influence diagram in Figure 4, displays the resource needs of individuals and populations of the species, and how those needs are affected by environmental or anthropogenic stressors (Appendix A). Heavier (darker) lines in the influence diagram (Figure 4) represent increased severity of the stressor acting upon the species. In addition, we used the influence diagram (Figure 4) to display our evaluation of the cumulative nature of the effects acting upon the species. We determined that while a number of stressors may act upon the species and its ecological needs, cumulatively they did not exert significant negative effects on the resiliency of the boreal toad. Based on our analyses, we conclude that only chytridiomycosis resulting from Bd infection has the potential to cause population-level effects to boreal toads. The other stressors (Appendix A) may affect individuals or individual breeding sites, but are not expected to impact the species as a whole. The remainder of this analysis focuses on this one primary stressor. The most significant driver of current population resiliency for the boreal toad is response to Bd infection. Other factors that could also influence the viability of the boreal toad are climate change and conservation management actions. These potential influences are described further below.

Figure 4. Conceptual Model of Effects of Environmental and Anthropogenic Stressors on Population Resiliency.



Batrachochytrium dendrobatidis, Chytrid Fungus (Bd)

The primary factor influencing some boreal toad populations is the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) [Bd]. Chytrid fungus can cause chytridiomycosis (the disease that can result from Bd infection). The exact mechanism by which Bd infection causes mortality in toads is not understood, but possible mechanisms include disruption of water, oxygen, and ion exchange and secretion of toxins from the Bd associated with chytridiomycosis (Berger et al. 1998, p. 9036).

Amphibian chytrid fungus was first identified and implicated in amphibian declines in Central America and Australia (Berger et al. 1998, entire), and has caused 90-100 percent mortality in other species of metamorphosed amphibians (McGee and Keinath 2004, pp. 43–44). Declines in boreal toads were noted in the mid-1980's in Colorado and south-central Wyoming, with disappearance of 17 percent (10 out of 59) previously known boreal toad localities [breeding sites] (Corn et al.1989, p. 10). These losses may have been caused by Bd-related mortality. In the southern Rocky Mountains, data suggest that boreal toads were likely extirpated from 6 of 20 known sites where Bd invaded between 2003 and 2013 (Crockett 2017a, p. 7). Although extirpations do occur, ongoing monitoring in the southern Rocky Mountains suggests that some

populations experience a significant decline in the number of toads followed by years of persistence at far lower numbers (Crockett 2017a, p. 7), rather than outright population extirpation. Our understanding of how Bd affects boreal toad populations has improved since we issued our 90-day finding. At that time, we concluded that Bd may be the major factor in the decline of the boreal toad, and that it may pose a significant threat to the Eastern Population of the boreal toad (Loeffler 2001, p. 13; Hogrefe et al. 2005, pp. 13–14) (77 FR 21922). At the time of our 90-day finding, we assumed that Bd could cause up to approximately 100 percent mortality of toads (77 FR 21932), and we had no indication, as we do now, that toads in other parts of the range appear unaffected by Bd, or are more resistant to Bd infection, compared to boreal toads in the southern Rocky Mountains.

Within the southern Rocky Mountains, our conclusion about the threat of Bd may still hold true. In 2014-2016, the Southern Rocky Mountains Conservation Team undertook an extensive structured decision-making process to update the existing conservation plan (Loeffler, C. [ed.] 2001, entire) for the boreal toad range in that region. The goals of this process were to maximize the likelihood of toad persistence across a wide distribution, identify the most effective management strategies, and allocate Team resources most efficiently. The methods and results of this endeavor are reported in detail in Converse et al. (2016, entire) and Gerber et al. (in review) as cited in Crockett (2017a, p. 2). As part of that process, the authors, with input from the Team, developed a model of boreal toad population persistence in the southern Rocky Mountains under various management scenarios (Boreal Toad Meta-population Viability Model 2016). The model and associated publications [Converse et al. (2016), Mosher et al. (in prep.), Gerber et al. (in review)] provide the most statistically rigorous assessment of viability of toads in the entire southern Rocky Mountains (Crockett 2017a, p. 2).

Boreal toad population viability in the southern Rocky Mountains is considered robust in the absence of Bd (Crockett 2017a, p. 2). Active breeding sites that tested negative for the presence of Bd had a probability of breeding the following year of 0.96 (+/- 0.02) according the Boreal Toad Meta-population Viability Model (2016, https://borealtoad.shinyapps.io/SRM-BT-MODEL-Public/). However, the probability of observing breeding the following year at sites that tested positive for Bd was reduced to 0.70 (+/- .08). The arrival of Bd at breeding sites has caused severe declines in boreal toad numbers followed by years of persistence with far fewer individuals (Crockett 2017a, p. 8). The model further predicts that the probability of toads persisting in the southern Rocky Mountains is 0.95 in the next 50 years. However, the number of active breeding sites within this geographic area will likely be reduced by approximately one-quarter to one-third from current conditions. Although we do not have data to confirm what may have caused extirpations in the southern Rocky Mountains, chytridiomycosis resulting from Bd infection could be responsible.

In other areas within the Eastern Population, Bd has been detected within breeding sites, but the response of toads differs from the response observed in the southern Rocky Mountains. Boreal toads in Utah showed little evidence tying Bd directly to any substantial boreal toad losses

(Seegert 2017, p. 7). In western Wyoming, populations infected by Bd declined 5 to 7 percent per year during a 6-year study, while uninfected areas (in Colorado) remained stable (Pilliod et al. 2010, p. 1264). Murphy et al. (2009, entire) found differences in resistance to Bd, and described behavioral differences between toads from Colorado and western Wyoming. In addition, Murphy et al. (2011, entire) found that dry conditions promoted increased survival of toads previously exposed to Bd, and that prior exposure to Bd boosted survival compared to Bd-naive toads. Currently we have no indication that the presence of Bd has caused breeding site extirpations in western Wyoming. The earliest known occurrences of Bd in the Eastern Population in Idaho were from samples collected between 2000 and 2004 (Muths et al. 2008, p. 1486-1487). The Idaho Department of Fish and Game indicated that it does not have information about the effects to boreal toad populations (e.g., survival probability, population growth/declines) at breeding sites where Bd has been detected (Idaho Department of Fish and Game 2017, p. 5). However, persistence of the boreal toad population at the Tincup Creek breeding site in Idaho in 2012 and 2015, suggests that this population may be coexisting with Bd, since the pathogen was detected at this site in 2000-2004, and in 2015 (Idaho Department of Fish and Game 2017, p. 5; Muths et al. 2008, p. 1486).

Climate Change

Climate change may have a variety of effects on boreal toads in the future. These effects are described in more detail in Appendix A, and are summarized here. As displayed in Figure 3, the availability of water is important to all life stages of the boreal toad. Climate change, which may potentially cause increased frequency, duration, and severity of droughts in the future, may result in changes to water availability, leading to drying of breeding ponds and subsequent desiccation of eggs and tadpoles. Climate change may also result in more precipitation in some areas, or remain similar to long-term averages (Lukas et al. 2014, pp. 1-4). A synergistic effect of climate change could be an increase in predators of toads (Pierce 2017, pers. comm., p. 1).

Drying of water sources may negatively affect dispersal and connectivity by reducing available water sources across the landscape that likely facilitate more extensive movements of toads. In addition, decreases in the number of breeding ponds or duration of water available for development of toads may decrease recruitment of toads into the adult breeding population (Lambert et al. 2016, pp. 1263, 1265).

Snowmelt has shifted 1-4 weeks earlier in the last 30 years due to climate change in Colorado (Lukas et al. 2014, p. 2). A shift to earlier snowmelt could mean there is potential for lack of late-summer water availability (Lukas et al. 2014, p. 4), reducing the chance of metamorphosis. However, the active season for boreal toads may also increase an additional 34 to 48 days between years 2040 to 2069 (Steen 2017a, pers. comm., p. 1; Steen 2017b) due to warmer daytime temperatures that allow toads to remain active longer. Consequently, the potentially detrimental effects of a shift to earlier spring snowmelt could be offset by a longer active season, allowing more time for metamorphosis and growth of toads, depending on the site-

specific structure of breeding ponds and water availability. Climate change may alter early or late season freezing temperatures, possibly causing mortality before or after hibernation (McGee and Keinath 2004, p. 41; Scherer et al. 2008, entire). Boreal toads typically hibernate in ground squirrel burrows or in woody debris that is covered by a persistent snowpack over most of the winter (approximately November through April) (Muths 2017, p. 6). Snowpack is expected to be reduced in the spring due to warmer temperatures, despite prediction of more winter precipitation (at least in Colorado) (Barnett et al. 2005, entire; Lukas et al. 2014, p 4). There is some evidence that boreal toads have lower survival probabilities in years with lower minimum daily temperatures in winter, and that low snowpack and warm temperatures could rouse toads out of hibernation and reduce survival (Scherer et al. 2005, p. 2159; Scherer et al. 2008, p. 516; Sinclair et al. 2013, p. 301; Wipf et al. 2009, p. 112).

However, adults have been found to be able to tolerate freeze transitions (daily fluctuation below and above freezing) (Lambert et al. 2016, p. 1262). Studies suggest that more active season days could increase body mass and result in increased survival of breeding adults (Lambert et al. 2016, p. 1262; Browne and Paszkowski 2010, p. 256).

Climate change could potentially influence the distribution of Bd. However, the temperature range in which Bd can survive (4° C or less to 30° C (39° F – 86° F)), and its optimal temperature range for growth (17° – 25° C (63° F - 77° F)), are relatively broad (Piotrowski et al. 2004, p. 14). Consequently, even the highest predicted increase in mid-century temperatures (3.6^{o} C (6.5^{o} F)) are not likely to cause extirpation of Bd, but could potentially reduce its prevalence at lower elevations of the toad's range, where temperatures could get warmer (and possibly drier) than is optimal for Bd, and the toads may be able to shed Bd more readily by basking in warmer and drier climates. Several of the highest known elevation breeding sites in the Eastern Population already have Bd (Lambert and Schneider 2016, pp. 6-38). Consequently, increased temperatures are unlikely to facilitate Bd exposure to higher elevation breeding sites, since Bd can already survive there.

Overall, there is the potential for climate change to affect boreal toads in the future, but the outcome of these effects is uncertain. Based on climate predictions, the active season for boreal toads (at least in the southern Rocky Mountains) is expected to increase with elevated temperatures, potentially increasing the length of time for development and increasing survivorship and abundance of toads. However, earlier spring snowmelt and drier conditions due to higher evapotranspiration could affect late-summer water availability and preclude metamorphosis of tadpoles, especially at higher elevations. Conversely, earlier snowmelt could also potentially shift the breeding season earlier with no additive mortality. Lower snowpack (due to predicted increases in rain and decreases in snow (Barnett et al. 2005, p. 305) will potentially increase the risk of exposure of overwintering boreal toads to freezing temperatures, and could decrease survival, but these effects would likely be site-specific and not affect the boreal toad population as a whole, since adult toads were found to tolerate freezing transitions (Lambert et al. 2016, p. 1262).

To date, we have no evidence that the current levels of precipitation, current increases in temperature, or current observations of earlier snowmelt have caused long-term or widespread effects to the life stages of boreal toads, or abundance of toads in the Eastern Population. There is potential for predicted future changes in climate to affect the boreal toad, particularly from reductions in available water, but based on existing information, the added recruitment from additional active season days could offset a reduction in areas with successful recruitment. Based on available information, there does not appear to be a synergistic effect of current or predicted future climate changes to Bd distribution or prevalence. In addition, we have no information to determine if predators of boreal toads have or will increase as a result of current or predicted changes in climate. In summary, there is potential for both positive and negative impacts as a result of climate change. However, we currently have no evidence that predicted changes in climate will result in significant range-wide changes that are likely to significantly affect the Eastern Population of the boreal toad at the population-wide level.

Management Actions and Conservation Efforts

Management actions have the potential to positively influence boreal toad viability. Two conservation plans have been developed by the Utah Boreal Toad Conservation Team (Hogrefe et al. 2005, entire), and the Southern Rocky Mountains Boreal Toad Recovery Team and Technical Advisory Group (Loeffler, C. [ed.] 2001, entire). Both plans contain a number of conservation actions that have been implemented to minimize stressors and maintain or improve the status of the Eastern Population of the boreal toad. Actions include species and disease monitoring, habitat management, and restoration of populations into historical and potential habitats. At this time, we have little information about the effectiveness of implementing these actions pursuant to these conservation plans. There is limited information from reintroduction efforts under the Southern Rocky Mountain Conservation Plan. Some reintroduction sites did not show recruitment success (survival to adult life-stage and breeding), but did provide information to help increase the chances of success of subsequent reintroductions or translocations (Muths et al. 2001, entire, Muths et al. 2014, entire). However, one reintroduction site in Colorado, known as the Zimmerman site, has experienced successful recruitment into the adult breeding population and subsequent reproduction for several years (Bailey 2017a, p. 3).

In addition, the species has different legal statuses in different states. Boreal toads are state listed as endangered in Colorado, a state sensitive species in Utah, a protected nongame species and a Species of Greatest Conservation Need in Idaho, state listed as endangered in New Mexico, and a native species status one in Wyoming. These state level designations are accompanied by regulations to either prohibit or restrict collection of boreal toads.

The U.S. Forest Service and the Bureau of Land Management consider boreal toads a “sensitive species.” The conservation provided by this status likely varies. Both agencies include policy guidance to avoid, minimize, or eliminate potential adverse effects caused by management

actions to sensitive species. At this time, we have no information to meaningfully assess or quantify the level of conservation imparted by the policies of these agencies.

Collectively, these efforts and legal statuses likely have some positive influence on boreal toad viability, but we do not have information to indicate that they have been major drivers of the toad's current status now or into the future.

Summary

Figure 4 depicts our understanding of the most important factors that influence boreal toad population resiliency. The most significant stressor to boreal toad populations is the occurrence of the pathogenic fungus, Bd. However, Bd affects toads differently within various parts of the range. It appears that the effects of Bd infection range from nearly undetectable in Utah to significant declines and some extirpations in the southern Rocky Mountains. Climate change may also play a role in water availability, but we have little evidence (positive or negative) of climate change effects in the Eastern Population.

Evaluation of Current Condition

We considered various methods for evaluating the current condition of boreal toad populations. For example, we considered categorizing the health of each population (represented by the HUC-12s) based on the presence of the available resources that individuals and populations need, such as water, vegetation, invertebrate prey, and hibernacula. We also considered categorizing the health of populations based on demographic factors, such as abundance, evidence of active breeding, recruitment, and connectivity (factors identified above). However, data are not available to measure these factors consistently across the range. Therefore, we decided to assess the current condition of populations based on occupancy of toads and the presence or potential absence of Bd. As discussed above, Bd can be a significant factor affecting population resiliency, and we have information across much of the range on Bd status and boreal toad occupancy.

Resilience

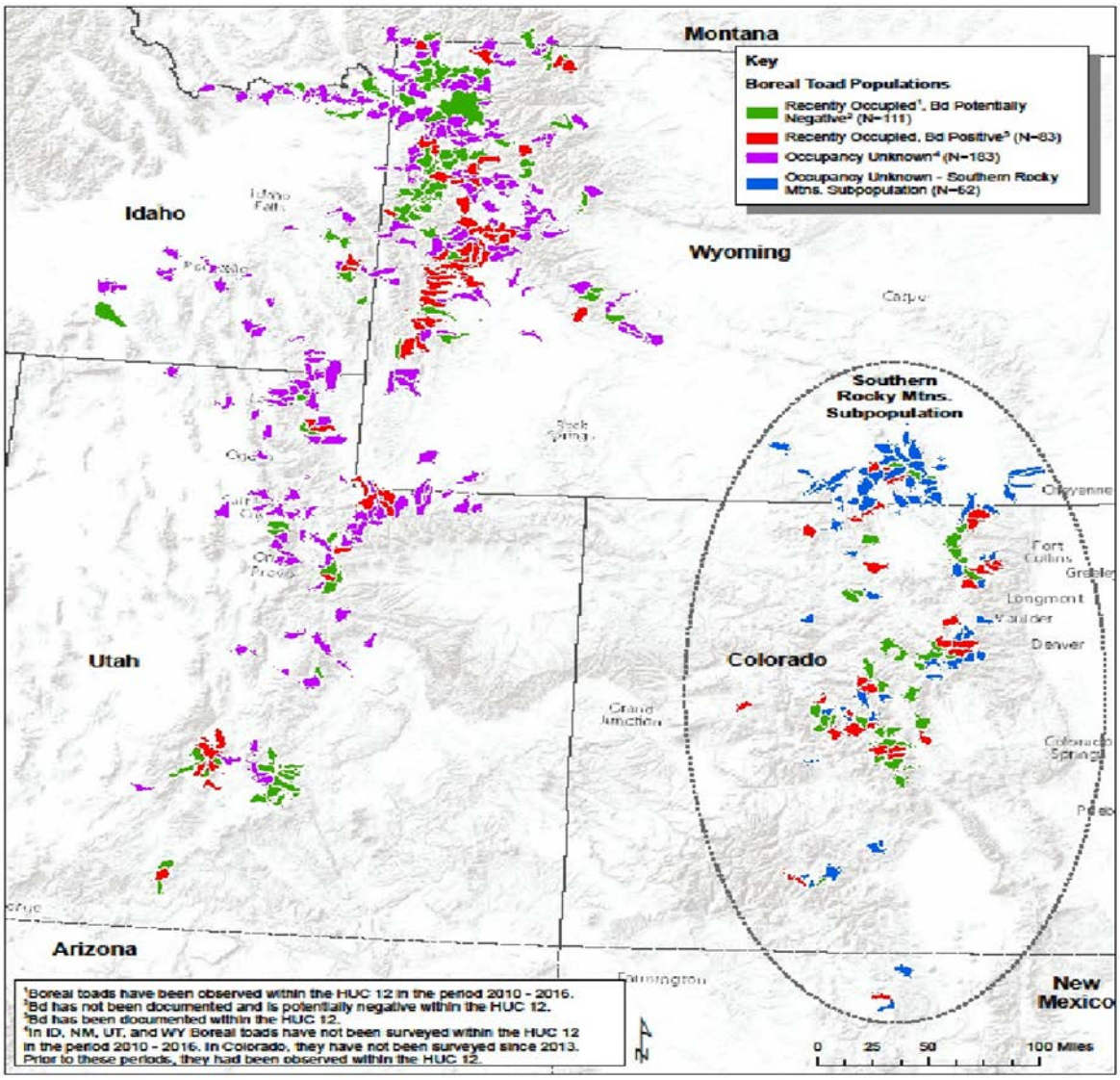
The presence of boreal toads and Bd status of each HUC-12 is displayed below (representing resiliency, Figure 5). The map displays the HUC-12 occupancy status, and the presence or potential absence of Bd, across the range of the species. We developed this map based on information provided by species experts through a questionnaire and request for available data on both species occupancy and the known presence of Bd. The map does not necessarily reflect the total possible range of the species, and it does not necessarily provide the full extent of Bd presence within the range of the species. Any omissions/limitation to either of these parameters within the species' range is based on the limited availability of data. In other words, not all possible areas that could contain boreal toads have been surveyed, or some areas may not have

been surveyed recently (since approximately 2009). In addition, survey efforts for the presence of Bd may not have been conducted recently.

We considered HUC-12s that are occupied by boreal toads, and are Bd negative, to have higher resiliency than HUC-12s that are occupied by boreal toads, and are Bd positive. The presence of Bd can significantly impact the resiliency of individual breeding populations, especially in the southern Rocky Mountain portion of the range. However, as discussed previously, Bd presence does not affect all boreal toad populations in the same way, i.e., boreal toads display diversity in response to Bd infection across its range. Although these differences are not well understood, one ongoing investigation suggests that toads from Utah do not appear to display any significant relationship between increasing Bd infection load and body condition (Corey-Rivas 2017, entire), whereas toads from Colorado displayed a strong negative relationship between body condition and increasing Bd infection (Corey-Rivas 2017, entire). Populations of boreal toads in Colorado have experienced declines in population numbers, and even extirpation with the arrival of Bd. In Utah, the presence of Bd does not appear to have caused significant declines in toad numbers. In western Wyoming and southeastern Idaho, population declines caused by Bd appear less pronounced than in the southern Rocky Mountains (Pilliod et al. 2010, p. 1264), displaying gradual population decline. Therefore, some boreal toad populations may be considered resilient even when Bd is present.

In our 90-Day finding, we concluded that Bd may be the major factor in the decline of the boreal toad and that it may pose a significant threat to the Eastern Population of the boreal toad (Loeffler 2001, p. 13; Hogrefe et al. 2005, pp. 13–14). However, recent ongoing investigations have improved our understanding of the species' response to Bd (Pilliod et al. 2010, entire; Corey-Rivas 2017, entire; Murphy et al. 2009, entire), which complicates our assessment of resiliency. We no longer necessarily assume that that the presence of Bd in a population will lead to significant declines or extirpation, but this likely depends on the geographic area.

Figure 5 Current Range and Bd Occupancy.



Species Redundancy and Representation

Redundancy means having sufficient numbers of resilient populations for the species to withstand catastrophic stochastic events. A catastrophic event is defined here as a rare destructive event or episode involving many populations. The most likely catastrophic events are larger scale extirpation of toad populations due to drought or possibly Bd. However, some populations of the boreal toad appear to display at least some tolerance to Bd (e.g. Utah and western Wyoming). Although not well understood, some toad populations in the southern Rocky Mountains are persisting despite significant population declines attributable to Bd infection.

We describe redundancy in boreal toads in terms of the number of resilient populations across the range. Healthy populations of boreal toads across the range should provide adequate redundancy for the Eastern Population to withstand large scale stochastic events. Table 3 displays the current status of HUC-12s across the range, and Figure 5, displays the distribution of Bd across the species range. Occupied HUC-12s with negative Bd status likely represent the most resilient populations. Within the SRM, approximately 27 percent of HUC-12s with known occupancy fall into this category. Twenty-four percent of the HUC-12s in SRM are currently occupied and are Bd positive. However, several populations in the SRM continue to persist, including breeding activity, for years after the appearance of Bd (Crockett 2017, p. 7).

Outside of the SRM, occupancy of approximately 58 percent of all HUC-12s is unknown, primarily due to the lack of recent data. Therefore, we cannot make any assumptions about the status of toad populations in these locations. In the southern Rocky Mountains, boreal toad occupancy in 62 HUC-12s were reported as unoccupied. Boreal toads in the SRM HUC-12s may have been extirpated due to the presence of Bd, but we have no data to support any conclusions about the possible cause of these suspected extirpations. The remaining resilient populations collectively contribute to redundancy, spreading the risk of population losses in case of a catastrophic stochastic event. We note that although many of the occupied HUC-12s are adjacent to one another, toads in adjacent HUCs may be isolated from one another due to distance between breeding sites. Given their relatively limited dispersal ability (Bartelt et al. 2004, p. 460; Bartelt et al. 2010, p. 2683, fig. 7A, 7B; Lambert 2003, p. 88; Seegert 2017, p. 5), and breeding site fidelity (Carey et al. 2005, pp.230, 235), repopulation of extirpated breeding sites from extant breeding sites may not readily occur without active management.

Table 3. HUC-12 Occupancy and Status of Bd

Population Areas	HUC-12 Recently Occupied and potentially Bd Negative		HUC-12 Recently Occupied and Bd Positive		-12 Occupancy Status		Total HUC-12
	Number	%	Number	%	Number	%	Number

					Likely Extirpated		
Southern Rocky Mountains Subpopulation	34	27%	30	24%	62	49%	126
					Unknown		
UT, WY, ID	77	25%	53	17%	183	58%	313
Total	111	25%	83	19%	245	56%	439

Representation means the species having the breadth of genetic and ecological diversity that allows the species the potential to adapt to changing environmental conditions. We assessed representation of boreal toads in terms of the diversity that occurs across the range, in genetic factors, ecological settings, stressors, behavioral differences, and morphological differences (Table 4). However, we are not aware of any behavioral or morphological differences between toads across the species range.

Table 4. Representative Diversity in the Boreal Toad

State/Area	Genetics	Ecological Setting	Stressors (Bd)	Behavioral Differences	Morphological Differences
S. Rockies (Colorado, south-central WY, northern NM)	Similar nDNA and mtDNA within S. Rockies. NM toads reintroduced from CO	2,590 - 3,728 m (8,500 - 12,232 ft)	Higher Mortality rates from Bd relative to other areas	None	None
Southeastern Idaho	Transition zone, containing haplotypes from both the NW Clade and the Eastern Clade	1,902 - 2,434 m (6,240 - 7,986 ft)	No known effects to toads from Bd. Persistence of toads in Bd positive areas	Unknown	Unknown
Northwest Wyoming	MtDNA, admixture zone, but not apparent in nDNA.	Not evaluated. 1,570 - 3,657 m (5,150 - 12,000 ft)	Slower rates of decline from Bd relative to S. Rockies	None	None
Utah	nDNA similar to rest of Eastern Population. mtDNA shows differences versus northwest WY.	2,000 - 3,657 m (6,550 - 12,000 ft)	Little evidence of population declines caused by Bd. Possible genetic adaptation showing resistance to Bd.	None	None

Current Condition Summary

The boreal toad likely occupies the majority of its known historical range within Colorado, Wyoming, Utah, northern New Mexico, and southeastern Idaho, although many HUC-12s lack recent survey data to confirm boreal toad occupancy. Boreal toads in the southern Rocky Mountains have experienced rapid, precipitous declines in breeding populations, or extirpations possibly caused by Bd. However, breeding continues to persist within some Bd infected sites for many years after being infected (Crockett 2017a, p.8). Boreal toads in Utah are not significantly affected by the presence of Bd (Corey-Rivas 2017, entire), with no evidence of precipitous declines or extirpations caused by Bd or any other stressor (Seegert 2017, p. 7). Boreal toads in western Wyoming are experiencing some population declines which have been attributed to Bd, but the infections have not resulted in declines in the number of breeding populations, and breeding occurs regularly at surveyed sites (Estes-Zumpf 2017, pp 13-14). Boreal toads in at least one population in southeastern Idaho continue to persist despite the presence of Bd (Idaho Department of Fish and Game 2017, p. 5).

The Eastern Population of boreal toads includes at least 111 populations we considered healthy due to the absence of Bd, and are considered the most resilient. These populations are distributed across the range of the species. In other populations, Bd is present, but some of these populations display some tolerance of Bd infection; however this is variable across the range.

Chytrid fungus infected toads in Utah may be as resilient as toad populations that are not infected by Bd elsewhere. Based on evidence of gradual population declines likely attributed to Bd, boreal toads in western Wyoming appear to be somewhat less tolerant of Bd compared to toads in Utah. Due to the persistence demonstrated by at least one population of toads in southeastern Idaho, we considered Bd infected populations in these areas moderately resilient. Boreal toad populations in the southern Rocky Mountains that are infected with Bd display no tolerance to Bd, and are considered to be of low resilience. Therefore, there is a gradation of resiliency within the Eastern Population, which also indicated representation across the range of the Eastern Population of boreal toads.

Approximately 85 percent of occupied (since 2010) boreal toad populations in the Eastern Population are considered highly and moderately resilient, and 15 percent of the occupied toad populations (e.g. toads in the southern Rocky Mountains) display low resilience. We are uncertain about the occupancy status of 183 populations that occur outside of the southern Rocky Mountains, because the occupancy of these populations is currently unknown. These populations occur in areas that show some level of tolerance to Bd. As stated above, we have no data or information that would lead us to assume that boreal toads have been extirpated from these unoccupied HUC-12s. If we assume that these HUC-12s are occupied, we would likely place them in the moderately resilient category since they occur in areas that display some tolerance to Bd. These differences in tolerance to Bd demonstrate representation across the range of the toad. Assuming that the HUC-12s where the occupancy is currently unknown are actually occupied, approximately 348 of 439

(79%) of all boreal toad populations (HUC-12s) would be considered in a moderate to high resiliency category, providing a high degree of redundancy across the range of the boreal toad. We acknowledge that our assessment is at a coarse scale, as explained previously.

CHAPTER 4 – SPECIES' FUTURE CONDITION

Assessing the future condition of the boreal toad is challenging. Our assessment of numerous stressors indicates that the primary factor that may affect the future viability of the Eastern Population of boreal toads is Bd infection as described above. Climate change may affect environmental conditions which may positively or negatively affect the resiliency of individual breeding populations, but is unlikely to significantly affect the distribution, presence or persistence of Bd. Additionally management actions, described in Chapter 3, also play a role in enhancing resiliency where boreal toad populations may not be resistant to BD. There is uncertainty regarding whether and how rates of Bd infection may change, and what management actions to conserve boreal toads may be implemented in the future.

As discussed in the previous chapter, there are multiple possible future climate change scenarios, and a high degree of uncertainty regarding future water availability under any of these scenarios. While the amount of precipitation may not change, its physical form is likely to change (e.g. more rain, less snow) (Barnett et al. 2005, entire; Lukas et al. 2014, pp. 1-4). A predicted increase in evapotranspiration could reduce available water sources (Steen 2017c, pers. comm.; Steen 2017d). However, the predicted increase in active season days could increase recruitment of toads (Steen pers. comm. 2017a; Steen 2017b).

Climate change is expected to have little effect on Bd distribution or prevalence of infection in boreal toads, based on the relatively broad temperature range tolerated by Bd. Projected precipitation scenarios (at least in Colorado, where Bd effects appear most severe) are variable (Lukas et al. 2014, pp. 1-4). The prevalence of Bd may be reduced at lower elevations of the boreal toad's range if environmental conditions become warmer and drier. The difference in the boreal toad's response to Bd at lower elevation sites in Utah, western Wyoming, and southeast Idaho could indicate Bd's lower tolerance for warmer and drier conditions, or the ability of toads to slough the fungus off. Therefore, climate change could influence boreal toad resilience both positively and negatively, but we remain uncertain about the trajectory and severity of the possible effects.
As described above, during 2014-2016, the Southern Rocky Mountains Conservation Team undertook an extensive structured decision-making process to update the existing conservation plan (Loeffler, C. [ed.] 2001, entire) for the boreal toad range in that region. A model of boreal toad population persistence in the southern Rocky Mountains was developed (Boreal Toad Meta-population Viability Model 2016).

The Boreal Toad Meta-population Viability Model (2016) (a web app) is available on the internet at: https://borealtoad.shinyapps.io/SRM-BT-MODEL-Public/. The web app was designed to allow researchers and managers to evaluate how potential management actions may influence boreal toad and Bd occurrence across a collection of historically occupied sites throughout the southern Rocky Mountains. Site occupancy is defined based on the presence of

boreal toad reproduction (e.g., egg masses, tadpoles) and the presence of Bd at any life stage. If toads are present at a site, but there is no breeding, the model considers the site unoccupied. Default model parameters (i.e., extirpation/colonization) were estimated based on data from 83 sites monitored over 10 years using a dynamic two-species (toad and Bd) occupancy model that accounts for false-negative detections (i.e., the species was present at a site but not detected). We note that climate change effects were not considered in this model.

The model predicts that even without additional management actions, the probability of persistence of boreal toads within the southern Rocky Mountains in 50 years will be quite high (greater than .95). This projection assumes a "worst case" scenario, where populations have no resistance to Bd. However, under this worst case scenario, the model predicts that the number of active breeding sites and occupied mountain ranges would both be lower than they are today, and lower than the objectives described in Loeffler (2001, p. 18) (i.e., approximately a 50% chance that there would be active breeding at 20 or fewer sites, with toads continuing to occupy 3 to 4 of the 11 historically occupied mountain ranges). When an optimal suite of management actions is added, the probability of persistence rises to essentially 1.00 (or 100%), with a 0.80 (80%) chance of having 20 or more active breeding sites, and toads likely persisting in 6 to 7 (of 11) historically occupied mountain ranges. The most important component of the optimal management scenario is maintaining, or ideally accelerating, the current pace of reintroductions (at present there are five sites where toads are being reintroduced at some level).

The predictions of this model represent the best available information on the potential future condition of boreal toads within the southern Rocky Mountains. We are not aware of any additional efforts to predict future population conditions for boreal toads in any other part of the Eastern Population; however we believe the model described above may still be informative when considering the rest of the species' range.

Our analysis in Chapter 3 suggests that the current condition (resiliency) of the boreal toad in the southern Rocky Mountains is lower than the current condition of boreal toads in western Wyoming/ southeastern Idaho, and Utah. Our basis for this conclusion is the apparent higher susceptibility of boreal toads to Bd infection within the southern Rocky Mountains compared to other areas in the Eastern Population. Therefore, the predicted future condition of boreal toads in the southern Rocky Mountains may represent a worst case scenario, since boreal toads in the remainder of the Eastern Population appear to fare better, and possibly exhibit some tolerance to Bd infection. Therefore, if we were to apply the model to similar data from the other geographic areas within the species' range, we would expect a higher probability of persistence in those areas compared to the projected outcome for the southern Rocky Mountains. However, we note that there is uncertainty regarding how climate change will factor into the future viability of the species across its range.

Status Assessment Summary

We used the best available information to forecast the likely future condition of the Eastern Population of the boreal toad. Our goal was to describe the viability of the species in a manner that will address the needs of the species in terms of resiliency, redundancy, and representation. We considered the possible future condition of the species. The predictive model described above considers a range of potential scenarios that we believe accurately predicts the probable status of the species in the southern Rocky Mountains. We believe the predictive model is based upon the worst case scenario (i.e. toads in the southern Rocky Mountains), where no management actions are directed toward conservation of the species, yet the predictive model anticipates a high probability of persistence of toads in 50 years within the southern Rocky Mountains. The model also predicts a reduction in the number of active breeding sites and occupied mountain ranges in the southern Rocky Mountains, which would indicate lower levels of redundancy and representation; however these numbers are predicted to improve with active management. Although we anticipate reduced levels of the 3Rs in the southern Rocky Mountains in the future, it appears that boreal toads in this area will have sufficient resiliency, redundancy, and representation to ensure a high probability of persistence. Given the apparent higher tolerance of Bd displayed by boreal toads across the rest of the range, we have high confidence that these populations will retain relatively higher levels of resiliency, representation, and redundancy compared to toads in the southern Rocky Mountains, and will have a higher probability of persistence.

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Appendix A

This table of Confidence Terminologies explains what we mean when we characterize our confidence levels in the cause and effects tables on the following pages.

Confidence Terminology	**Explanation**
Highly Confident	We are more than **90% sure** that this relationship or assumption accurately reflects the reality in the wild as supported by documented accounts or research and/or strongly consistent with accepted conservation biology principles.
Moderately Confident	We are **70 to 90% sure** that this relationship or assumption accurately reflects the reality in the wild as supported by some available information and/or consistent with accepted conservation biology principles.
Somewhat Confident	We are **50 to 70% sure** that this relationship or assumption accurately reflects the reality in the wild as supported by some available information and/or consistent with accepted conservation biology principles.
Low Confidence	We are less than **50% sure** that this relationship or assumption accurately reflects the reality in the wild, as there is little or no supporting available information and/or uncertainty consistency with accepted conservation biology principles. Indicates areas of high uncertainty.

Evaluating Cause and Effects for the Eastern Population of Boreal Toad

THEME: Water Management			
ESA Factor(s): E	**Analysis**	Confidence /Uncertainty	Supporting Information
SOURCE(S)	**Water Management**		
Activity(ies)	Urban and agricultural water use.	Highly Confident	Scott 1996, pp. 45–46; Skelly1996, pp. 599–604; Semlitsch2002, pp. 621–623; McGee and Keinath 2004, p. 37; Campbell1970a, p. 7; Hammerson 1999,p. 92
Stressor(s)	Water manipulation causing loss of reproduction and recruitment, loss of subadult, and adult foraging and wintering habitat.	Highly Confident	Scott 1996, pp. 45–46; Skelly1996, pp. 599–604; Semlitsch2002, pp. 621–623; McGee and Keinath 2004, p. 37; Campbell1970a, p. 7; Hammerson 1999,p. 92
Affected Resource(s)	Ponded, stable, water for egg and tadpole survival and recruitment, moist dispersal corridors for subadult hydration, water bodies for subadult and adult hydration.	Highly Confident	McGee and Keinath 2004, pp. 9-10.
Exposure of Stressor(s)	Breeding habitat, post-breeding dispersal habitat, summer habitat, and winter habitat. Breeding affected by unstable water levels (e.g. too shallow or too deep), in natural or man-made water bodies. Post-breeding and summer habitat affected if streams or ponds have little or no water due to diversions or drawdowns such that hydration opportunities are reduced or eliminated.	Highly Confident	Lambert and Schneider 2016, pp. 6-8, 12, 24, 37
Immediacy of Stressor(s)	Past, present, and future.	Highly Confident	Campbell 1970a, p. 7; Hammerson 1999, p. 92

Changes in Resource(s)	Dams and water control structures can cause water levels to rise above natural levels potentially flooding summer foraging and wintering areas for the toad. Water can be released from the reservoirs for agricultural/urban use, potentially dewatering sites.	Moderately confident	Campbell 1970a, p. 7; Hammerson 1999, p. 92; Stoddard 2005, p. 40, fig. 15.
Response to Stressors: Individuals	Loss of breeding sites. Mortality of eggs and tadpoles. Desiccation and mortality of juvenile toads and subadults. Potential loss of juveniles, subadults, and adults from unsuitable hibernacular microclimate. Loss of summer habitat for thermoregulation, shelter, and loss or reduction of (prey) that rely on water or moist soil for their life cycles.	Highly Confident	Campbell 1970a, p. 7; Hammerson 1999, p. 92
POPULATION & SPECIES RESPONSES			
Effects of Stressors: Population [resiliency]	There is at least one example in the literature of toads losing habitat and being displaced. Unfortunately, there is no known directly assessing the influence of dam building, wetland dredging and/or flooding, and water manipulation for urban and agricultural use throughout species range. Not all breeding sites are affected by waterbody manipulation; alteration of water availability could reduce survival and abundance of toads, and impact recruitment into the adult population. If water levels are reduced to an extent that dispersal or immigration is not possible to the closest neighboring breeding population then genetic connectivity may be lost for a year or more. Lack of comprehensive analysis of historically occupied breeding areas lost to water manipulation reduced our confidence in the extent of the effects despite local impacts.	Low Confidence	Campbell 1970a, p. 7; Hammerson 1999, p. 92

GEOGRAPHIC SCOPE	Changes to natural wetlands and water bodies are widely distributed in Colorado/Southern Rocky Mountains (SRM) and likely throughout the Eastern population. The percentage of natural wetland areas/water bodies directly modified in the Eastern population and indirect effects to still-existing natural water bodies by timing of water retention and release in historical boreal toad habitat is unknown.	Somewhat Confident	
MAGNITUDE	The geographic scope likely encompasses the Eastern Population but retention of natural habitats and managed water bodies continuing to provide functional habitat, it is likely that the magnitude of effects of this stressor are low to moderate.	Moderately Confident	
SUMMARY	Water management has been documented causing at least localized effects and widespread but local effects are likely. However, the magnitude of effects is likely low to moderate (see magnitude). Currently, water management is not known to be a primary stressor but localized effects are expected to persist and could become more common with increasing human population growth. This stressor will not be evaluated further in our analysis of current or future conditions.		

THEME: Roads and Driving			
[ESA Factor(s): A,E]	Analysis	Confidence / Uncertainty	Supporting Information
SOURCE(S)	Roads and Driving		Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p. 26.
Activity(ies)	Crushing and change in dispersal behavior.	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p. 2
STRESSOR(S)	Direct mortality and loss of breeding population connectivity.	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p. 2
Affected Resource(s)	Dispersal of young toads and immigration of adults.	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p. 2
Exposure of Stressor(s)	Post-metamorphosis dispersal by juveniles and dispersal of subadults and immigration of adults could be disrupted in late summer/fall for juveniles and all summer for subadults and adults.	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p 26.
Immediacy of Stressor(s)	Past, present, and future. Roads have likely impacted boreal toads for 100 years and are expected to into the future.	Highly Confident	Greenwald et al. 2011, p 26.
Changes in Resource(s)	Some summer foraging habitat is lost by roads. Roads can destroy or alter wetlands and shift hydrology. Pollution or sedimentation can occur that	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p 26.

	could affect survival of eggs and tadpoles.		
Response to Stressors: INDIVIDUALS	Direct mortality, possible disruption of dispersal, even without direct (crushing) mortality of individuals. May cause indirect mortality (e.g. dehydration, increased chance of predation). Direct mortality has been observed in several areas.	Highly Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p 26; Lehtinen et al. 1999, entire.
POPULATION & SPECIES RESPONSES			
Effects of Stressors:- POPULATIONS [RESILIENCY]	Reduced survival and abundance. A limited ability to disperse or immigrate can result in reduced connectivity, potentially resulting in deleterious alleles. In the short-term, traffic on roads can lead to isolation and lower abundance increasing the risk caused by stochastic events possibly leading to the eventual loss of a breeding population.	Somewhat Confident	Hogrefe et al. 2005, p. 17; Greenwald et al. 2011, p 26; Lehtinen et al. 1999, entire.
GEOGRAPHIC SCOPE	Roads and the potential for direct and indirect mortality are Eastern population-wide.	Highly Confident	Greenwald et al. 2011, p 26.
MAGNITUDE	There may be more potential from road impacts in the Salt Lake City area up to Idaho and in southeastern Idaho. Roads likely affect local populations to some extent annually.	Somewhat Confident	Greenwald et al. 2011, p. 72.

SUMMARY	Roads can affect dispersing or immigrating toads throughout the Eastern Population and could cause isolation of some breeding sites or populations. Road impacts appear localized and do not appear to affect the Eastern Population as a whole. Localized effects of roads are expected to persist and become more common if the human population continues to increase. This stressor will not be evaluated further in our analysis of current or future conditions.	Somewhat Confident	

THEME: Livestock Grazing			
[ESA Factor(s): A,E]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Livestock Grazing**		
Activity(ies)	Grazing, watering.	Highly Confident	
STRESSOR(S)	Removal of vegetation, trampling, water quality degradation.	Highly Confident	
Affected Resource(s)	Eggs and tadpoles may be susceptible to disease as a result of poor water quality. Eggs, tadpoles, and juveniles are susceptible to trampling. Water manipulation for grazing purposes may change water availability and levels. Subadult and adult toads may be affected if large areas are cleared to promote livestock forage.	Highly Confident	Bartelt and Peterson 1996, p. 14; Bartelt 2000, pp. 20-27, 74-77, 98; Hogrefe et al. 2005, p. 15; Fleischner 1994, pp. 631–632; Loeffler 1998, p. 54; McGee and Keinath 2004, pp. 33–34.

Exposure of Stressor(s)	Eggs and tadpoles may be susceptible to disease in summer during development as a result of poor water quality. Eggs, tadpoles, and juveniles are susceptible to trampling in summer. Water manipulation for grazing purposes may alter water availability and levels. Subadults and adults could be trampled in summer but also affected in summer due to vegetation removal. Adults could be trampled around breeding ponds in summer. Livestock could transmit Bd to ponds that were previously Bd negative	Highly Confident	Bartelt and Peterson 1996, p. 14; Bartelt 2000, p. 20-27, 74-77, 98; Hogrefe et al. 2005, p.15; Fleischner 1994, pp. 631–632; Loeffler 1998, p. 54; McGee and Keinath 2004, pp. 33–34.
Immediacy of Stressor(s)	Past, present, and future. Livestock grazing has occurred for 150 years, is still occurring, and is expected to continue to occur within the range of the Eastern population into the future.	Highly Confident	
Changes in Resource(s)	Water quality can be affected. Vegetation around breeding ponds can be removed by grazing. Vegetation may be cleared in large areas to promote grasses.	Highly Confident	Bartelt and Peterson 1996, p. 14; Bartelt 2000, p. 20-27, 74-77, 98; Hogrefe et al. 2005, p.15; Fleischner 1994, pp. 631–632; Loeffler 1998, p. 54; McGee and Keinath 2004, pp. 33–34.
Response to Stressors: INDIVIDUALS	Mortality by trampling, dehydration due to lack of shade or water.	Highly Confident	Bartelt et al. 2004, p. 464
POPULATION & SPECIES RESPONSES			

Effects of Stressors: POPULATIONS [RESILIENCY]	Abundance, recruitment, and possibly connectivity could be affected.	Somewhat Confident	Bartelt and Peterson 1996, p. 14; Bartelt 2000, p. 20-27, 74-77, 98; Hogrefe et al. 2005, p.15; Fleischner 1994, pp. 631–632; Loeffler 1998, p. 54; McGee and Keinath 2004, pp.33–34.
GEOGRAPHICSCOPE	Livestock grazing occurs throughout the Eastern Population.	Highly Confident	
MAGNITUDE	Although livestock grazing is widespread it does not impact all breeding sites or summer foraging areas.	Moderately Confident	
SUMMARY	Localized effects of grazing can occur from water quality degradation, water level manipulation, trampling, vegetation removal by grazing, and vegetation removal for forage production. Grazing does not currently appear to affect the boreal toad at the population level and effects from grazing are expected to remain similar in the future. This stressor will not be evaluated further in our analysis of current or future conditions.	Somewhat Confident	

THEME: Recreation			
[ESA Factor(s):A,E]	**ANALYSIS**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Recreation**		
Activity(ies)	Camping, hiking, biking, fishing, and off-highway vehicle use.	Highly Confident	Loeffler 1998, p. 51
STRESSOR(S)	Increased predation and the chance of pathogen introduction, trampling, loss of vegetation, reduced water quality.	Somewhat Confident	Hogrefe et al. 2005, pp. 15, 17
Affected Resource(s)	All life stages.	Somewhat Confident	Loeffler 1998, p. 51; Hogrefe et al. 2005, pp. 15, 17
Exposure of Stressor(s)	Eggs could be crushed (vehicle) or trampled (feet) in ponds. Tadpoles could be crushed; pathogens such as Bd could be introduced to ponds. Juveniles could be crushed or trampled. Vegetation providing summer shade could be trampled affecting juveniles, subadults, and adults. Off-highway vehicle use in wetland areas may lead to poor water quality and could increase pathogens to eggsand tadpoles. Crushing of all life-stages.	Somewhat Confident	Lambert and Schneider 2016, p. 11, 12, 28; Loeffler 1998, p.51; Hogrefe et al. 2005, pp. 15,17
Immediacy of Stressor(s)	Past, present, future. Fifty-year history of recreational use within boreal toad habitat, and is likely remain stable or increase in the future.	Highly Confident	

Changes in Resource(s)	Possibility of long-term localized impacts to boreal toad habitat from annual use of areas by humans. Predator abundance may increase due to increased human use. Effects to toads and their habitat can occur annually or occasionally.	Somewhat Confident	Lambert and Schneider 2016, p. 6, 9, 13, 15, 28
Response to Stressors: INDIVIDUALS	All life stages may be crushed or trampled and disease introduction can kill individuals or a breeding population. Attempts at breeding could be interrupted. Vegetation could be trampled eliminating shaded areas, increasing dehydration risk, especially to juveniles or subadults. Survival, abundance, and recruitment could all be negatively affected.	Highly Confident	Loeffler 1998, p. 51; Hogrefe et al. 2005, pp. 15, 17
POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	It is unlikely that individuals at breeding sites are affected by recreation. Bd can be spread by recreation equipment and clothing. However, the extent of human transfer versus animal or other natural means in the Eastern Population is unknown. One large breeding population in Colorado is located near recreation amenities. The site recently became infected with Bd after many years of remaining Bd-negative despite relatively high human traffic. This suggests that recreation may not always correlate with spread of Bd.	Somewhat Confident	Crockett 2017b, Bd summary spreadsheet; Lambert and Schneider 2016, p. 6, 9, 13, 15, 28
GEOGRAPHIC SCOPE	Recreational impacts are widespread.	Highly Confident	

MAGNITUDE	Direct effects to individuals or breeding populations and vegetation are most likely localized. Bd can potentially spread from recreating humans but extent is unknown. Direct effects of recreation appear localized and are of low to moderate magnitude.	Somewhat Confident	Lambert and Schneider 2016, p. 6, 9, 13, 15, 28
SUMMARY	Recreation can cause localized impacts to individual toads and habitat. Bd transmission could occur from recreational activities but the severity of transmission compared to animal or other natural transmission is unknown. A large toad population area in Colorado was one of the latest to become infected with Bd, indicating that recreation does not always correlate with spread of Bd. Habitat impact from recreation is a localized issue that is expected to remain similar in the future. Recreation will not be evaluated further in our analysis of current or future conditions.	Highly Confident	

THEME: Timber Harvest			
[ESA Factor(s): A,E]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Timber Harvest**		
Activity(ies)	Tree cutting, access road building, equipment driving, slash-pile burning		
STRESSOR(S)	Removal of trees and vegetation, destruction and alteration of upland, riparian, and aquatic habitat by access road construction, crushing of understory vegetation by equipment, direct mortality by equipment, slash-pile burning that may be used by toads as shelter, sedimentation of streams and ponds by erosion of access roads or harvest areas.	Highly Confident	Bartelt and Peterson 1994, pp. 18–19; Loeffler 1998, pp. 56–57; Bartelt 2000, pp. 20–27, 74–77; McGee and Keinath 2004, pp. 32–33

Affected Resource(s)	Eggs and tadpoles possibly affected by stream (water source) alteration and sedimentation. Juveniles possibly affected by equipment crushing, slash burning. Juveniles, subadults, and adults potentially affected by all stressors.	Highly Confident	
Exposure of Stressor(s)	Eggs and tadpoles possibly affected in summer by stream (water source) alteration and sedimentation. Juveniles possibly affected by equipment crushing, slash burning in late summer through winter. Juveniles, subadults, and adults' dispersal corridors affected in summer by tree cutting, removal of understory by equipment, direct crushing by equipment, slash burning year-round, soil compaction by equipment may reduce wintering hibernacula.	Highly Confident	Loeffler 1998, pp. 56–57; McGee and Keinath 2004, pp. 32–33; Bartelt 2000, pp. 20–27, 74–77
Immediacy of Stressor(s)	Past, present, future. Timber harvest has been going on for around 150 years in boreal toad habitat. It has likely slowed in the last 2 decades but with tree die-offs due to disease in large areas, at least in the SRM, tree harvest may increase.	Highly Confident	
Changes in Resource(s)	Removal of trees and vegetation, destruction and alteration of upland, riparian, and aquatic habitat by access road construction, crushing of understory vegetation by equipment, direct mortality by equipment, slash-pile burning that may be used by toads as shelter, sedimentation of streams and ponds by erosion of access roads or harvest areas.	Highly Confident	Loeffler 1998, pp. 56–57; McGee and Keinath 2004, pp. 32–33
Response to Stressors: - INDIVIDUALS	Individual mortality from crushing, dehydration in clear-cut areas and physiological cost by interruption of dispersal corridors.	Highly Confident	Bartelt and Peterson 1994, pp. 18–19; Loeffler 1998, pp. 56–57; Bartelt 2000, pp. 20–27, 74–77; McGee and Keinath 2004, pp. 32–33

POPULATION & SPECIESRESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Individual and localized breeding population effects can occur but the level of effect are dependent on location, method, etc. Boreal toads do occur in open meadows, especially for breeding. Consequently, if understory vegetation is maintained and/or a partially closed canopy is maintained, timber harvest is likely to only have localized and minimal impact to toads.	Moderately Confident	McGee and Keinath 2004, pp. 32–33
GEOGRAPHIC SCOPE	Timber harvest is widespread throughout the Eastern population.	Highly Confident	
MAGNITUDE	Despite widespread occurrence, timber harvest activities are likely to only act on a localized level so effects are of low to moderate magnitude.	Moderately Confident	
SUMMARY	Localized effects to individuals and breeding sites or populations could occur, but there is not enough timber harvest to affect the toad at an Eastern population-wide level. Tree die- offs, at least in the SRM, have potential to lead to policies to increase timber harvest above levels in the last 20 years, but not at a level that would cause population-wide effects. Timber harvest in itself does not appear to be a stressor that requires further evaluation in our analysis of current or future conditions.	Moderately Confident	

THEME: Urbanization			
[ESA Factor(s): A]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Urbanization**		

Activity(ies)	Residential and commercial development. Blading and leveling of topography, changes to hydrology due to filling of wetlands, channeling of streams. Associated road and utility construction for access and power to homes or commercial buildings. Also, potential for pollution from herbicide application; road salt, sand; as well as vehicle oils and chemicals. Pet and human related mortality.		
STRESSOR(S)	Removal and alteration of vegetation and vegetation types, changes in water sources/hydrology.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257; Muths 2003, p. 163
Affected Resource(s)	Eggs and tadpoles potentially affected by changes to hydrology. Juveniles, subadults, and adults impacted by changes/loss of vegetation, crushing by construction equipment and vehicles, disruption of dispersal/migration corridors.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257; Muths 2003, p. 163; Lehtinen et al. 1999, entire
Exposure of Stressor(s)	All life stages in summer/fall could be impacted by all activities	High Confidence	
Immediacy of Stressor(s)	Past, present, future. Effects likely for about 100 years, continued development in some areas could impact toad presently and into future.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257

Changes in Resource(s)	In areas of development, especially during and soon after construction, there may be little to no vegetation suitable for boreal toads. Permanent loss of vegetation and wetlands supporting breeding habitat is also certain. Stream alteration affecting sources of water to breeding habitat is also possible.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257
Response to Stressors: INDIVIDUALS	Mortality from crushing by equipment, vehicles. Digging up and resulting mortality of wintering toads. Disruption of dispersal/migration corridors. Potentially lack of habitat for individual needs for the entire year.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257; Muths 2003, p. 163; Lehtinen et al. 1999, entire
POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Survival, recruitment, and abundance could all be affected on a local breeding site or breeding population level. There is evidence that development in the Wasatch Front north and south of Salt Lake City has caused loss of habitat and hindered the ability of toads to carry out their life cycle. Development elsewhere in the Eastern boreal toad population has occurred but appears smaller in impact.	High Confidence	Lee 2001, p. 4; Thompson 2004, p. 257; Greenwald et al. 2011, p. 71
GEOGRAPHIC SCOPE	Residential and commercial development is widely distributed in the Eastern population.	High Confidence	Greenwald et al. 2011, p. 71

MAGNITUDE	Despite widespread development, much boreal toad habitat in the Eastern population occurs on land that won't receive extensive development pressure due to it being federally managed land and/or having snow pack or topography that is not suitable for extensive development. The magnitude is therefore moderate to low.	Somewhat Confident	Greenwald et al. 2011, p. 71
SUMMARY	Due to low to moderate magnitude of residential or commercial development and expectation that extensive development will not encroach further into boreal toad habitat, housing and commercial development do not appear to affect the boreal toad at an Eastern population level and this stressor does not need to be evaluated further in our analysis of current or future conditions.	Moderately Confident	

THEME: Pollutants			
[ESA Factor(s): E]	**Analysis**	Confidence /Uncertainty	Supporting Information
SOURCE(S)	Pollutants		
Activity(ies)	Mining, regional deposition of chemicals producing acidic snowmelt, road pollution, pesticides, herbicides, fire retardant application, piscicide application, road salt, sands, other road-related pollutants from vehicles.		
STRESSOR(S)	Effects to water quality. Possibly prey base.		
Affected Resource(s)	Most likely effects to water quality affecting egg and tadpole survival, growth, and development.		
Exposure of Stressor(s)	Breeding site effects to egg and tadpoles.		

Immediacy of Stressor(s)	Past, present, future.		
Changes in Resource(s)	Water quality has been observed to change.	Somewhat Confident	Porter and Hakanson 1976, pp. 327–331; Corn et al. 1989, entire; Corn and Vertucci 1992, entire; Loeffler 1999, pp. 31– 32; Jackson 2006, pp. 58–59; Berrill et al. 1994, p. 663; Hayes et al. 2002, pp. 5476– 5479; Fellers et al. 2004, p. 2176; Relyea 2005, p. 626
Response to Stressors: INDIVIDUALS	If mine drainage or other acidic inputs cause pH to be below 4. 9 then eggs and tadpoles are expected to be impacted.	High Confidence	Corn et al. 1989, pp. 19, 20, 28; Corn and Vertucci 1992, p. 365
POPULATION & SPECIESRESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Acid mine runoff does occur but is not known to affect boreal toads on a Eastern population- wide basis. Acidification of snow/snowmelt, which could occur, Eastern population-wide, was found not to be an issue. It is not known that pesticides or herbicides are affecting toads in the Eastern population but further study may be warranted. Piscicides could kill tadpoles but their use is also infrequent enough in toad breeding habitat that effects are likely rare. Salt levels as a result of road runoff or otherwise are not thought to be an issue, though roads with salt application do occur adjacent to some toad breeding sites. Other road related pollutants from vehicles, etc. are not known to affect toads but we are unaware of research on other road pollutants and boreal toads.	High Confidence	Corn and Vertucci 1992, p. 367; Loeffler 1999, pp. 31–32; Jackson 2006, pp. 58–59; Stoddard 2005, p. 40, fig. 15
GEOGRAPHIC SCOPE	Pollutants occur locally and perhaps over large areas throughout the Eastern population.	High Confidence	Porter and Hakanson 1976, pp. 327–331; Corn et al. 1989, entire; Corn and Vertucci 1992, entire; Loeffler 1999, pp. 31– 32; Jackson 2006, pp. 58–59; Berrill et al. 1994, p. 663; Hayes et al. 2002, pp. 5476– 5479; Fellers et al. 2004, p. 2176; Relyea 2005, p. 626

MAGNITUDE	Although pollutants occur locally and could occur throughout the Eastern population the magnitude of effects to the boreal toad appears low.	Moderately Confident	Corn and Vertucci 1992, p. 367; Stoddard 2005, p. 40, fig. 15
SUMMARY	Pollutants currently occur locally and are expected to in the future primarily from road runoff. The apparent low magnitude of pollutant effects to the boreal toad from existing information indicates that this stressor does not need to be evaluated further in our analysis of current or future conditions.	Somewhat Confident	

THEME: Energy Development			
[ESA Factor(s):A,E]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Energy Development**		
Activity(ies)	Exploration, seismic activity, well pad drilling, pipelines, utilities, access road building, mining. Hard rock mining/acid mine drainage is addressed under pollutants.		
STRESSOR(S)	Habitat loss, possible water quality issues.		
Affected Resource(s)	All life stages		
Exposure of Stressor(s)	Eggs and tadpoles could be exposed to contaminated water. Juveniles, subadults, adults could be crushed by equipment, or their habitat lost to pipelines, pads, and facilities.		
Immediacy of Stressor(s)	Present, future.		
Changes in Resource(s)	Water quality could decline, loss of vegetation.		
Response to Stressors: INDIVIDUALS	Eggs and tadpoles could suffer mortality. Juveniles, subadults, adults could suffer mortality.		
POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	To our knowledge there has been no impact to the SRM population by energy development activities. Oil and gas reserves and coal are generally not extensive in the SRM. Other areas are not known to have high potential for oil and gas or coal.	Somewhat confident	Gunnison Sage-grouse Rangewide Steering Committee 2005, p. 130; Colorado Greater Sage-grouse Steering Committee 2008, p. 112
GEOGRAPHIC SCOPE	Pipelines could occur in areas with toads. Some oil and gas well pad development could also occur or coal mines in some areas.		
MAGNITUDE	Low at present and expected to be low in the future.		

SUMMARY	There are few if any oil and gas developments or coal mines (or other energy related mining) that are known to impact boreal toads throughout the Eastern population. Localized impacts could occur but are not expected to affect the entire Eastern population. If natural gas and oil development increases in the future and regulation of activities is relaxed a low amount of additional habitat and pollutant impacts could occur from pipelines, well pads, and associated roads, utility corridors, and other energy-related infrastructure. There is no need to evaluate this stressor further in our analysis of current or future conditions.	Moderately Confident	

THEME: Collection			
[ESA Factor(s): B]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Collection**		
Activity(ies)	Personal, commercial, or scientific collection.		
STRESSOR(S)	Removal of individuals, disease transmission.		
Affected Resource(s)	All life stages.		
Exposure of Stressor(s)	Spring to fall collection of egg masses, individuals.		
Immediacy of Stressor(s)	Past, present, future.		
Changes in Resource(s)	Reduced number of individuals, disease introduction.		
Response to Stressors: INDIVIDUALS	Mortality, loss of breeding potential.		

POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Little to no effect. There is no known personal or commercial collection. Scientific collection for genetic analyses, research, or captive propagation for reintroduction efforts occurs, but removal of individuals, typically tadpoles, are limited and do not cause effects to breeding sites, breeding populations or the Eastern population. Procedures to avoid disease transmission have been employed so boreal toad researchers/surveyors should not be introducing Bd or other diseases.	High Confidence	McGee and Keinath 2004, p. 37; Scherff–Norris 1997, entire; Loeffler 2001, pp. 36– 53; Hogrefe et al. 2005, pp. 28–38
GEOGRAPHIC SCOPE	Collection has occurred Eastern population- wide.	High Confidence	
MAGNITUDE	Magnitude of effects of collection is low.	High Confidence	
SUMMARY	Personal and commercial collection is not known to occur. Scientific collection for research occurs but the number collected are designed to not impact the boreal toad on a breeding site or breeding population level and do not affect the toad on an Eastern population-wide level. Procedures have been implemented to avoid disease transmission by researchers. It is not expected that collection will increase in the future. Consequently, there is no effect to the Eastern Population and this stressor does not need to be evaluated further in our analysis of current or future conditions.	High Confidence	

THEME: Chytrid Fungus Bd			
[ESA Factor(s):C]		Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Bd**		
Activity(ies)	Transmission and infection.		
STRESSOR(S)	Infection, causing chytridiomycosis.	Highly Confident	Muths and Nanjappa 2005, p. 395

Affected Resource(s)	All life stages except eggs.	Highly Confident	
Exposure of Stressor(s)	Bd can infect tadpoles. Bd can cause mortality in juveniles, subadults, and adults.	Highly Confident	Garner et al 2009, entire
Immediacy of Stressor(s)	Past, present, future. Bd appears to have been introduced into North America and the SRM area in the Eastern population of boreal toad in the 1960s. It is currently causing loss of toads and is expected to in the future.	Highly Confident	Hogrefe et al. 2005, p. 14; Ouellet et al. 2005, pp. 1436, 1438
Changes in Resource(s)	Boreal toads may decline to the point that breeding sites or breeding populations are not sustainable.	Highly Confident	Crockett 2017a, p. 4; Jackson 2008, pp. 12–91, 94
Response to Stressors: INDIVIDUALS	Tadpoles can be infected with Bd on their mouthparts and possibly cause negative effects on growth or development, but mortality is not known to result at the tadpole stage. Mortality occurs in subadult and adult life stages.	Highly Confident	Garner et al 2009, entire
POPULATION & SPECIES RESPONSES			

Effects of Stressors: POPULATIONS [RESILIENCY]	The SRM area has experienced significant declines in large and small breeding populations and declines can be from 90 to 100 percent of the breeding site/population. Bd may have caused up to a 49 percent reduction in occupied HUCs in the SRM since the 1960's. For the SRM, Bailey (2017b) stated that Gerber (2017, in review) created a Bd-effect model based on 2001 to 2010 data from 125 breeding sites in 11 mountain ranges. The model suggests a declining trend in toad breeding occurrence and an increase in Bd occurrence at the breeding sites. Despite the declining breeding occurrence and increasing Bd occurrence the model predicted a 97 percent chance of persistence (3 percent probability of extinction) of the SRM in the next 50 years with no additional boreal toad management and a 98 percent chance or higher of persistence with management. Some breeding populations have become extirpated, but some have remained with low number of individuals despite presence of Bd. Some formerly large or moderate sized breeding sites/populations in the SRM have had a low number of adult toads, egg masses, and tadpoles after a few years of apparent extirpation, though survival and recruitment of tadpoles and juveniles appears very low. It is unknown if toads found were resident toads that had not bred for a couple years and were, therefore, not detected due to them being away from breeding areas, or if toads from other drainages/breeding populations immigrated into the sites.	Highly Confident	McGee and Keinath 2004, pp. 43–44; Bailey 2017b, pp. 1-3; Crockett 2017a, p. 4; Crockett 2017b
GEOGRAPHIC SCOPE	Bd occurs or can occur throughout the entire Eastern population of boreal toad.	Highly Confident	Jackson 2008, pp. 12–91, 94; UDWR 2010, pp. II-4, III–3, IV- 4

MAGNITUDE	Bd does not appear to cause significant declines outside of the SRM. Despite the declining breeding occurrence and increasing Bd occurrence the model predicted a 97 percent chance of persistence (3 percent probability of extinction) of the SRM in the next 50 years with no additional boreal toad management. Based on known monitoring information and predicted Bd occurrence and demographic trends the magnitude of effects from Bd in the next 50 years appears to be moderate.	Highly Confident	Hammerson (1999, p. 91); Bailey 2017b, pp. 1- 3; Crockett 2017a, p. 4; Crockett 2017b
SUMMARY	Bd can cause high mortality and cause extirpation of breeding sites/populations. The SRM area appears to be affected the most by Bd within the Eastern population. Despite the declines in the SRM, it is expected that this population has a 97 percent chance of persistence in the next 50 years without additional management and a 98 percent chance or higher of persistence with management. Lack of significant declines from Bd in the rest of the Eastern population suggest that, in combination with low probability of extinction in the SRM in the next 50 years, even without additional management, that the Eastern population is not expected to become extinct in the next 50 years. Bd is the primary cause of boreal toad mortality and extirpation and, as such, this stressor should be evaluated in current and future scenarios.	Highly Confident	

THEME: Other Diseases			
[ESA Factor(s):C]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Diseases**		
Activity(ies)			
STRESSOR(S)	*Saprolegnia ferax* was spread to boreal toads from rainbow trout (*Oncorhynchus mykiss*) experimentally infected with *S. ferax*. *Saprolegnia* spp. or other fungus could be caused by poor water quality from a variety of sources. However, it also can occur in pristine waters. Other diseases or trematode parasites or other parasites could occasionally affect the toad but none are known to affect the toad to any degree in the wild so only Saprolegnia (or other aquatic fungus) is addressed here.		Johnson et al. 2001, p. 370; Kiesecker et al. 2001, p. 1064; Watry 2017, p. 1.
Affected Resource(s)	Eggs		
Exposure of Stressor(s)	Eggs during breeding typically noticed in poor quality water, and possibly invasive species		
Immediacy of Stressor(s)	Past, present, future.		
Changes in Resource(s)	None that are known.		
Response to Stressors: INDIVIDUALS	Mortality of eggs		
POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	No population level responses known.		
GEOGRAPHIC SCOPE	Likely ubiquitous.		
MAGNITUDE	Low, not known to be major factor in mortality.		
SUMMARY	Saprolegnia or other fungi and other diseases may occasionally affect survival of individual		

	boreal toads but none are a major source of mortality. Other diseases are not expected to increase in the future but new diseases or newly introduced diseases are always possible. Based on current information, other diseases (besides Bd) do not need to be evaluated further in our analysis of current or future conditions.		

THEME: Predation			
[ESA Factor(s):C]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Predation**		
Activity(ies)	Natural or human induced predation.		
STRESSOR(S)	Avian, mammalian, reptilian, amphibian, and arthropod predators, possibly invasive species.	Highly Confident	Olson 1989, entire; Hammerson 1999,p. 97, 98; Livo 1998, pp. 117-119; Livo 1999, p. 1; Beiswenger 1981, entire; Lambert 2003, pp. 22, 24, 77; Bradford 1989, pp. 776–777; Pierce 2017, p. 1.
AffectedResource(s)	All life stages, but eggs appear to have toxins that reduce predation.		Licht 1969, p. 296; Voris and Bacon 1966, p. 597
Exposure of Stressor(s)	All life stages, from spring to fall.	Highly Confident	Olson 1989, entire; Hammerson 1999,p. 97, 98; Livo 1998, pp. 117-119; Livo 1999, p. 1; Beiswenger 1981, entire; Lambert 2003, pp. 22, 24, 77; Bradford 1989, pp. 776–777
Immediacy of Stressor(s)	Past, present, future.	Highly Confident	
Changes in Resource(s)	Fewer tadpoles (especially) may survive as well as other life stages.		
Response to Stressors: INDIVIDUALS	Individual mortality or injury can result.		
POPULATION & SPECIES RESPONSES			

Effects of Stressors: POPULATIONS [RESILIENCY]	Some predators can cause poor survivorship at some breeding sites but it is likely that large or total loss from predation is infrequent and does not cause breeding populations to decline.		Lambert 2003, pp. 22, 24, 77; Hammerson 1999, p. 98
GEOGRAPHIC SCOPE	Throughout the Eastern population.		
MAGNITUDE	Though undoubtedly widespread, the magnitude of effects to Eastern population is likely low.		
SUMMARY	Natural or human induced predation can and does occur. It has not been demonstrated to be a major factor in boreal toad decline currently and is not expected to increase in the future. This stressor does not need to be evaluated further in our analysis of current or future conditions.	Moderately Confident	

THEME: Isolation			
[ESA Factor(s):E]		Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Isolation**		
Activity(ies)	Geographic separation.		
STRESSOR(S)	Separation of breeding sites/populations by natural features, separation due to anthropogenic changes (roads and other development) or induced climate change.		Hogrefe et al. 2005, pp. 14–15; Lehtinen et al. 1999, entire.
Affected Resource (s)	All		

Exposure of Stressor(s)	Natural or anthropogenic causes of water loss, either directly or indirectly (climate change) can cause separation if breeding ponds dry up or are destroyed by human means affecting egg deposition and survival, and tadpole survival and recruitment. Juveniles can have mortality if natal pond water dries up or water sources dry up causing lack of survival and dispersal. Subadults and adults may not be able to find water sources to disperse/migrate to and would therefore be restricted to more permanent water sources.		Hogrefe et al. 2005, pp. 14–15; Lehtinen et al. 1999, entire.
Immediacy of Stressor(s)	Past, present, future. Past isolation (more than 100-150 years ago) was likely all due to natural environmental forces with large land blocks. Present isolation in the last 100 years has likely been added to by roads, other habitat loss, and water manipulation by humans. Bd has likely caused present-day isolation of some breeding sites/populations since the late 1960's.		
Changes in Resource(s)	Present-day isolation will likely continue into the future without translocations or other management.		
Response to Stressors: INDIVIDUALS	Mortality may result to eggs and tadpoles if ponds dry up. Juveniles, subadults, and adults may not be able to disperse/migrate to other areas or as many areas as previously and would have no or limited opportunity for breeding with other toads.		
POPULATION & SPECIES RESPONSES			

Effects of Stressors: POPULATIONS [RESILIENCY]	Nuclear microsatellite DNA analyses in 2009 suggest that breeding populations within the Eastern population are isolated from one another, with little gene flow, and could cause genetic problems. However, mitochondrial DNA analyses from the same author and another author predominantly found similar mitochondrial DNA across the Eastern population (despite large geographic separation of the SRM from the western part of the Eastern population) with suggestion of genetic differentiation in one area in southern Utah. Recent nuclear DNA analyses suggest cohesive genetics throughout the Eastern population (including southern Utah) though boundaries are slightly different in a couple areas from the 2009 mitochondrial DNA analyses. Genetic differentiation does not necessarily mean that boreal toads are experiencing negative isolative effects however. Additional ongoing genomic/single nuclear polymorphic (SNP) genetic analyses may add to knowledge of whether areas within the Eastern population are genetically isolated. Genetic differentiation can occur without negative effects if population levels remain stable or increase.		Goebel et al. 2009, entire; Switzer et al. 2009, entire; Goebel 2017, entire.
GEOGRAPHIC SCOPE	Eastern population-wide.		

MAGNITUDE	The loss of up to 49 percent of previously occupied HUCs in the SRM and distance of some remaining HUCs to other HUCs suggests that isolation is occurring in some areas. It is possible, without management, that populations in the SRM may become more isolated. Roads and other anthropogenic factors such as development and hydrologic changes in some areas may also work to isolate some toad breeding sites/populations especially in the SRM without management. Natural climate change appears to have caused separation of some populations/areas over thousands of years (between the SRM and rest of range and in Utah). Anthropogenically induced climate change has the potential to alter snowpack and timing of spring snowmelt in the SRM area but precipitation may remain about the same. The magnitude of isolation effects to boreal toad loss based on existing genetic and climatic information is low to moderate.	Somewhat Confident	Crockett 2017b; Carey et al. 2005, p. 235; Goebel et al. 2009, entire; Switzer et al. 2009 entire; Goebel 2017, entire; Ray et al. 2008, pp. 1-2; Livo and Loeffler 2003, pp. 10-11; Lukas et al. 2014, pp 1-4.
SUMMARY	There are both natural and anthropogenically induced causes of isolation. Existing information suggests isolation between some areas within the Eastern population, but the degree of isolation is unclear when looking at the different genetic analyses. Especially lower elevations in the SRM as well as the rest of the Eastern Population could experience climate induced effects that may isolate some currently occupied areas. Bd can depress populations, subjecting them to complete loss by itself or through additive stochastic events (predation, drought, etc.), thereby isolating remaining breeding sites/populations. Further research and analysis is needed to explore this issue, but current information does not suggest this is an issue in the next 50 years. As such this issue will not be evaluated further in our analysis of current or future conditions.	Somewhat Confident (that isolation is not a problem within 50 years, even without management).	

THEME: Ultraviolet Radiation			
[ESA Factor(s):E]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Ultraviolet Radiation**		
Activity(ies)	Human induced loss of the ozone layer and resultant increased level of UV-B radiation.		
STRESSOR(S)	Radiation	Low Confidence	Blaustein et al. 1994, pp. 1791, 1793–1794
Affected Resource(s)	Eggs, tadpoles.		
Exposure of Stressor(s)	Survival and development during egg and tadpole stage.		
Immediacy of Stressor(s)	Past, present, future.		
Changes in Resource(s)	More UV-B radiation than would occur naturally.		
Response to Stressors: INDIVIDUALS	Egg and tadpole mortality.	Low Confidence	Blaustein et al. 1994, pp. 1791, 1793–1794; Corn 1998, pp. 18, 21–25; Corn and Muths 2002, pp. 2961-2962
POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Although one study we are aware of indicated UV-B radiation could affect boreal toad survival, subsequent studies showed no effects to boreal toad or other amphibian species survival. One study recorded increases in UV-B levels in Colorado in the 1980s through early 2000s, but found no evidence of UV-B radiation affects, and hypothesized that, temperature extremes may have more effect on survival of boreal toads. If UV-B radiation was a factor, large areas or the entire Eastern population of the boreal toad would be experiencing egg and tadpole mortality in the absence of Bd. This has not been the case. Significant losses have only been positively correlated with the presence of Bd.	Low Confidence	Blaustein et al. 1994, pp. 1791, 1793–1794; Corn 1998, pp. 18, 21–25; Corn and Muths 2002, pp. 2961-2962
GEOGRAPHIC SCOPE	If UV-B were a factor, it would likely affect most or all of the Eastern population.	Low Confidence	
MAGNITUDE	Low or non-existent in the Eastern population of the boreal toad.		

SUMMARY	UV-B radiation does not appear to cause losses of individual toads within the Eastern population of the boreal toad and it is unlikely to become a factor in the future. This stressor does not need to be evaluated further in our analysis of current or future conditions.	Low Confidence	

THEME: Climate Change and Drought			
[ESA Factor(s): E]	**Analysis**	Confidence / Uncertainty	Supporting Information
SOURCE(S)	**Climate Change and Drought**		
Activity(ies)	Natural and human-induced climatic changes.		
STRESSOR(S)	Lack of water (drought), higher temperatures, altered snowpack.		
Affected Resource(s)	all life stages.		
Exposure of Stressor(s)	Breeding ponds can dry up, reducing opportunities for egg deposition, or if eggs are deposited, ponds may subsequently dry up causing mortality to eggs and tadpoles. Drought may reduce water availability for juveniles, subadults, and adults during the summer/fall resulting in possible mortality. Lower snowpack over hibernacula could cause toads to rouse in late winter or early spring and cause decreases in individual toad survival.	Highly Confident	Barnett et al. 2005, entire; Livo and Loeffler 2003, pp. 10 - 11; Muths 2017 pp. 6-7; Lukas et al. 2014, pp 1-4; Lambert et al. 2016, entire.
Immediacy of Stressor(s)	Natural periods of drought have and will continue to occur. Human-induced droughts and other changes to climate could also occur in the future.	Highly Confident	Barnett et al. 2005, entire; Livo and Loeffler 2003, pp. 10 - 11; Muths 2017 pp. 6-7; Lukas et al. 2014, pp 1-4; Lambert et al. 2016, entire; Steen 2017 pers. comm.

Changes in Resource(s)	Breeding ponds can dry up for one or more years depending on severity and length of drought. Water sources for daily hydration of juveniles, subadults, and adults could also dry up. Food resources may also decline because of drought. For the Southern Rocky Mountains above 8,000 feet, the estimate is an extension of the active season for toads between 34 and 48 days from 2040 to 2069. However, with warmer temperatures evapotranspiration is expected to outpace any potential increase in precipitation leading to 17 to 56 percent less water availability by mid-century.	Highly Confident	Livo and Loeffler 2003, pp. 10 -11; Muths 2017 pp. 6-7; Lukas et al. 2014, pp 1-4; Steen 2017, active season and precipitation bar plots
Response to Stressors: INDIVIDUALS	Mortality or reduced survival of all life stages. Preclusion of breeding by adults. Climate change may alter early or late season freezing temperatures, possibly causing mortality before or after hibernation. Climate change may also alter the timing of breeding in a way that reduces survivability to subsequent life stages. For example, early breeding may increase the likelihood of freezing eggs, tadpoles, or metamorphs. Late breeding could reduce the amount of feeding time during growth phases, and lead to mortality from inadequate winter fat stores and/or small body size. Boreal toads may hibernate in small mammal burrows or in woody debris that is covered by a persistent snowpack over most of the winter. Boreal toads have been shown to have lower survival probabilities in years with lower minimum daily temperatures in winter and shorter growing seasons. Additional findings supported the idea that survival was correlated with snow depth, but the relationship was weaker. These studies suggest that without the insulation properties of an adequate snowpack, the daily temperatures experienced by hibernating toads	Highly Confident	Livo and Loeffler 2003, pp. 10 -11; McGee and Keinath 2004, p. 41; Scherer et al. 2008, entire; Muths 2017, p. 6; Scherer et al. 2005, p. 2159; Scherer et al. 2008, p. 516; Williams et al. 2014, pp. 12- 13, entire; Wipf et al. 2009, p. 112; Sinclair et al. 2013, p. 301; Browne and Paszkowski 2010, p. 256; Lambert et al. 2016, p. 1262

	could be cold enough to reduce their probability of survival. These findings are in line with broader studies that find that adequate snowpack provides stability, and that reduced snowpack may signal more days of frost, lower soil temperatures, and earlier snowmelt. Changes in winter snow cover have been shown to affect community- and individual-level attributes. Lack of snowpack and warm temperatures in winter could facilitate inappropriate rousing of toads from hibernation, which is also energetically expensive and potentially detrimental to survival in amphibians. However, larger body size adults were found to arrive at hibernacula later in the fall and are able to forage later into the fall which likely increases body reserves important for overwinter survival as well as reproductive ability. Lambert et al. (2016) found that adults were able to cope with freeze transitions (daily fluctuation below and above freezing). The number of freeze transitions seemed to correlate to higher survival, repudiating previous articles that suggested earlier snow melt could cause freeze transitions resulting in increased mortality. Body mass of adults also influenced survival positively at 2 of their 3 study sites. However, in contrast to survival, recruitment models suggested the active season length was bounded by single freeze events. In other words adult toads can survive multiple days of freeze transitions (multiple cold days) but single early fall freezes can prevent metamorphosis and cause mass mortalities of recently metamorphosed toads. Results of study on snowpack, weakly supported the assertion that snowpack affects conditions in the hibernacula and may lead to better adult survival.		

POPULATION & SPECIES RESPONSES			
Effects of Stressors: POPULATIONS [RESILIENCY]	Precipitation monitoring has detected no long-term trend in Colorado even considering the relatively dry period since 2000. Snowmelt has been 1 to 4 weeks earlier during the last 30 years in Colorado due to lower snow-water equivalency since 2000, a warming trend in spring temperatures, and enhanced solar absorption from dust on snow. Average temperatures in Colorado have increased 2 degrees in the last 30 years (which follows the global average). Drought in 2002 in Colorado was noted as causing breeding ponds to dry up but little or no indication of drought effects since then. To date there has been no known Eastern population-wide decline as a result of drought. The southern part of Colorado is more at risk of less precipitation in the future. Temperatures are expected to rise 2.5°F to 5°F by 2050 relative to a 1971–2000 baseline under a medium-low emissions scenario, and rise 3.5°F to 6.5°F in a high emissions scenario Current temperature predictions suggest that the active season for boreal toads may increase 34 to 48 days between 2040 to 2069. The active season length was found to be a strong positive correlate for both survival and recruitment of toads. Precipitation is expected to increase in the winter in Colorado but due to increased temperatures more of it may come as rain and less as snow. Most projections of Colorado's spring snowpack (April 1 snow- water equivalency) show declines for the mid-21st century due to the projected warming. Heat waves, droughts and wildfires are expected to increase in frequency and severity in Colorado by 2050 due to the projected warming. If precipitation remains the same or increases in the winter there may be little or no effect to boreal toads in Colorado or the SRM. However, earlier spring snow melt and warming temperatures may result in cause less water availability in late	Moderately Confident	Barnett et al. 2005, entire; Livo and Loeffler 2003, pp. 10-11; Muths 2017 pp. 6-7; Steen pers. comm. 2017; Steen 2017, Barplot_Average_PPET.png ; Steen 2017, BarPlot_Average_Add_ACT LE N2.png; Lukas et al. 2014, pp 1-4; Lambert et al. 2016, entire; Livo 1998, p. 117-119; Pierce 2017, p. 1;

	summer, possibly affecting survival of tadpoles. An analysis using climate models for the Southern Rocky Mountains above 8,000 feet found that water availability is expected to decrease 17 to 56 percent due to higher evapotranspiration from higher temperatures sometime between 2040 and 2069. A study on environmental and climatic conditions in relation to boreal toad survival and recruitment found that hydro- period (i.e. stable water condition) was a key factor in recruitment of boreal toads and may be the biggest positive factor influencing recruitment of toads into the adult population. Consequently, expected decreases in the number of breeding ponds or duration of water available for development of toads may decrease recruitment of toads into the adult breeding population. Predation by dragonfly larvae could possibly increase with increased water temperatures but effects are likely localized Ironically, year-round and relatively stable water conditions, beneficial for egg and tadpole survival and development, may also facilitate more predatory aquatic insects that eat tadpoles such as predaceous diving beetles. Though predation by the beetles has been observed it is likely compensated for by high fecundity of toads (lots of eggs/tadpoles) and is likely a variable and localized occurrence not affecting long-term toad abundance at breeding colonies or across the Eastern Population.		
GEOGRAPHIC SCOPE	Temperature increases and precipitation pattern changes are likely in the SRM and across the entire Eastern population. Changes may affect lower elevation boreal toads to a greater extent due to greater evapotranspiration of water but this is uncertain.	Somewhat Confident	Barnett et al. 2005, entire; Ray et al. 2008, pp. 1-2; Lukas et al. 2014, pp 1-4

MAGNITUDE	Although drought has been noted in areas, severity across differing areas and across the entirety of the Eastern population can change year to year. We have little current evidence of population-wide effects to boreal toads. The magnitude of effects of climate change and drought based on monitoring has been low. Human-induced climate change may cause year-round or seasonal drought conditions to become more severe, widespread, and/or increase in duration. However, the magnitudes of these effects are difficult to predict based on current data and observation.	Somewhat Confident	Barnett et al. 2005, entire; Ray et al. 2008, pp. 1-2; Livo and Loeffler 2003, pp. 10-11; Muths 2017 pp. 6-7; Lukas et al. 2014, pp 1-4; Lambert et al. 2016, entire; Steen 2017 pers. comm.
SUMMARY	Drought-induced drying of breeding ponds has been observed in some areas, but is not known to have caused long-term effects to those breeding populations, the rest of the Eastern population. Human-induced climate change could increase the severity and length of droughts and possibly increase the size of areas stricken with drought. As predicted, temperature has increased in the last 30-years, and is expected to get warmer in the future resulting in earlier snowmelt. Winter precipitation is projected to increase, at least in the SRM, with the expectation of less water availability in late summer/fall when tadpoles are still developing, especially at higher elevations within the toad's range. However, variable precipitation projections in spring, summer, and fall, coupled with no current evidence of climate impacts to the toad, leaves it currently impossible to quantify water availability and what effect reduced water availability may have. Based on current information climate change may affect toads, both positively and negatively. These effects may act upon the toads as a result of longer active season to less available water, or from potential positive and negative Bd interaction, habitat changes, and possibly changed predator interactions. Based on our assessment, climate change effects are not likely to cause extirpation of Bd, but could	Somewhat Confident	

	potentially reduce its prevalence at lower elevations of the toad's range, where temperatures could get warmer (and may be drier) than desired for Bd, and the toads are able to shed Bd more readily if they bask in warmer and drier climates.		

Appendix B

This appendix contains a Performance Report to the US Fish and Wildlife Service and Wyoming Game and Fish Department. The Report is entitled: Phylogeographic distribution of the Eastern clade of *Anaxyrus boreas* based on sequence data from the mitochondrial control region and nuclear genes, Dr. Anna Goebel, April 3, 2017.

Final Performance Report to: US Fish and Wildlife Service and Wyoming Game and Fish
Date: Final report dated 31 March 17 – This version modified 3 April17 in minor ways.
Title: Phylogeographic distribution of the Eastern clade of *Anaxyrus boreas* based on sequence data from the mitochondrial control region and nuclear genes.

Grant ID information:

1. USFSW Organization Code: FF06E24000
   Identifying genetic groups in the eastern population of the boreal toad species group (*Anaxyrus boreas*) using a tiered approach with select nuclear and mitochondrial DNA sequences.
   FWS Project Officer: Terry Ireland FWS Research Coordinator: Greg Watson Principal Investigator: Anna M. Goebel, FGCU.

2. Wyoming Game and Fish Commission:
   DNA analyses of Boreal toads (*Anaxyrus [Bufo]boreas)* in Wyoming, Ammended.
   Mark Smith, WGF WGFC Deputy Director: John Kennedy

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Although the report was written by Anna Goebel and she is solely responsible for its content, this project could not have been conducted without substantive input by several people including: Terrry Ireland, USFWS, Ecological Services, Grand Junction CO, terry_ireland@fws.gov
Dr. Sara Oyler-McCance, Research Geneticist, USGS, Fort Collins Science Center,Fort Collins, CO 80526, soyler@usgs.gov
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This performance report includes:
1) a comparison of actual accomplishments with the goals and objectives of the award as detailed in the approved scope of work;
2) a description of reasons why established goals were not met, if appropriate; and
3) any other pertinent information relevant to the project results.

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Abstract

Analyses confirm the existence of a genetically distinct Eastern population of the *Anaxyrus boreas* species group based on molecular data from mtDNA and seven nuclear genes. This group is found in Colorado, Utah, Wyoming, and eastern Idaho. The exact geographic boundaries of the group are unclear in two areas where divergent groups overlap in northwest Utah and southwest Montana. Nuclear DNA data strongly supports the presence of the Eastern group across all or most of Wyoming, which differs from analyses based on mtDNA alone. The presence of the Eastern group throughout Wyoming is strongly supported because species delimitations based solely on a single gene, especially a maternally inherited mitochondrial gene, can be strongly affected by lineage sorting and thus data from multiple nuclear genes provides much strong support for group delimitation. Data here do not identify the SRM population as a monophyletic group and this may be due to the limits of data. I report here on the first analyses of the data, and further analyses are in progress.

Introduction

Purpose/Problem to be solved – Conservation organizations petitioned the USFWS to consider the status of the Southern Rocky Mountain Population of the boreal toad (*Anaxyrus boreas*) in May 2011 (Greenwald et al. 2011). This petition resulted in the USFWS issuing a 90-day finding in April 2012 (USFWS 2012) in which it was concluded that substantial information exists to indicated that the listing of the Eastern Population, originally defined by mitochondrial DNA (Goebel 2000, Goebel et al. 2009), as a distinct population segment (DPS) may be warranted. The Eastern Population includes the southern Rocky Mountain (SRM) population (which includes all of the range in CO, NM and southeastern WY) as well as toad populations in Utah, southwest Wyoming, southeast Idaho, northeast Nevada (USFWS 2012). The purpose of this genetic analysis of nuclear genes is to identify the potential genetic divergence and geographic distribution of the Eastern Population to facilitate the US Fish and Wildlife Service (USFWS) in its listing determination.

Background of listing and status - The boreal toad in Colorado suffered serious declines in population size first noted in the 1970s (Corn, 2003, Muths and Nanjappa, 2005). Although the cause was not clear in the early years the most likely cause remains the high mortality caused by chytrid fungus (*Batrachochytrium dendrobatidis*). The toad was listed as Endangered by the states of Colorado and remains so to date. The toad was extirpated in New Mexico (Stuart and Painter, 1994). The Colorado Division of Wildlife (CDOW) has an active conservation program. The SRM population (CO, NM and southeastern WY) was on the US Fish and Wildlife Service's candidate species list from 1995 to 2005 when it was removed due to a lack of conclusive genetic data (USFWS 2005). Listing of either the Eastern or SRM populations will provide assistance for the protection of toads in Colorado. Thus, a second purpose of this study is to identify the degree of genetic diversity and biogeographic distribution of the diversity within the Eastern group and whether the SRM population should be recognized as a distinct unit separate from the rest of range of the Eastern Population (discussed by Hammerson 1999).

Background of genetic analyses - Analyses of mitochondrial DNA (mtDNA) are concordant, despite the examination of different mitochondrial genes and sampling strategies as describe by Graybeal (1993), Goebel (1996, 2000), Goebel et al. (2009), Shaffer et al., (2000), Stephens (2001), Switzer et al. (2009) and unpublished work by Dr. Richard Tracy (University of Nevada, Reno). All analyses are concordant with the existence of an Eastern Population that is distinct from boreal toads to the north and west, from other subspecies of the western toad, and from other closely related species in the boreal toad complex (Goebel et al. 2009, Switzer et al. 2009). Similar conclusions were made for nuclear DNA microsatellites (Switzer et al., 2009). However, nuclear data were still lacking to identify the status of the Eastern group within the boreal toad complex, the genetic diversity of populations within the eastern group, especially the SRM population as well as the divergent population in the Paunsaugunt Plateau, East Fork Sevier, Kane Co., UT, and degree of potential mixing of mtDNA clades within Wyoming.

Comparison of actual accomplishments with the goals and objectives and description of reasons why established goals were not met, if appropriate.
The specific goals of the project were to facilitate the US Fish and Wildlife Service (USFWS) in its listing determination by:

1. Identifying the potential genetic divergence of the Eastern Population from the rest of the *A.boreas* group.
2. Identifying the degree of genetic diversity and biogeographic distribution of that diversity within the Eastern group.
3. Determining whether the SRM population should be recognized as a distinct unit separate from the rest of range of the Eastern Population (discussed by Hammerson 1999).

To meet these goals, the objective was to examine the DNA sequence diversity of multiple nuclear genes using a tiered approach to data collection, with each tier informing the best approach to the next level:

1. The first analysis was to examine a small set of individuals across the full range of the group to identify the utility of each nuclear gene in addressing the problem.
2. The second analysis was to examine additional individuals to identify the genetic diversity of the eastern group and identify its geographic distribution.
3. A third analysis was to examine individuals near the borders of the eastern group to further delimit its distribution.

These goals were met (as described fully in the report below) although the approach was modified slightly during the data collection process. Rather than examining three or four nuclear genes, we examined seven in order to increase confidence in the data (e.g., Fujitan, et al., 2012, Fennesey et al., 2016). Note that this study had a companion study with similar goals but a different approach directed by Dr.Sara Oyler McCance, USGS) the goal of which was to identify the utility of SNP data using the process of RAD-seq to identify the genetic groups within the *A.*

boreas toad complex. The initial budget from that project included funds for some of the sequence data collection. However, after initial analyses of both DNA sequences and SNP data we determined that it was better to double the amount of SNP data and reduce the amount of sequence data. It was apparent that the SNP data could indeed identify the diversity within the eastern group, and could identify diversity at a finer level than that DNA sequence data. Thus, we reduced the goal of examining a total of 200 samples with sequence data to examining about 120, and here we report the results from 99 samples. The results from an additional 20 or so samples are expected to be completed before publication. These samples are not expected to change the conclusions presented here, but may increase the strength of the conclusions.

Methods

Sample collection. Although many samples were on hand from previous analyses (N= 266, Goebel 1996, Goebel 1999, Goebel et al., 2009) additional samples needed to be collected. Terry Ireland (USFWS) contacted state agencies across the western US and requested they identify possible collectors in their regions (Table 1). A collection protocol (Appendix 1) was distributed as needed. When possible, previous collections were identified and provided for analyses (Wyoming, Montana, Washington), but not all of these had enough DNA for all analyses. I also included samples that had been collected between 1992 and 2016 and used in previous studies (e. g., Goebel et al., 2009). Although localized genetic turnover has been seen among close populations with microsatellite data, genetic differences remained consistent between major population centers (Holmes, 2015). Thus, collection over a 25-year time span is not expected to affect the slowly diverging nuclear genes over broad geographic areas as examined here.

DNA extraction, amplification and sequencing. DNA was extracted by Jennifer Fike and Dr. Sara Oyler McCance at the Fort Collins Science Center, CO, USA (USGS), using an ammonium acetate protocol (modified from the PUREGENE kit; Gentra Systems) with variable hydration of the final DNA pellet between 25-100ul depending on pellet size. DNA was quantified with the Qubit fluorometric quantification system (Molecular Probes/Life Technologies/Thermo Fisher). Aliquots sufficient to analyze with genomic methods were reserved at USGS. Additional DNA from samples, as well as the full aliquot for samples for which there was not enough DNA for genomic analyses, was shipped to FGCU for sequence analysis.
DNA was amplified from eight DNA regions including the mitochondrial DNA control region and seven nuclear genes and/or gene introns (Table 1). Genes were chosen from preliminary analyses (Goebel, unpublished data 2006 to present) of a variety of published genes and those that were most variable among five divergent lineages (based on mtDNA analyses) were chosen for the final analyses. Initial amplification was initially conducted with published primers and internal primers were developed to sequence through long fragments (C-YMC, RAG1, SOX9) as well as to sequence through difficult spots such as runs of bases or heterozygous sites with insertions and deletions that make downstream sequence data unreadable. Species specific primers were developed in some case for better initial amplification. Primers for one gene

(REV3RI were developed internally by Tyler Jacobson, University of Wisconsin, Madison). The sequencing was performed using Big Dye Terminator v3.1 chemistry on an ABI 3730 Genetic Analyzer at the Florida State University Sequencing Facility with the help of Dr. Steven Miller. DNA electropherograms were assembled using Geneious 9.1.6 (http://www.geneious.com, Kearse et al., 2012) from both forward and reverse sequences. Heterozygous individuals were resolved both by eye and with Indelligent (Dmitriev and Rakitov, 2008). Samples that may require allele specific primers were set aside for future analyses.

Table 1. Genes Analyzed.

Name	Number of samples analyzed in total.	Source
C-MYC Cellular Myelocytomatosis	122	Chen et al, 2009. Internals designed in this paper.
DBI introns 2 and 3 Diazepam binding inhibitor	148	Zhan and Fu, 2011, and this paper.
RAG-1 Recombination-activating gene-1	160	Pramuck et al., 2008; Biju and Bossuyt, 2003, and this paper.
REV3I including introns REV3 like, DNA directed polymerase zeta catalytic subunit.	124	This paper. Primers designed by Tyler Jacobson.
RHOD Rhodopsin including intron 3	163	Hoegg et al., 2004, and this paper.
RPL3 including intron 5 Ribosomal protein L3	168	Pinho et al, 2009, and this paper.
SOX9 including intron 2 Transcription factor SOX-9, in the sex determining region of the Y chromosome. Note that in amphibians this gene is found on the Z chromosome and is diploid (equivalent to the X chromosomes in humans).	163	Zhan and Fu, 2011. Internals designed in this paper.
Mitochondrial DNA - Control region	352	Goebel et al 1999, Goebel et al., 2009.

DNA alignment and tree analyses. Sequences were aligned using MUSCLE (2004) and corrected by eye as needed. All sequences were trimmed within alignments to exclude missing data and gaps were excluded from analyses. Although some nuclear DNA sequences were obtained from up to seven outgroup samples from the Nearctic clade (Pauly et al., 2004) including those from the *Anaxyrus americanus* species group as well as *A. retiformis, A. debilis, A. cognatus, A. speciousus*, and *A. punctatus*, a composite of two *A. cognatus* samples had the fewest indels and was therefore used as the outgroup in analyses. Models of evolution were chosen using jModelTest 2.1.1 (Darriba et al., 2012). Preliminary analyses were conducted from within Geneious using Bayesian (MrBayes v3.2.6, Huelsenbeck and Ronquist, 2001) and maximum likelihood (RAxML v8.2.4, Stamatakis, 2014) methods and the

GTR+I+G model. For Bayesian analyses, the HKY+I+G model was used, with default priors, 1,100,000 generations, burnin of 100,000 and sampling every 2000 generations. Convergence was analyzed with Tracer v1.6 (Rambaut et al, 2014).

Population analyses. Haplotype trees for mtDNA haplotypes were constructed using TCS v1.2.1 (Clemment et al., 2000) with a connection probability of 0.95. Haplotypes for nuclear DNA sequences were obtained for both individual genes and the concatenation of all seven nuclear genes with DNAsp v5 (Librado and Rozas, 2009) using a threshold of 0.9 and allowing for recombination. This program identifies the two alleles (each allele is now a haplotype; each individual has two states in any analysis). Bayesian phylogenetic analyses were conducted on the alleles/haplotypes for the concatenated sequences to identify zones of hybridization among divergent lineages. In order to identify admixture and genetic clustering within the *A.boreas* group, we used the Bayesian clustering algorithm within STRUCTURE v2.3.4 (Pritchard et al., 2000). Individuals were coded by alleles deduced within DNAsp treating each of seven genes as a single locus. Within STRUCTURE we sampled 40,000 steps following a burnin of 50,000 steps, for K=1-10 with 20 replicates each. The results were averaged with CLUMPP (Jakobsson and Rosenberg, 2007) and the most likely K (number of genetic clusters) was inferred by Structure Harvester (Earl and vonHoldt, 2011).

Analyses still in progress include the calculation of Fst values (Arlequin v3.5.2.1, Excoffer and Lischer, 2010). PCAs using the package R package adegenet (Jombart, 2008) will be used to assess the degree of similarity between apriori defined populations. Species delineation will be performed with Bayesian phylogenetic and phylogeographic analyses within BPP 3.2 (Yang 2015). Although the timing of divergence of identified clades might be estimated using MCMCtree in PAML (Yang, 2007), fossils to use as base references are still lacking due to the soft and not easily fossilized bones in anurans. Further analyses in STRCTURE will be conducted that will include locality data for samples.

A tiered approach was used for data collection for efficiency. In a first analysis of about 40 samples, priority was given to samples covering the broadest geographic distribution, all known species, and known diversity from previous analyses to delimit the geographic distribution of the eastern group. Sample collection was the highest close to the known eastern group as identified by mtDNA. Sample collection from the rest of the known distribution of the toad was sparse and used to identify the diversity within the group as possible. As the geographic distribution was refined, a second set of samples was prioritized to identify the outer boundaries of the eastern group. Finally, a third set of samples was identified to include 30 samples in the major divergent groups and at least two samples, if possible, for all unique and divergent lineages. Our goal was to identify and delimit the geographic distribution of the eastern group, not provide a phylogenetic analysis of the complete *A. boreas* species group, nor confirms speciation within the group.

Results and Discussion

Samples- Between Summer 2014 and November 2016, 312 samples were received from

Colorado, Utah, Wyoming, Idaho, Montana, Washington, Oregon, Nevada, Alaska and British Columbia (Appendix 2). Several samples were excluded from analyses because they were determined to be non-target species (probably *Acris gryllis* and *Rana sp.* based on comparisons to sequence data in GenBank and Dr. Wendy Estes Zumpf, WGF, personal communication), could not be amplified, or had too little DNA to analyze all nuclear genes. The latter were saved for future analyses. The mtDNA control region was amplified for about 200 new samples resulting in an analysis of 421 total samples. Data for one or more nuclear genes was collected for 170 samples (newly and previously collected) and all seven nuclear genes were successfully collected for 99 samples (newly and previously collected). The fragment length, as well as the information content (as measured by the number of variable sites, number of haplotypes, and haplotype diversity) varied among genes (Table 2).

Table 2. Information content of DNA regions.

Gene	Fragment length (bp range)	Aligned length (bp) (with outgroup)	Total sites analyzed (bp)	Number of variable sites (ingroup)*	Number of haplotypes (ingroup)	Haplotype Diversity (ingroup)
CYMC	816-826	826	809	22	16	0.70
DBI	503-560	610	495	49	18	0.70
RAG	936	936	936	27	17	0.49
REV	602-652	658	640	30	19	0.50
RHO	696-725	726	724	29	30	0.87
RPL	621-656	665	619	39	26	0.58
SOX	1263-1340	1355	1333	38	47	0.82
7 nuclear genes combined	5557-5687	5776	5605 (ingroup)	233 (ingroup)	118 (ingroup)	0.97 (ingroup)
mtDNA Control Region	536	NA (ingroup only)	536	86	-	-

*Excluding gaps and sites with missing data.

Phylogenetic analyses of mitochondrial DNA. Mitochondrial analyses of the small fragment of the control region (421 samples, 665bp) were consistent with all previous analyses of mitochondrial DNA (Figures 1 and 2) in identifying the major clades (Northwest, Southwest, Eastern) as described in Goebel et al. (2009). Analyses also identified a previously described clade (Richard Tracy, University of Nevada, Reno) named the Lahontan clade and found within the NW clade.
Note several features of the analysis of mtDNA (see Figures 1 and 2 below):

1) *The Eastern mtDNA clade* consists of toads found in eastern Nevada, most of Utah, western Idaho, Wyoming and all of Colorado.

2) *Within the eastern clade divergent lineages* are found in Kane Co, UT, Hot Creek NV/ Box Elder Co, UT, Railroad Valley, NV, and Piute/Wayne Cos, UT.

3) *The Southern Rocky Mountain Population (SRMP)* was not identified as a monophyletic group with the control region fragment analyzed here. This does not exclude monophyly of the SRMP, which might be found with a greater length of mtDNA (for example a combination of the full control region and the cytochrome b gene).

4) *The divergent mtDNA clades overlap in both Wyoming and NW Utah / NE Nevada.* Wyoming toads have populations that consist of both NW and Eastern haplotypes within the same populations. Box Elder Co, UT and NN Co, NV, both have populations that consist of toads with haplotypes from the NW and Eastern mtDNA clades.

5) *The Middle Rocky Mountain Group* is found within the NW major clade (identified in blue) and is not closely related to the Eastern Clade (identified in red). This contrasts with the nuclear DNA analyses presented below.

6) *This tree is consistent with all previous mtDNA analyses* including Graybeal (1993), Goebel (1996, 2000), Goebel et al. (2009), Shaffer et al., (2000) Switzer et al. (2009), Stephens (2001), and unpublished work by Dr. Richard Tracy (University of Nevada, Reno). Further work is in progress by Dr. Greg Pauly (NHM) in the southwest US and Mike Lucid (Idaho Department of Fish and Game) in Idaho.

7) *This tree is consistent with work by Richard Tracy, but puts his analyses of toads in Nevada into a larger picture of the whole A. boreas group.* Specifically, he describes a Lahuntan clade, closely related to the NW clade, and found largely in Nevada. The clade Tracy calls the "Mojave group" is consistent with the SW mtDNA clade described by Goebel et al., (2009). The three new species described by Tracy's lab (in press or recently published?) are found within the Middle Rocky Mountain group (Dixie Valley) or in the Eastern mtDNA clade (Hot Creek and Railroad Valley).

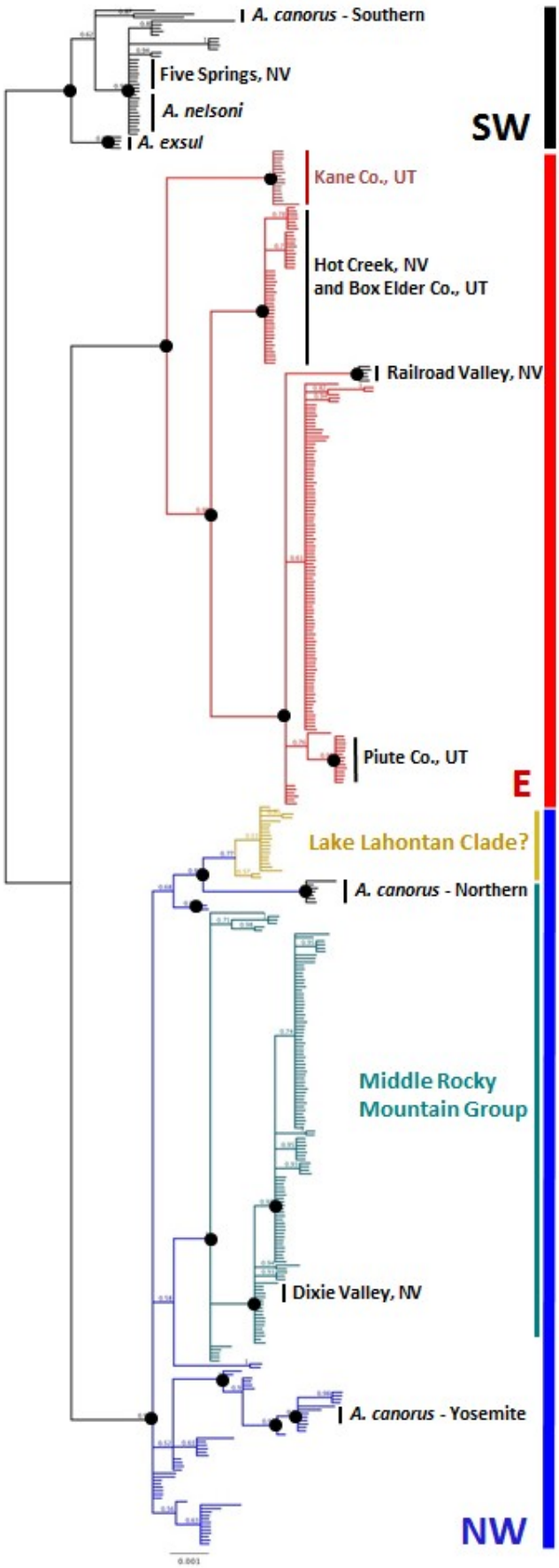


Figure 1. Phylogenetic analysis of mtDNA haplotypes of the *Anaxyrus boreas* species group (421 samples). Tree is unrooted. Tree constructed with Bayesian methods and dots on branches identify Bayesian probability values 0.95 to 1, showing high support for those clades. Trees constructed with maximum likelihood methods were identical or differed only in minor ways.
The analysis recovers the previously described major mtDNA clades: Eastern (red) Northwest (blue) and Southwest (black) as well as the Middle Rocky Mountain subgroup (blue-green). The geographic distribution of the groups is in Figure 2.

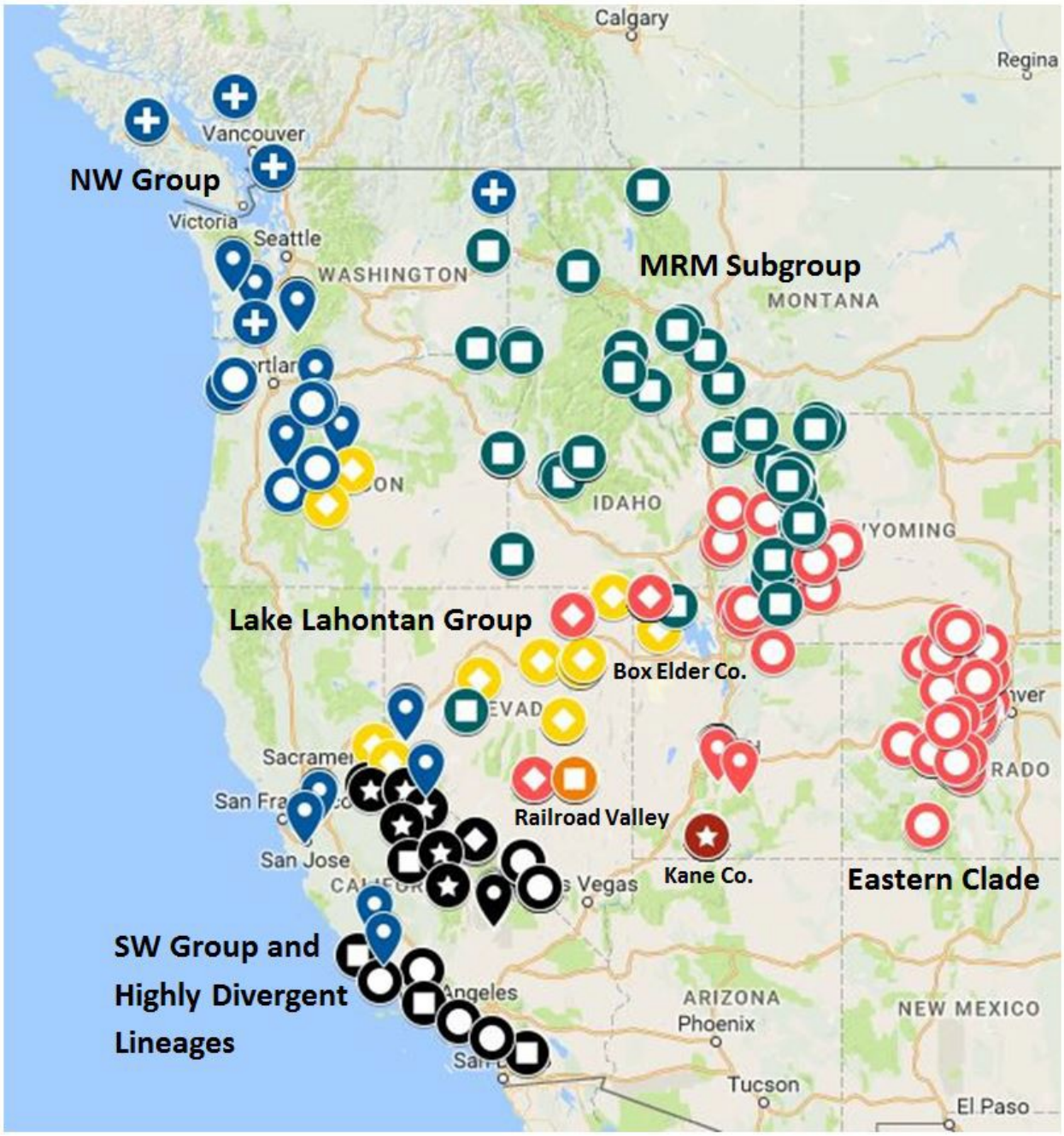


Figure 2. Phylogeographic distribution of the major clades of mtDNA. The groups are: Red-Eastern clade, Blue- Northwest group, Bluegreen – Middle Rocky Mountain group, Yellow-Lake Lahontan group, Black-Southwest group and divergent groups including new groups and previously recognized species in the group. Note that the Railroad Valley population is in the Eastern group but is divergent within the group and so is depicted here as orange. The

population in Kane Co UT, is similarly in the Eastern Groups and depicted as dark red.

Network analyses of mitochondrial DNA haplotypes. Network analyses (identified with TCS v1.2.1, Clemment et al., 2000) of mitochondrial analyses identified four clusters (Appendix 3). The highly divergent population in Kane Co., UT, the Eastern group excluding the population in Kane Co. UT, the SW mtDNA clade, and a highly interbranched and unresolved network of haplotypes from the NW mtDNA clade. These data suggest that the smaller fragment of mtDNA is informative and sufficient to resolve the identity of the four clades, but may not be sufficient to resolve all relationships within the divergent clades.

Phylogenetic analyses of nuclear genes (and comparison to mtDNA analyses)

The number of samples and the pattern of inheritance differ between mtDNA and nuclear gene analyses. Analyses from seven nuclear genes (Figures 3, 4 and 5), and their geographic distribution, was largely similar to the analyses based on mtDNA (Figures 1, 2), but differed in significant ways. The analyses differed in sample number; over 400 samples were included in the mtDNA analysis, while nuclear gene analyses were based on 99 samples and focused on the delimitation of the Eastern group, so sampling across the western portion of the toad's distribution was sparse. The analyses differ because mtDNA and nuclear DNA are inherited differently. MtDNA is inherited effectively as a single large locus that does not recombine and is passed on maternally only. In contrast, each nuclear gene is a separate locus, it is possible for the loci to recombine, and both parents pass on one copy of the genome to the offspring. Thus, each individual has only one copy of the mtDNA, individuals have two copies of each nuclear gene and can be heterozygous for one or more genes. In other words, they can be "hybrids" having one allele from one clade and another allele from a divergent clade. Therefore, we provide two analyses of nuclear genes, one of the individuals represented as heterozygous individuals (Figures 3,4,5), and one of the alleles (each allele is effectively an "individual" or tip on the tree (Figure 6). Separate analyses are important because heterozygous individuals can affect the identification clades. For example, if an individual has alleles from two divergent groups, the individual may be identified as intermediate between two clades. However, if alleles are analyzed, one allele will be found in one clade, and the other allele will be found in the other clade; the individual will be represented in two clades.

Note several features of the analyses based on heterozygous individuals (see Figures 3, 4 and 5 below):

1) *Analyses based on nuclear genes found similar groups to those based on mitochondrial genes*. The two major groups, Southwest and Eastern are recognized as clades or genetically close groups. Subgroups within the Northwest group are recognized (Lake Lahontan and Middle Rocky Mountain). Nuclear genes also identify many of the smaller divergent populations (*A. canorus*, *A. nelsoni*., *A. exsul*) however, these were not the focus of the study so the limited samples analyzed may not be representative of the diversity within those species.

2) *Analyses based on nuclear genes found a similar geographic distribution of groups compared to mtDNA anal*yses. In general, the distribution of the NW, SW, Lake Lahontan, are very

similar. The distributions of the Middle Rocky Mountain and Eastern groups are largely similar (but see below). In both analyses the population in Box Elder Co, UT, is comprised of genotypes from three groups (Eastern, Middle Rocky Mountain, and Lake Lahontan).

3) *Analyses of nuclear genes differ in that the relationships among the groups differ.* Relationships vary among the larger groups; within nuclear analyses both the Middle Rocky Mountain and Lake Lahontan subgroups are closely related to the Eastern group rather than being found in the NW major group. Relationships of some of the minor groups also vary; within the mtDNA analyses the Kane Co, UT, population is highly divergent, but far less so with nuclear DNA sequence analyses.

4) *Analyses of nuclear genes differ in that the geographic distribution of the groups differ in significant ways in Wyoming.* Within mtDNA analyses, both the NW and E major clades are found within Wyoming but in nuclear analyses, the E group is identified (Figure 7A and B).

5) *For listing purposes, the most significant findings were:*
    a. The Eastern group is identified in all genetic analyses, both here and in previous analyses, with mtDNA and with nuclear genes. Although its status as a species has not yet been determined, it is a genetically distinct group.
    b. The geographic distribution of the Eastern group, based on nuclear genes, includes toads in Utah, SE Idaho, all of Colorado and probably all of Wyoming. Alleles seem to occur in Montana, but its distribution in Montana is unclear due to sparse sampling.

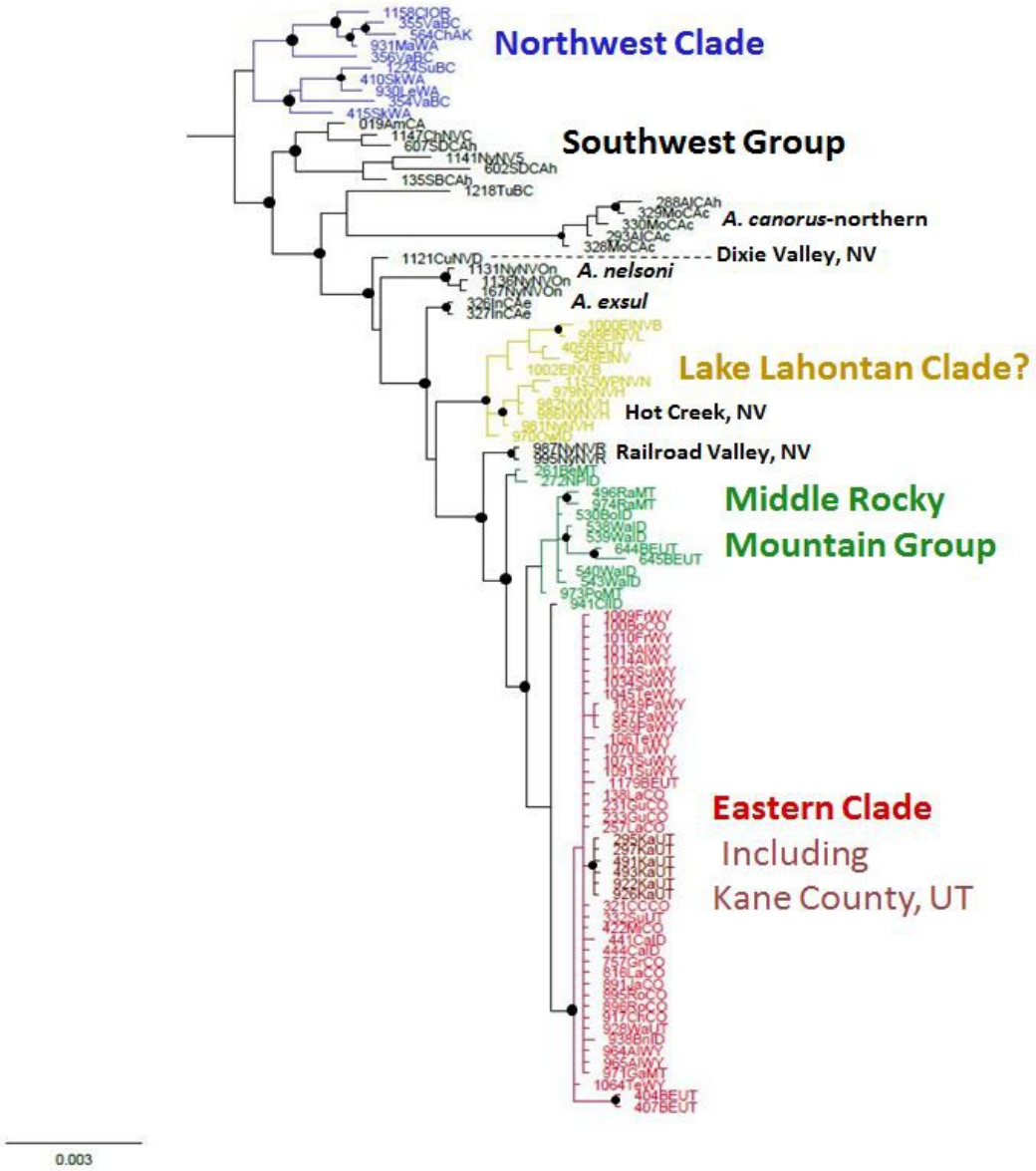


Figure 3. Phylogenetic analysis of the *Anaxyrus boreas* group based on seven nuclear genes, rooted. Support for the nodes (posterior probability values 0.95-1) are identified as dots on the tree. The tree is rooted with *Anaxyrus cognatus*. Trees constructed with maximum likelihood methods were identical or differed only in minor ways. The groups are: Red- Eastern clade, Green – Middle Rocky Mountain group, Blue- Northwest group, Yellow- Lake Lahontan group, Black-Southwest group and divergent groups including new and old named species in the group.

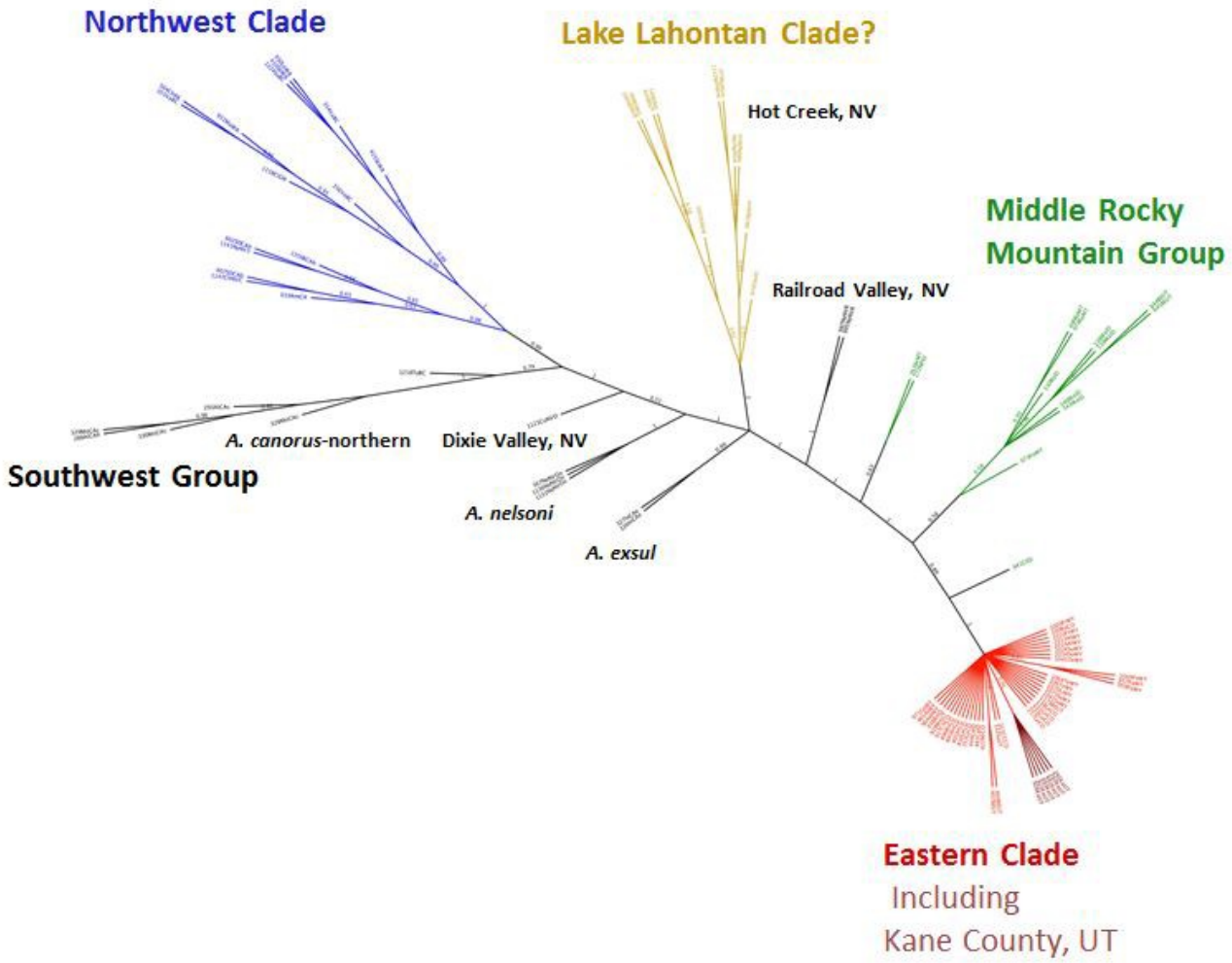


Figure 4. Phylogenetic analysis of the *Anaxyrus boreas* group based on seven nuclear genes. The tree is unrooted. The groups are: Red- Eastern clade, Green – Middle Rocky Mountain group, Blue- Northwest group, Yellow- Lake Lahontan group, Black- Southwest group and divergent groups including new and old named species in the group. The same samples, except for the outgroup, were used in this analysis as in the analysis for Figure 3 and the tree identifies the same groups.

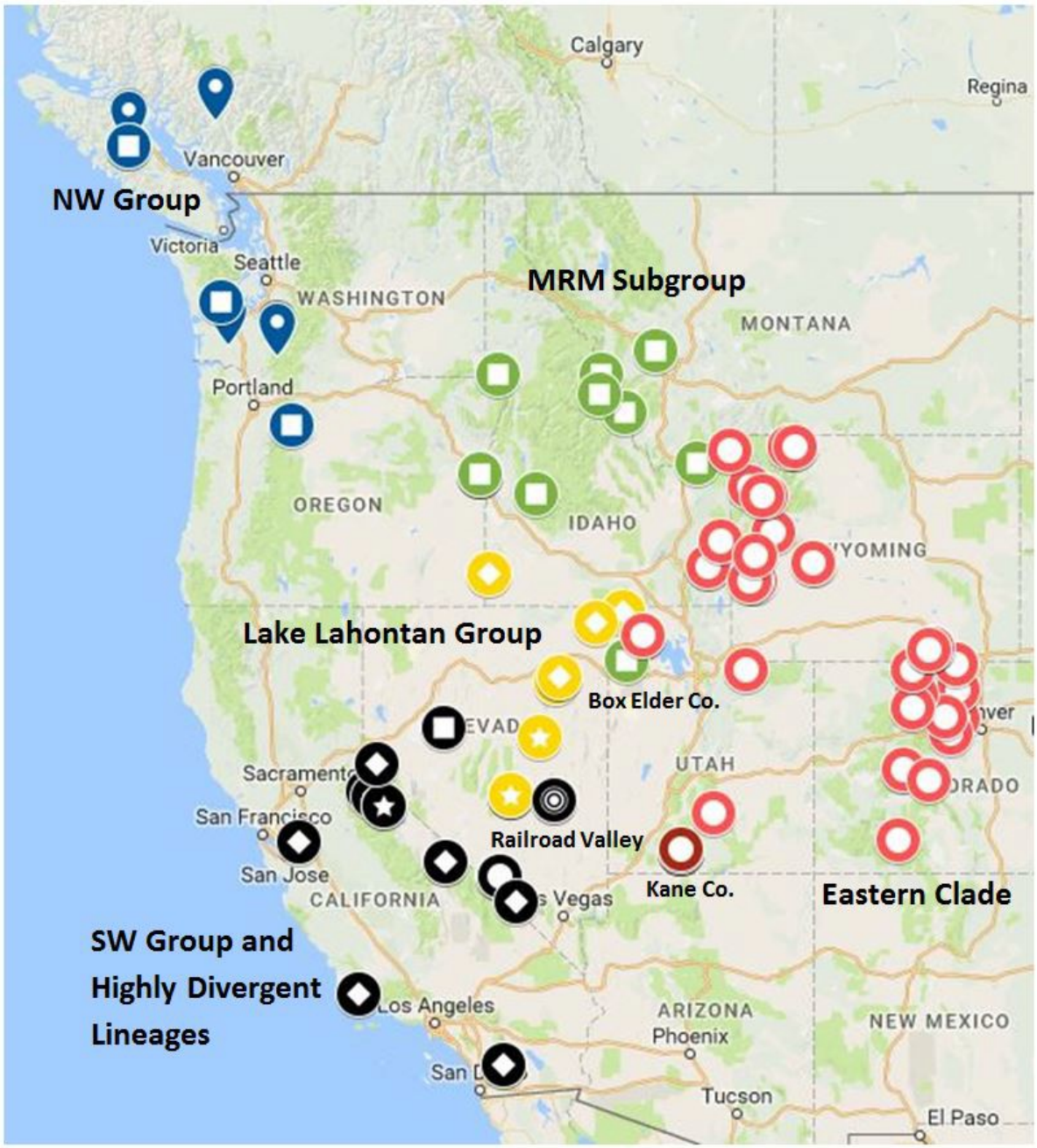


Figure 5. Phylogeographic distribution of the major groups and clades based on seven nuclear genes. The groups are: Red- Eastern clade, Green – Middle Rocky Mountain group, Blue- Northwest group, Yellow- Lake Lahontan group, Black-Southwest group and divergent groups including new and old named species in the group. Note that Railroad valley is depicted as black

because it is highly divergent, but it is not within the SW group. Samples from Alaska are in the NW group and not shown in this figure.

Note several features of the analyses based on heterozygous individuals (see Figure 6 below):

1. *Analyses based on heterozygous individuals found similar groups to those based on haplotypes (alleles) alone*. In most individuals both haplotypes were found within the same group (NW, SW, Lake Lahontan, Middle Rocky Mountain, and Eastern) and they were found in the same relationships found with the analyses of heterozygous individuals.
2. *Analyses of nuclear haplotypes (alleles) identified some individuals that were heterozygous for alleles from divergent group*s. This is most significant for the samples within and close to Wyoming where there may be a zone of overlap between the Middle Rocky Mountain and Eastern Clades. Specifically, note that three samples 971GaMT (from Gallatin MT), 973PoMT (from Powel Co MT) and 1064TeWY (Teton Co WY) had haplotypes found in both the Middle Rock Mountain and Eastern groups; they had divergent alleles within single individuals (Figure 7 C). In contrast, sample 941ClID (Clark Co ID), had both alleles from the Eastern clade, supporting the presence of the Eastern clade into north east Idaho. It was previously thought to be in southeast Idaho only.
3. *For listing purposes, the most significant finding was:* The geographic distribution of alleles in Wyoming and Montana suggest a broad region of overlap of nuclear alleles from both the Eastern and Middle Rocky Mountain groups. Alleles of the Eastern group predominate in Wyoming (only a single Middle Rocky Mountain allele was found). The zone of overlap most likely exists between Wyoming and middle Montana.

Identification of genetic groups with STRUCTURE. Genetic structure within the data set was examined for the nuclear DNA haplotypes/alleles (Appendix 4). The preliminary analyses presented here determined a K of 2, suggesting that the *A. boreas* group can best be described as consisting of two subgroups. These can be largely defined as one group consisting of Northwest, Southwest, *A. nelsoni*, *A. canoris* and Hot Creek populations with substantive admixture for the Lake Lahuntan, *A. exsul*, Railroad Valley groups, and the second group consisting of the Eastern, and Middle Rocky Mountains with admixture identified as largely green with some red in the Middle Rocky Mountains. These results are suspect, and may not make biological sense, as *A. canorus*, a highly divergent and monophyletic lineage was not recognized as a distinct genetic group. STRUCTURE fails to recognize groups with small samples sizes (and most of our divergent populations had small sample sizes). STRUCTURE also fails to recognize subdivision when there is a genetic pattern of isolation by distance. While I do not have great confidence in the current analyses from STRUCTURE presented here (further analyses are needed), it is interesting to note that increasing the groups by one (K=3) recognizes three groups; the Eastern group, samples in the Northwest, Southwest, *A. canorus*-northern, *A. nelsoni*, and Dixie Valley Populations, and a group consisting of samples in the *A. exsul*, Lake Lahontan, Railroad Valley, and Middle Rocky Mountain groups. Clear admixture is seen in samples from Box Elder Co., UT and samples in NW Wyoming and SE Montana (haplotyes in both the E and MRM groups).

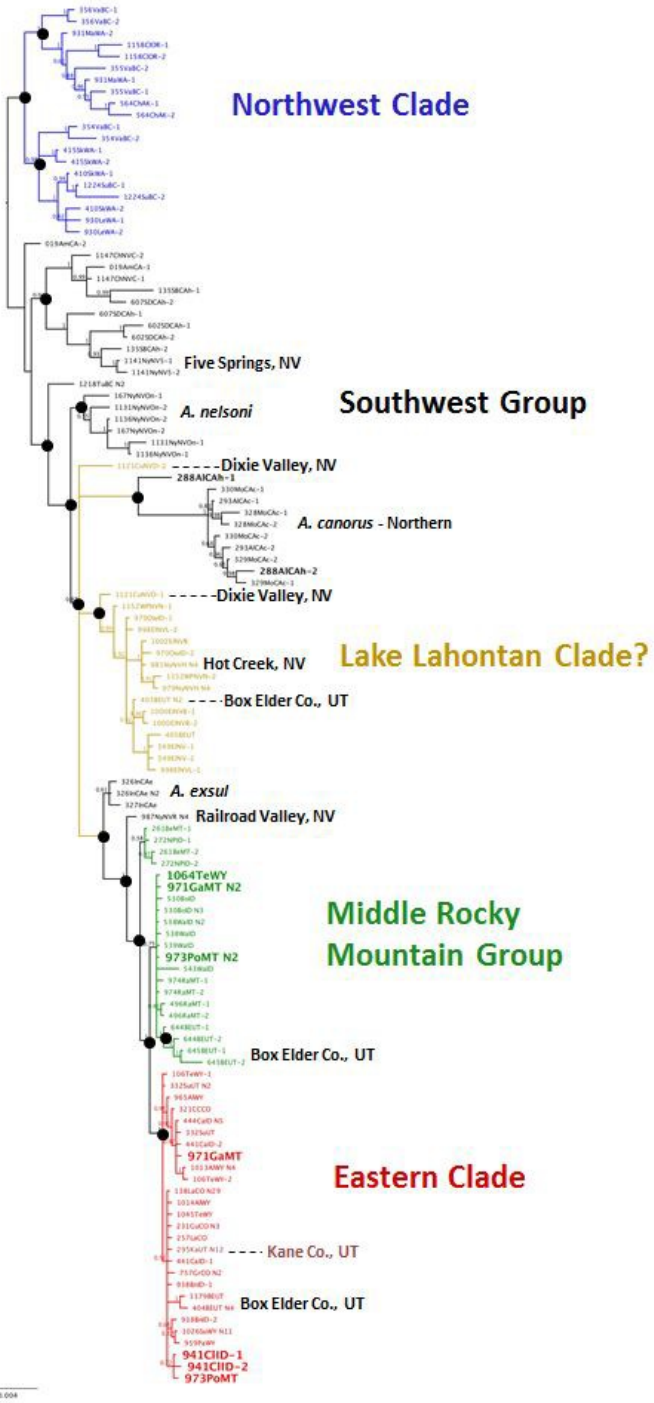


Figure 6. Phylogenetic analysis of nDNA alleles (haplotypes). Haplotypes (N=118) were deduced from PHASE with the program DNAsp from the same 99 individuals included in previous nuclear DNA analyses (Figures 3 and 4). The tree is unrooted and Bayesian support values (0.95 to 1) are indicated on the branches as black dots. Note that the samples 971GaMT and 973PoMT have alleles that appear in both the Eastern

and Middle Rocky Mountain groups. In contrast, both alleles from sample 941ClID are found in the Eastern clade (See figure 7C).

A

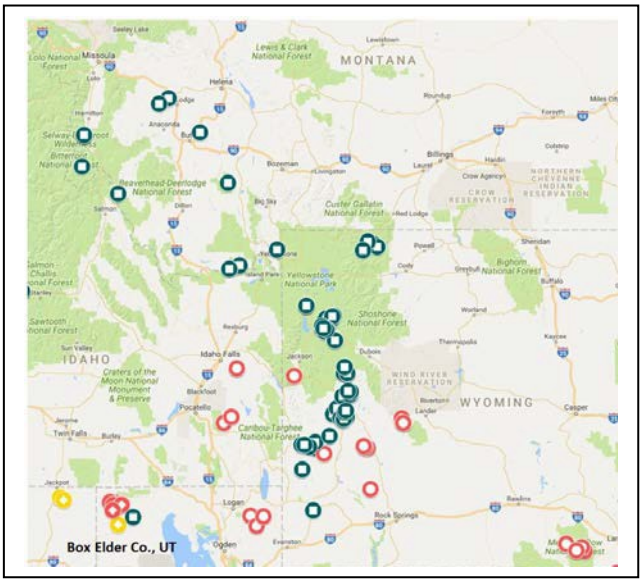
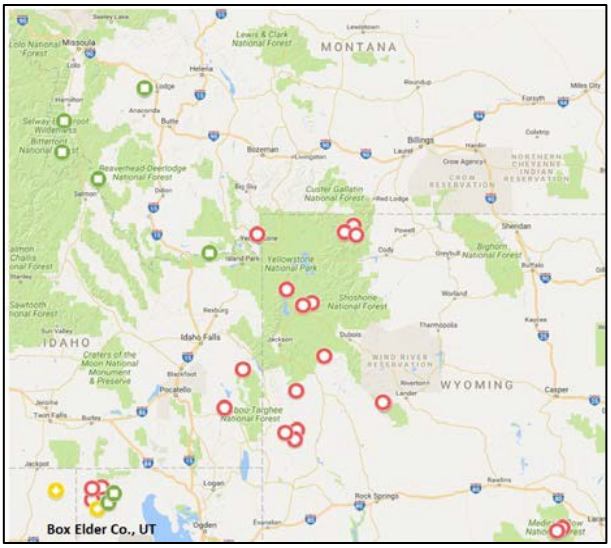


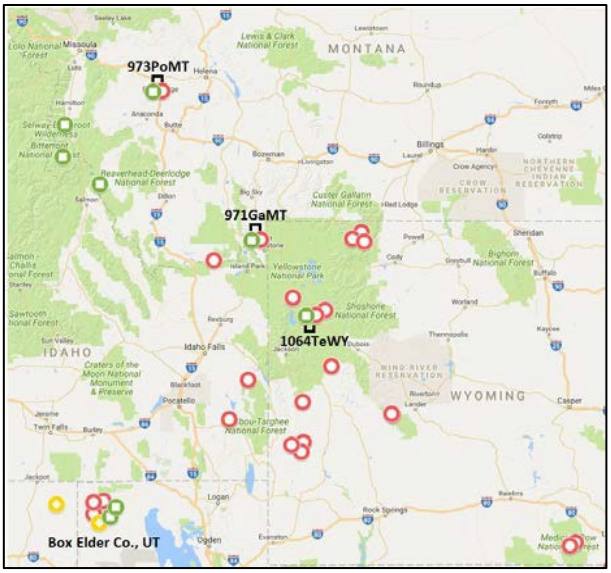
Figure 7. A. Distribution of major clades of mtDNA in Wyoming and surrounding states. Bluegreen- Middle Rocky mountain group of the NW clade. Red- Eastern clade. Yellow-Lake Lahontan group. A region of intergradation can be seen in western Wyoming (red and blue-green) as well as in Box Elder Co, UT (red, blue-green, yellow).

Lahontan group. A region of intergradation can be seen n Box Elder Co., UT (red, green, yellow).

B.



B. Distribution of major groups of nuclear DNA in Wyoming and surrounding states based on genotypes of heterozygous individuals. Green- Middle Rocky Mountain group. Red- Eastern clade. Yellow-Lake



C. Distribution of major groups of nDNA alleles in Wyoming and surrounding states based on inferred nDNA haplotypes. Green- Middle Rocky Mountain group. Red- Eastern clade. Yellow-Lake Lahontan group. Note three individuals with alleles (nDNA haplotypes) from both the Middle Rocky Mountain group and Eastern clade (973, 971 and 1064). When only alleles are considered, the sample from northeast Idaho falls in the Eastern group. Samples in Box Elder Co, UT have several divergent alleles.

Conclusions

Current genetic data suggests:

1) A genetically distinct group of boreal toads exists in the eastern portion of the range and is found within all of Colorado, most of Utah, Wyoming and southeastern Idaho. Part of this group also may occur in southwestern Montana.

2) The geographic boundaries of the eastern group are unclear in several regions.

- First, northwestern Utah and northeastern Nevada (Box Elder Co. UT and Elko Co. NV) have a mix of distinct eastern and western alleles. However, a combined analysis of multiple nuclear genes suggests that the population of toads in this region have differentiated in allele type and frequencies from the eastern group.
- Second, populations in middle-eastern Nevada (Hot Creek and Railroad Valley, NV) have unique mtDNA haplotypes within the Eastern mtDNA clade, but analyses of nuclear genes show that they have differentiated from the Eastern Clade and are more closely related to toads in Nevada.
- The third region where boundaries are unclear is in southwestern Montana. Sampling was sparse but individuals with alleles from both the eastern and western middle-Rocky Mountain groups occur. For example, three toads (973, 971, 1064; Figure 7C) have nuclear haplotype from both the Eastern and Middle Rocky Mountain groups. While sampling in Wyoming was denser, sampling in Montana was sparse. It is unclear whether there is a clear break, or whether this will be a zone of intergradations between the two groups.

3) Results of mitochondrial DNA analyses are slightly different than the combined results of seven-nuclear genes analyzed.

- While mitochondrial DNA suggested that Wyoming consisted of a large zone of intergradation of the northwestern (Middle Rocky Mountain) and eastern mtDNA haplotypes, the analysis of multiple nuclear genes suggests that Wyoming is comprised of toads most genetically similar to the eastern group. This can best be explained by the comparatively rapid introgression (gene flow into another genetically differentiated group) of mtDNA into the populations of Wyoming, but the introgression of nuclear alleles was undetected in most genes and at a low frequency in a single nuclear gene. A differential rate of mitochondrial and nuclear gene introgression is common due to different patterns of inheritance. Mitochondrial DNA can introgress rapidly as it is inherited strictly maternally, is passed on to all offspring of a female, and does not undergo recombination. Nuclear genes are inherited bi-parentally and undergo abundant recombination, thus introgression into another population takes longer to occur and may, therefore, remain undetectable for a long period, especially with limited sampling.
- The nuclear DNA analyses presented here do not identify as much genetic differentiation within the Eastern Clade as the mitochondrial DNA analyses. Toads in southern Utah (Kane Co.) form a distinct mtDNA clade. However, the nuclear DNA genes examined differentiate them within the Eastern clade, but only show moderate divergence. Both mitochondrial and nuclear genes identify all toads in Utah as being in the eastern group, except those in the

northwest corner (Box Elder Co.) and northeastern Nevada.

- The analyses of seven nuclear genes demonstrate that the Box Elder Co. group may be another area of intergradation between Middle Rocky Mountain Group, the Lake Lahontan Group, and the Eastern Clade. While similar, these toads are likely distinct from the Eastern Clade.

Further Comments

The goal of this project was to identify the genetic distinctness of the Eastern group, as previously identified by mtDNA alone, and delineate its geographic distribution. The goal was not to identify speciation within the group, nor to identify all diversity in the *A. boreas* group as a whole. We did examine all previously described species in the group and as broad a geographic distribution for which we could obtain samples. Analyses to identify speciation are not yet complete, nor are analyses to identify genetic substructure (e. g., Carstons, et al, 2013, Fujita et al., 2012, and Fennessy, 2016).

Acknowledgements

This project could not have been completed without the assistance of many people. This includes the many that collected samples for analyses (See Appendix 2). Thanks to Dr. Greg Pauly who provided the samples of A. canorus (south and some north) included in mtDNA analyses in Goebel et al., (2009) and shown Figure 1. Thanks to Quillan Arico, lab manager at FGCU, for conducting the lab work, supervising student assistants, continuously drawing maps and providing input at all levels. Thanks for the laboratory assistance to: Dr. Steven Miller at the Florida State University DNA Sequencing facility provided invaluable assistance with sequencing. Tyler Jacobson, University of Wisconsin, Madison, developed the primers for the REV gene for his senior project at Florida Gulf Coast University (FGCU). Daniel Bowen, Victoria Grille, and Salvatore Grasso, identified the utility of the RAG1, SOX and DBI genes respectively as part of their senior projects at FGCU. Brittany Storiale, Anandi Ehman, and Jamie Simmons volunteered in the lab. Jennifer Fike and Dr. Sara Oyler McCance at the Fort Collins Science Center, CO, USA (USGS), received the DNA from collectors and extracted and quantified the DNA before sequencing at FGCU. Ms. Fike and Dr. Oyler McCance also provided ongoing professional assistance with the complimentary project analyzing the genomic DNA (RAD-seq) of a subset of the samples analyzed here. A number of people provided intellectual input and professional assistance with the project including Terry Ireland, USFWS, who assisted with grant writing, sample collection, and proofing reports; Dr. Sara Oyler McCance (USGS) who provided assistance with interpreting data analyses and Dr. Adam Leache (Burke Museum, University of Washington) who suggested an approach using coalescent-based species delimitation.

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Appendix 1. Sampling protocol for *Anaxyrus (Bufo) boreas* for DNA analyses for Sanger sequencing and RAD sequencing.

Version 2 June 15

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Introduction/Definitions

Purpose of the Study: To identify the evolutionary units for conservation in *Anaxyrus boreas*, specifically the status of the Eastern mtDNA clade and the SRMP, to assist the USFWS in their determination of the need for listing of the toad.

The **Eastern mtDNA clade** (Goebel et al., 2009; Switzer et al., 2009) consists of toads in Colorado, New Mexico, Utah, and parts of Wyoming, Nevada, and Idaho. **The Southern Rocky Mountain Population (SRMP)** consists of toads in Colorado, south central Wyoming, and New Mexico where they were introduced from Colorado. We need to obtain tissues within and immediately surrounding the Eastern Clade region. We have samples from some of these areas but we need help filling in the gaps.

Definitions of collection sites.

We need some samples collected over a narrow spatial scale, and some over a broader geographic distribution. For the purposes of collection, we define the terms below, but recognize that they will vary based on the state and local geography.

Locality - anything with a unique GPS; the unique data that belongs with each collected sample.

Local population - for practical purposes this will be defined as toads that are interbreeding at the time of collection. We define this as toads within a few miles of each other. Examples include a pond, or set of close ponds.

Breeding population– for practical purposes this will be defined as toads that could reasonably interbreed with each other in any specific year. Since toads can move 5 miles over the summer, we define a breeding population as any group where individuals are within about 20 miles of each other. Common examples might be drainages.

Region – A group of local populations distributed across the state(s), but within the same recognized genetic group. These will vary greatly in size depending on how the toads are distributed. For example, the SRMP is considered one region.

Genetic group – Previous analyses of mtDNA have identified several genetic groups as in Goebel (2009). These include three major groups; Eastern, Southwestern and Northwestern. Some minor groups were also identified as well as unique populations (for example, toads in Kane County, UT).

State – because state-specific permits may be necessary we have divided the collections by state. We recognize that the above categories may not be state-specific in some cases. For example, the SRMP is located in Colorado, as well as in south central Wyoming and now New Mexico.

Definitions of individual samples or "adult equivalents".

The number of samples collected from each site will depend on the tissue type. The number of samples that should be collected is also a judgment call by the managers in the state and the collectors in the field.

"Adult equivalents" – Ideally, we want to represent viable adults in each population however, it is not appropriate to kill a large number of adults. Tissue from any single adult (toe clip, blood, body parts from dead toad, buccal swab) is an "adult equivalent". Remember, mitochondrial DNA is passed from a mother to all offspring, so sampling siblings will only identify the same mitochondrial DNA. Similarly, parents pass half their

nuclear DNA to each offspring, so siblings typically share half their DNA. Therefore, for the purposes of this study all siblings (tadpoles, metamorphs or juveniles from the same egg mass) represent the same "adult equivalent". In some cases, the tadpoles from a single egg mass can be identified (e. g., they are all in an isolated pond) but in other cases the potential to be from multiple egg masses should be noted on the labels. Individual one-year-olds may or may not be "adult equivalents". However, if the toads are very rare and you have reason to believe that all one-year-olds in a locality are siblings, collect tissue representing one sample.

Tadpoles – collect at least one, or tissue from one, for DNA analysis. If there are a large number of tadpoles, you might consider collecting for future analyses by collecting up to but no more than 10. One tadpole can be preserved in a museum as a voucher specimen for the tadpole series, one can be used in this study, and the others can be maintained for any future analyses that may need to be conducted. Although each study requires very little tissue, each study may need to extract the DNA in slightly different ways.
Therefore, preserving small bits of tissue is better than preserving the DNA. If you need to be conservative collect three tadpoles (one for a voucher, one for DNA analysis, and one as a backup in case the first sample is degraded or unusable for unpredictable reasons). Note that the tail is the preferred tissue from the tadpole as the body is largely intestines full of food (non-toad DNA). Therefore, tadpoles with large tails are preferred. All tadpoles from the same egg mass will represent a single sample or "adult equivalent".

Metamorphs – collect at least one for DNA analyses. I do not know of any described method to collect tissue from metamorphs; if the tail is still visible you can take a tail clip. If there are large numbers of metamorphs you might consider collecting up to three (one to be deposited in a museum as a voucher for the metamorph series, one as a spare, and one for multiple DNA analyses). All metamorphs from the same egg mass will represent a single sample or "adult equivalent".

Tissue from adults and juveniles – Collect one tissue clip, or one large drop of blood. Body parts from dead adults are also fine if they are fresh or dried. No voucher will be possible from tissue alone; if possible, take a photograph from both the ventral and dorsal sides as vouchers.

We have two different sampling strategies for the two different kinds of DNA data that will be collected. **The kinds of data are:**

Data type 1- Sanger sequencing

- Sanger sequencing consists of PCR amplifying specific nuclear and mitochondrial genes from the genome using gene-specific primers. Once amplified the complete DNA sequence will be determined through traditional Sanger sequencing methods. Phylogenetic trees will be inferred from the data and frequency of genotypes will be analyzed to determine structure within and among divergent groups.
- We will be collecting the cheaper sequencing data on as many samples as possible to identify the geographic distribution of mitochondrial and nuclear DNA clades. In general, the best sampling strategy is to collect a few samples (one or two per locality or local population) from the broadest geographic distribution as possible.
- Tissue collected for Sanger sequencing can be quite degraded (e. g., dead toads in the field, particularly if they have dried quickly) but high quality tissue is best (e. g., whole tadpoles, toe/tadpole tail clips, immediately preserved in alcohol). Because we want to compare Sanger sequencing methods and RADseq methods below, we want to use both methods on some samples.

Data type 2- RAD sequencing (RADseq or specifically ddRADseq)

- Consists of digesting the whole genome with restriction enzymes, then PCR amplifying a reduced portion of the genome using primers that will amplify the DNA beginning at the restriction sites. Because many small fragments will then be sequenced, tens to hundreds of thousands of polymorphisms (Single Nucleotide Polymorphisms or SNPs, pronounced "snips") can be identified (double digest RAD sequencing; Peterson et al., 2012). Multiple analyses can be conducted from the SNPs including phylogenetic analyses, identification of population substructure, and potentially identification of

selection/adaptation.

- Using the more expensive RADseq methods we will be looking at the diversity within a very few breeding populations and all genetic groups identified from the cheaper Sanger sequencing above. We will need 10 samples (adult equivalents) per group (either the specifically identified breeding populations or larger genetic groups).
- Tissue for RAD sequencing must be high quality (not decayed) and not highly contaminated with other species (e. g, dead animals and buccal swabs may contain many bacteria and fungi and are less useful).

Sample Collection:

Collecting strategies: The collecting strategies will be unique for each state and will depend on the size and distribution of the local and breeding populations and genetic groups. Strategies will be discussed in detail with the collection coordinators in each state. The collection strategies will also be based on results of previous genetic studies. Because we are trying to determine the presence and extent of overlap between divergent mitochondrial and nuclear gene lineages, in general more samples will need to be analyzed from regions of known overlap between divergent genes. Collection numbers will also be based on the number of previously collected samples (this study and other collections) that can be used for this analysis.

Because the toads are Endangered in Colorado and New Mexico, and may be declining in other states also, we need to limit the effects of sampling on the population. We fully recognize that managers within each state must balance the need for knowledge with any possible harm to individual populations. Therefore, we provide guidelines but recognize that managers must make decisions while they are in the field.

You may also wish to decontaminate all boots and equipment in between populations in order to reduce the spread of diseases. Your states may have guidelines for best practices, but in general scrubbing boots off with a bleach solution is a good habit.

General sampling strategy as described above: In order to get a few samples from the broadest geographic distribution, sample as many local populations as possible over the broadest possible geographic distribution, but no more than 10 samples per breeding population. This will allow for both RADseq and Sanger sequencing to be conducted as needed. If the local or breeding population is the only one in the region, then sample up to 10 adult equivalents. In general:

- The number of samples you might collect from a region or genetic group will vary depending on the size of the region and the number of breeding populations that exist in the area and the status of the population. Sample at least 10 adult equivalents, if possible, for any group that you believe is genetically distinct (for example, toads in Kane County, UT).
- Sampling should be heavier in areas where the known genetic groups overlap.

Tissue types

Preferred: fresh tissue for use in both Sanger and RAD sequencing

- Toe clips if you feel they will not cause undue distress or infection
- Tadpoles if they are available.
- Tadpole tail clips if you cannot collect whole tadpoles.
- Metamorphs if they are abundant and you feel they will not reduce the population size.
- Blood tissue if you are trained to draw blood

Less preferred tissue that can be used in Sanger sequencing but not RADseq:

- Buccal swabs
- Dead toads found in field, especially if they are not too decayed or if they dried quickly.

Samples must be tissue or dead toads. IACUC approval is not needed to receive dead animals or tissue for analyses.

Please call Anna Goebel, Sara Oyler-McCance, or your state coordinator at any time if you have questions.

Preservation methods

DNA can degrade quickly if it is wet and warm (enzymes in the tissue degrade the DNA quickly in wet warm conditions). The best way to stop enzymatic degradation is to put the tissue in alcohol and store it at -20C. Tissue can be stored in alcohol at room temperature but the cold temperature reduces evaporation of the alcohol and acts as a secondary preventative to degradation. Alternate methods are to get the tissue as cold as possible as soon as possible. It can then be transferred to alcohol at a later date. There is no degradation in tissue in live animals, and these can be transported to your facility for later processing.

Preserving tissue in alcohol:

- **Type of alcohol** - most DNA labs use 100% ethanol for analyses, but this is not readily available because it is also drinkable. Try to put the tissue into 90% isopropanol; it is not drinkable and can be purchased from just about any grocery, pharmacy, and even small stores in the first-aid section. 70% isopropanol is less desirable but can be used if it is the only alcohol available (Some tissues collected in the early 1800s were initially preserved in whisky). Do not use standard lab ethanol that has been denatured to make it undrinkable; the denaturing agents often interfere with downstream DNA analyses. Because alcohol evaporates, especially in warm temperatures and with some tubes, using the highest alcohol concentration will ensure tissue preservation even if there is some evaporation.
- **Preserving in alcohol** - The alcohol must soak into all of the tissue in order to deactivate the enzymes and stop degradation. In this process the alcohol will be diluted by liquid in the tissue. Therefore, ideally, the tissue should be put into a volume of alcohol that is 10 times the tissue volume. If it is put in less volume, then the alcohol can be poured out and fresh alcohol added after 24 hours. It can then be stored safely for long times at -20C or for short times at room temperature.
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- **Containers** - any clean container will do, but new, plastic, screw-top tubes (falcon tubes) are most convenient. These come in a variety of sizes. Smaller (Eppendorf) tubes are also excellent for small tissues
  and these come with screw caps also with o-rings that reduce leakage and evaporation. These are available from Fisher Scientific and other scientific suppliers.

15- 50 ml Falcon tubes: 1.5 ml Eppendorf tubes: 1.5 or 2 ml screw caps:

Fisher Cat No: NC9031845 Fisher Cat No: 05-402-18 Fisher Cat No: 02-682-558

To avoid contamination from one sample to another clean the scissors, razor blades, and tweezers in between taking each sample by wiping supplies with alcohol. This can be done easily with pre-packaged alcohol wipes purchased from grocery or pharmacy stores. Each wipe can be used once and disposed of. It is best to purchase a set of razor blades from a hardware store and use a fresh one for each sample, rather than risk cutting yourself in between taking samples. Be sure to store used razor blades in a sold container such as a can and dispose of them as hazardous waste.

- **Number of samples per container** - Put only one tadpole, toe clip, or other/multiple tissue fragments from one individual in each test tube. This is because DNA leaches into the alcohol and cross-contamination between samples can occur. Even tadpoles, metamorphs, or juveniles from the same egg mass should be in different containers.

- **All containers must be labeled. Pre-labeling tubes with numbers and recording data on separate sheets works well.** Print out a series of numbers and tape the numbers to the outside of the tube. You can also insert a piece of paper with the printed number inside the tube (Make sure the ink you use does not dissolve in alcohol).
- **Alcohol can dissolve the writing on the tube.** If the tube has a white block on the outside you can write on the tube with pencil, cover the pencil markings with tape so the writing will not be rubbed off, and the label will be secure. Often tubes do not have a white block and labels are written with a "sharpie". <u>However, the alcohol that is in the tube can also dissolve the writing on the tube.</u> You can do two things to limit this. First, wrap any brand of clear tape (or lab parafilm) tightly around the label. This will prevent incidental dissolving of the label. However, it will not prevent the writing from dissolving if the tube is soaked in alcohol. The second thing to do is to keep the tubes in such a way that they will not leak alcohol. Secure them in an upright position, or put individual tubes in zipped plastic bags and write the locality again on the outside of the sealed bags. Stretching parafilm around the seal between the cap and the tube will help prevent leakage. Alcohol resistant pens exist, but these are less resistant to 90% alcohols. Screw-cap tubes are better than the "flip-top" tubes, but both can leak. Most printer ink does not dissolve in alcohol, so pre-labeled tubes work well (tape the numbers to the outside of the tube).
- **Store the tissue in alcohol at -20C or in a freezer until shipment.**
- **Do not put the samples in formalin.** This dramatically reduces the kinds of DNA analyses that can be conducted.
- **Please call Anna Goebel, Sara Oyler-McCance, or your state coordinator at any time if you have questions.**

Labeling. Samples need to have the following information on the tube and/or on a separate sheet. A sample sheet is provided at the end of this document.

- Locality - GPS data is best (latitude and longitude is easiest for mapping), as well as population name (e. g. local drainage, etc.) and state. Elevation if possible.
- Collector – Names of individuals that collected the samples, these would be the individuals that have permits to collect.
- Date – date of collection. Written in a form that cannot be confused is best (e. g., 10 June 2015)
- Tissue type (e. g., "toe clip").
- Sex if known.
- Information on collection permits (under who's permit were the samples collected?)
- Any additional notes that could be relevant later (e. g., time of death for a dead toad, unusual collection methods).

Permits:

All collectors must have permits to collect animals within each state. This is because animals that were not collected with proper permits cannot be received by or analyzed by the University nor can the data

be published as many journals now require authors to include permit information within the publication. Note that multiple permits may be required (federal, state, and part permits). Your state collection coordinator can advise you on what permits are needed.

Samples shipped to the Fort Collins Science Center or any University must be tissue or dead toads. IACUC approval is not needed to receive dead animals or tissue for analyses.

Shipping samples:

Be sure to ship the tubes within a hard box or other hard container; samples that are shipped without protection can get crushed in the mail. It is a good idea to include paper towels or tissue within and surrounding the hard box to

absorb any leaked alcohol. Placing the wrapped box in a zip lock bag will add additional protection.

If possible, send copies of the sample data to both Anna Goebel and Sara Oyler-McCance.
Send all tissue samples to Sara Oyler-McCance at the address below.

Ship samples to:
Dr. Sara Oyler-McCance
USGS, Fort Collins Science Center
Center 2150 Centre Ave., Bldg C
Fort Collins CO 80526
970-226-9197 soyler@usgs.gov

Attention to:
Jennifer Fike, Lab Manger
USGS, Fort Collins Science
2150 Centre Ave., Bldg C
Fort Collins CO 80526
970-226-9199 fikej@usgs.gov

Feel free to use the sample data sheet below. If you already use a different data sheet please feel free to use your own and send us a copy of it instead.

Sample data sheet:

Sample_____

Species: *Anaxyrus boreas* Collector (Permits): _____

Name/ Number:_____

Date _____State _____Tissue Type _____

Locality description:

_____

_____

_____

Latitude_Longitude _____

Age Class (Adult, juvenile, tadpole)_____ Sex _____

Photo Taken? Yes No Altitude_____ SVL _____

Additional Collector (s):

_____

Comments:

_____

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Appendix 2. Samples used in the analysis.

Table N. Samples collected in 2014-2016 and previously collected samples for which nuclear DNA data were newly collected. Mitochondrial control region was analyzed for all samples. Mitochondrial DNA sequence data from previous analyses () were included also but not included in the table below. *Abbreviations E:Eggs, L:Larva, T:Tadpoles, M:Metamorph, TL:Toadlet also reported as Froglet, J:Juvenile, A:Adult, TC:Toe Clip, TAC:Tail Clip, B:Blood, LV:Liver.

Locality	**GPS**	**Map Datum**	**N**	**Sample Names**	**Original Name**	**Tissue Type***	**Collector as is available/agency**
Paunsaugunt Plateau, East Fork Sevier, Kane Co., UT	37.461104° N -112.3198745° W	unknown	4	920KaUT* 921KaUT* 922KaUT* 923KaUT*	Tank 3A Tank 3B Tank 3C Tank 3D	T, F	Sender: Paul Badame Observer: Wahweap
Paunsaugunt Plateau, Left Fork	37.489825° N	unknown	3	924KaUT*	Tank 4	T, F	Sender: Paul Badame
Kanab Creek, Kane Co., UT	-112.3378441° W			925KaUT* 926KaUT*	Tank 5A Tank 5B		Observer: Wahweap
Thousand Lake Mtn, Snow Lake, Wayne Co., UT	38.441698° N -111.461267° W	unknown	1	927WaUT*	UT1	T	Sender: Paul Badame Observers: MG,JS,ST
Boulder Mtn, Baker Spring, Wayne Co., UT	38.195173° N -111.5533867° W	unknown	1	928WaUT*	UT2	T	Sender: Paul Badame Observer: KKW
Allen Springs, Grouse Creeks, Box Elder Co., UT	41.538871° N -113.7751593° W	NAD 83	1	1179BEUT*	UTAS1A	T	Sender: Paul Badame
Keg Springs, Grouse Creeks, Box Elder Co., UT	41.585366° N -113.790166° W	NAD 83	2	1188BEUT 1189BEUT*	UTKS6A UTKS6B	E	Sender: Paul Badame
Chehalis River, Lewis Co., WA	46.54089° N -123.29823° W	unknown	2	929LeWA* 930LeWA*	CH806 CH807	TI - T	Sender: Julie Tyson Observer: Stephanie Lewis
Eastfork Satsop River, Mason Co., WA	47.11967° N -123.33917° W	unknown	1	931MaWA*	CH88	TC - J	Sender: Julie Tyson Observer: Caitlin McIntyre
Wynoochee River, Grays Harbor Co., WA	47.004070° N -123.649957° W	unknown	2	1193GHWA* 1194GHWA*	CH1679 CH1623	T	Sender: Julie Tyson Observers: Julie Tyson, Nicholas Wegener
Spokane Co., WA	47.69972222° N -117.5488889° W	unknown	1	1197SpWA*	3139-098	TC - A	Sender: Stacy James Observer: Stacy James
Pend Oreille Co., WA	48.63795° N -117.420967° W	unknown	1	1200PEWA*	WA 3	TC	Sender: C. Loggers Observer: Michele Isaksen
Clear Creek, Bonneville Co., ID	43.183527° N -111.3671059° W	NAD 83	1	936BnID*	BT-1502-6C	TC - T	Sender: Charlotte Snoberger Observer: D. Green
Clear Creek, Bonneville Co., ID	43.183708° N -111.3669101° W	NAD 83	1	938BnID	BT-1502-8A	TC - T	Sender: Charlotte Snoberger Observer: D. Green
Salamander Lake, Clark Co., ID	44.551967° N -111.908455° W	NAD 83	3	941ClID* 942ClID* 943ClID*	BT-1502-2C BT-1502-3A BT-1502-5A	TC	Sender: Charlotte Snoberger Observer: SND
Owyhee River Campground, Owyhee Co., ID	42.59246667° N -116.98295° W	unknown	3	968OwID* 969OwID* 970OwID*	ID1 ID2 ID3	TI - T	Sender: Caren Goldberg Observer: Matt Smith
Hot Creek Canyon, Nye Co., NV	38.538195° N -116.458521° W	unknown	5	978NyNVH* 979NyNVH* 981NyNVH* 982NyNVH* 986NyNVH*	NV2 NV3 NV5 NV6 NV10	TC - A	Sender:Kevin Guadalupe
RailRoad Valley Lockes Ranch, Nye Co., NV	38.554023° N -115.774163° W	unknown	5	987NyNVR* 991NyNVR* 992NyNVR* 993NyNVR* 995NyNVR*	NV1b NV5b NV6b NV7b NV9b	TC – A, J	Sender: Kevin Guadalupe Observers: Jennie Grill (Volunteer), Kevin Guadalupe (NDOW), Chris Burg (NDOW)
Lamoille Canyon, Elko Co., NV	40.629594° N -115.3686748° W	NAD 83	1	997ElNVL*	NV15LC1	TC	Sender: Jeff Petersen Observer: NDOW-Jessica

Lamoille Canyon, Elko Co., NV	40.643554° N -115.378056° W	NAD 83	2	998ElNVL* 999ElNVL*	NV15LC2 NV15LC3	TC	Sender: Jeff Petersen Observer: NDOW-Jessica
Ruby Valley Drainage, Robinson Lake, Elko Co., NV	40.728412° N -115.2810427° W	NAD 83	1	1000ElNVB*	NV15RL1	TC	Sender: Jeff Petersen Observer: NDOW-Petersen
Ruby Valley Drainage, Robinson Lake, Elko Co., NV	40.729116° N -115.2817114° W	NAD 83	1	1001ElNVB*	NV15RL2	TC	Sender: Jeff Petersen Observer: NDOW-Petersen
Ruby Valley Drainage, Robinson Lake, Elko Co., NV	40.728606° N -115.2789773° W	NAD 83	1	1002ElNVB*	NV15RL3	TC	Sender: Jeff Petersen Observer: NDOW-Petersen
McCoy Ranch, Pershing Co., NV	40.087578° N -117.6118767° W	NAD 83	1	1117PeNV*	M-1	TI - T	Sender: Kris Urquhurt
Dixie Meadows, Churchill Co., NV	39.794088° N -118.0631486° W	NAD 83	1	1121CuNVD*	D-2	TC - J	Sender: Kris Urquhurt
Dixie Meadows, Churchill Co., NV	39.7938° N -118.0630858° W	NAD 83	1	1122CuNVD*	D-4	TC - J	Sender: Kris Urquhurt
Dixie Meadows, Churchill Co., NV	39.79379° N -118.0631908° W	NAD 83	1	1123CuNVD*	D-5	TC - J	Sender: Kris Urquhurt
Bonneville Drainage, Rock Springs, Elko Co., NV	41.723518° N -114.376414° W	NAD 83	2	1124ElNVS 1125ElNVS*	NV16RCS1 NV16RCS2	TC	Sender: Jeff Petersen
Parker Ranch, Oasis Valley, Nye	36.964969° N	NAD 83	1	1127NyNVOn	NVPR1	TC - A	Sender: Kevin Guadalupe
Co., NV (*nelsoni*)	-116.7235808° W		0	1128NyNVOn 1129NyNVOn 1130NyNVOn 1131NyNVOn* 1132NyNVOn 1133NyNVOn 1134NyNVOn* 1135NyNVOn 1136NyNVOn*	NVPR2 NVPR3 NVPR4 NVPR5 NVPR6 NVPR7 NVPR8 NVPR9 NVPR10		Observers: Alejandra Cortes, Kevin Guadalupe, Gregory Munson
Ash Meadows National Wildlife Refuge, Five Springs, Nye Co., NV	36.464589° N -116.3240483° W	NAD 83		1137NyNV5	NVAM1	TC - A	Sender: Kevin Guadalupe Observers: Alejandra Cortes, Gregory Munson
Ash Meadows National Wildlife Refuge, Five Springs, Nye Co., NV	36.464558° N -116.3239705° W	NAD 83		1138NyNV5	NVAM2	TC - A	Sender: Kevin Guadalupe Observers: Alejandra Cortes, Gregory Munson
Ash Meadows National Wildlife Refuge, Five Springs, Nye Co., NV	36.464564° N -116.3238476° W	NAD 83	1	1139NyNV5*	NVAM3	TC - A	Sender: Kevin Guadalupe Observers: Alejandra Cortes, Gregory Munson
Ash Meadows National Wildlife Refuge, Five Springs, Nye Co., NV	36.464503° N -116.324875° W	NAD 83	4	1140NyNV5 1141NyNV5* 1142NyNV5* 1143NyNV5	NVAM4 NVAM5 NVAM6 NVAM7	TC -A	Sender: Kevin Guadalupe Observers: Alejandra Cortes, Gregory Munson
Ash Meadows National Wildlife Refuge, Five Springs, Nye Co., NV	36.464601° N -116.3253652° W	NAD 83	3	1144NyNV5 1145NyNV5* 1146NyNV5	NVAM8 NVAM9 NVAM10	TC - A	Sender: Kevin Guadalupe Observers: Alejandra Cortes, Gregory Munson
Carson River Internal Basin, Churchill Co., NV	39.100206° N -119.7279403° W	NAD 83	4	1147ChNVC* 1148ChNVC 1149ChNVC* 1150ChNVC*	NV16CR1 NV16CR2 NV16CR3 NV16CR4	TC	Sender: Jeff Petersen Observer: NDOW-Allen
Newark Valley Internal Basin, White Pine Co., NV	39.624532° N -115.7649405° W	NAD 83	4	1151WPNVN* 1152WPNVN* 1153WPNVN 1154WPNVN*	NV16NW1 NV16NW2 NV16NW3 NV16NW4	TC	Sender: Jeff Petersen Observer: NDOW-Korell
Frog Lake, Mt Hood National Forest, Wasco Co., OR	45.21673639° N -121.6835494° W	NAD 83	2	1155WaOR* 1156WaOR	Corkran 1 Corkran 2	TI - T	Sender: Char Corkran
Fryingpan Lake, Mt Hood National Forest, Clackamas Co., OR	45.18340639° N -121.7834836° W	NAD83	1	1158ClOR*	Corkran 4	T	Sender: Char Corkran
Crosswater #13 Fairway Pond, Deschutes Co., OR	43.8586° N -121.44285° W	unknown	1	953DeOR*	OR5	J	Sender: ANBO Observer: Jay Bowerman
Tillamook Co., OR	45.45006389° N -123.8000556° W	NAD 83	2	1165TiOR 1166TiOR*	Site 1 Group 3A Site 1 Group 3B	L	Sender: Chris Rombough Observer: Jay Bowerman

Tillamook Co., OR	45.46668056° N -123.8001556° W	NAD 83	1	1168TiOR*	Site 2B	L	Sender: Chris Rombough Observer: Jay Bowerman
Tillamook Co., OR	45.60000833° N -123.6000722° W	NAD 83	2	1169TiOR* 1170TiOR	7-2-A1 7-2-A2	L	Sender: Chris Rombough Observer: Jay Bowerman
Lake, 2.5 miles ENE of Mount Bradley, Madison Co., MT	45.53424016° N -111.9278722° W	unknown	1	945MaMT*	BUBO_06_04 7_017A1	TC	Sender: Bryce Maxell
Hagan Pond, 4.0 miles SW of Garrison, Powell Co., MT	46.47907342° N -112.8836517° W	unknown	1	946PoMT*	BUBO_18708 A1-C1	TC	Sender: Bryce Maxell
Haugan, pond SE of Sauenac Nursery, Mineral Co., MT	47.3841° N -115.39509° W	unknown	1	948MiMT*	BUBO_MI#1 712	TC	Sender: Bryce Maxell
Gigantic wetland, 2.5 miles SE of Corey Springs, Gallatin Co., MT	44.77478522° N -111.1408218° W	unknown	1	971GaMT*	BUBO_06_04 4A1	TC	Sender: Bryce Maxell
Inactive beaver pond, 2.1 miles ENE of Elk Park Fire Hall, Jefferson Co., MT	46.0980286° N -112.3771548° W	unknown	1	972JeMT*	BUBO_06_05 2A1	TC	Sender: Bryce Maxell
Wetland, 100m SE of SW corner of Doney Lake, Powell Co., MT	46.44512002° N -112.9558342° W	unknown	1	973PoMT*	BUBO_15_40 0_004A1-2	TC	Sender: Bryce Maxell
Pond just below dam on Painted Rocks Reservoir, Ravalli Co., MT	45.72030252° N -114.2801192° W	unknown	1	974RaMT*	BUBO_15_42 8_002A1-2	TC	Sender: Bryce Maxell
Delta pond on Tutshi Lake, where Tutshi River flows in. Breeding site, Tutshi Lake, Stikine Co., BC	59.81913° N -134.80627° W	unknown	1	1218TuBC*	BS1	TC - M	Sender: Brian Slough Observer: Brian Slough
Summit Lake, Sunshine Coast Co., BC	50.146137° N -117.6061775° W	NAD 83	1	1224SuBC*	BC 1	S – A?	Sender: Jakub Dulisse Observer: Jakub Dulisse
Thayer Lake, Hoonah-Angoon Co., AK	57.62523° N -134.46269° W	unknown	1	1222TLAK*	ANM5 A	TC?	Sender: Tallmon
Swamp Lake, Park Co., WY	44.839169° N -109.5765137° W	NAD 83	3	957PaWY* 959PaWY* 961PaWY*	G-2015-772 G-2015-774 G-2015-776	TC, TI – A, L, M	Sender: Charlotte Snoberger Observer: SA
Fall Creek Albany Co., WY	41.27614° N -106.2259° W	WGS 84	1	964AlWY*	UW2015-339	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD
Fall Creek Albany Co., WY	41.27662° N -106.2228° W	WGS 84	1	965AlWY*	UW2015-416	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD
Ryan Park Albany Co., WY	41.31615° N -106.4832° W	WGS 84	1	967AlWY*	UW2015-487	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD
North Fork Popo Agie River, Fremont Co., WY	42.764453° N -109.1135218° W	NAD 27		1004FrWY	G-2015-819	TC - A	Sender: Charlotte Snoberger Observer: David Brady, Cory Abrams (WGFD)
North Fork Popo Agie River, Fremont Co., WY	42.76396° N -109.113659° W	NAD 27		1005FrWY	G-2015-833	TC - J	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Fremont Co., WY	42.78784° N -109.1026271° W	unknown	1	1006FrWY*	Gen14-05	TC - J	Sender: Charlotte Snoberger
Fremont Co., WY	42.787544° N -109.1076244° W	unknown		1007FrWY	Gen14-12	TC - M	Sender: Charlotte Snoberger
Fremont Co., WY	42.786099° N -109.0959317° W	unknown		1008FrWY	Gen14-20	TC - J	Sender: Charlotte Snoberger
Fremont Co., WY	42.785925° N -109.0909366° W	unknown	1	1009FrWY*	Gen14-22	TC - M	Sender: Charlotte Snoberger
Fremont Co., WY	42.788045° N -109.1112281° W	unknown	1	1010FrWY*	Gen14-25	TC - M	Sender: Charlotte Snoberger
Fremont Co., WY	42.78833° N -109.112711° W	unknown		1011FrWY	Gen14-46	TC - J	Sender: Charlotte Snoberger
Fremont Co., WY	42.786858° N -109.103733° W	unknown		1012FrWY	Gen14-48	TC - M	Sender: Charlotte Snoberger
Albany Co., WY	41.23247° N -106.31654° W	NAD83	1	1013AlWY*	UW2015-587	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD
Albany Co., WY	41.23332° N -106.319219° W	NAD 83	1	1014AlWY*	UW2015-588	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD

Albany Co., WY	41.233311° N -106.318997° W	NAD 83		1015AlWY	UW2015-589	TC	Sender: Wendy Estes-Zumpf Observer: WYNDD
Chall Creek, Lincoln Co., WY	43.026791° N -110.4438772° W	NAD 83	1	1018LiWY*	BT1407-10H	TC - A	Sender: Charlotte Snoberger Observer: L. Yandow
Indian Trail Ponds, Sublette Co., WY	42.91618° N -110.4307° W	Unknown		1019SuWY	BT1407-1G	TC - A	Sender: Charlotte Snoberger Observer: M. Osipchuk
Indian Trail Ponds, Sublette Co., WY	42.9137° N -110.42957° W	unknown		1021SuWY	BT1407-5G	TC - A	Sender: Charlotte Snoberger Observer: L. Yandow
Snider Basin, Sublette Co., WY	42.45928° N -110.506727° W	unknown		1022SuWY	BT1407-9E	TC - A	Sender: Charlotte Snoberger Observer: M. Lillrose
Snider Basin, Sublette Co., WY	42.45904° N -110.50932° W	WGS 84		1023SuWY	BT-1501-10B	TC - A	Sender: Charlotte Snoberger Observer: N.D. Turner
Upper Green River, Sublette Co., WY	43.345577° N -110.0054573° W	NAD 83		1024SuWY	BT-1501-1A	TC - A	Sender: Charlotte Snoberger Observer: N.D. Turner
Big Twin, Sublette Co., WY	43.165679° N -110.1648952° W	NAD 83	1	1025SuWY*	BT-1501-1C	TC - A	Sender: Charlotte Snoberger Observer: D. Hoshizaki
Tosie Creek, Sublette Co., WY	43.347185° N -110.0614591° W	NAD 83	1	1026SuWY*	BT-1501-1D	TC - T	Sender: Charlotte Snoberger Observer: D. Hoshizaki
Upper Green River, Sublette	43.346528° N	NAD 83		1027SuWY	BT-1501-2A	TC - A	Sender: Charlotte Snoberger
Co., WY	-110.0081068° W						Observer: N.D. Turner
Tosie Creek, Sublette Co., WY	43.347417° N -110.0611471° W	NAD 83		1028SuWY	BT-1501-4B	TC - T	Sender: Charlotte Snoberger Observer: N.D. Turner
Big Twin, Sublette Co., WY	43.165769° N -110.1648694° W	NAD 83		1029SuWY	BT-1501-4C	TC - A	Sender: Charlotte Snoberger Observer: D. Hoshizaki
Indian Trail Ponds, Sublette Co., WY	42.915014° N -110.429924° W	NAD 83		1030SuWY	BT-1501-4G	TC - A	Sender: Charlotte Snoberger Observer: D. Hoshizaki
Indian Trail Ponds, Sublette Co., WY	42.914139° N -110.4296135° W	NAD 83		1031SuWY	BT-1501-5E	TC - A	Sender: Charlotte Snoberger Observer: N.D. Turner
Tosie Creek, Sublette Co., WY	43.347758° N -110.0609938° W	NAD 83		1032SuWY	BT-1501-6B	TC - A	Sender: Charlotte Snoberger Observer: N.D. Turner
Snider Basin, Sublette Co., WY	42.45886° N -110.50346° W	WGS 84		1033SuWY	BT-1501-6C	TC - A	Sender: Charlotte Snoberger Observer: N.D. Turner
Snider Basin, Sublette Co., WY	42.45848° N -110.50248° W	WGS 84	1	1034SuWY*	BT-1501-8C	TC - T	Sender: Charlotte Snoberger Observer: N.D. Turner
Red Castles, Sublette Co., WY	42.706695° N -110.5121439° W	NAD 83		1035SuWY	BT-1501-9F	TC - T	Sender: Charlotte Snoberger Observer: D. Hoshizaki
North Dollar Lake, Sublette Co., WY	43.347218° N -110.0067013° W	NAD 83		1036SuWY	BT1507-1I	TC - T	Sender: Charlotte Snoberger Observer: M. Lillrose
Snider Basin, Sublette Co., WY	42.95904° N -110.5093° W	unknown		1037SuWY	BT1507-2C	TC - A	Sender: Charlotte Snoberger Observer: M. Osipchuk
North Dollar Lake, Sublette Co., WY	43.345485° N -110.005212° W	NAD 83		1038SuWY	BT1507-2F	TC - T	Sender: Charlotte Snoberger Observer: M. Lillrose
Upper Wagon 3, Sublette Co., WY	43.41594° N -110.0491944° W	NAD 83	1	1041SuWY*	BT1507-3E	TC - A	Sender: Charlotte Snoberger Observer: M. Lillrose
Slide Creek Pond, Sublette Co., WY	43.197025° N -110.2590918° W	NAD 83		1042SuWY	BT1507-4A	TC - A	Sender: Charlotte Snoberger Observer: M. Lillrose
Trail near Pacific Creek, Teton Co., WY	44.009871° N -110.2359118° W	NAD 27		1044TeWY	G-2015-555	TC - A	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Enos Creek, Teton Co., WY	43.975119° N -110.2580512° W	NAD 27	1	1045TeWY*	G-2015-569	TC - M	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Enos Creek, Teton Co., WY	43.986413° N -110.2984255° W	NAD 27		1046TeWY	G-2015-579	TC - L	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Crandall Creek, Park Co., WY	44.834175° N -109.6730912° W	NAD 27	1	1049PaWY*	G-2015-683	TC - A	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
North Fork Popo Agie River, Fremont Co., WY	42.766583° N -109.0850532° W	NAD 27	1	1051FrWY*	G-2015-713	TC - J	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)

Cathedral Lake, Fremont Co., WY	42.816913° N -109.1369172° W	NAD 27		1052FrWY	G-2015-714	TC - L	Sender:Charlotte Snoberger Observers: Cory Abrams, Cara Thompson (WGFD)
Enos Creek, Teton Co., WY	43.986582° N -110.298199° W	NAD 27		1054TeWY	G-2015-718	TC - M	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Green River, Sublette Co., WY	43.075353° N -110.0782828° W	NAD83		1056SuWY	G-2015-722	TC - T	Sender: Charlotte Snoberger Observer: DFG Pindale
North Fork Popo Agie River, Fremont Co., WY	42.761624° N -109.0960338° W	NAD 27		1059FrWY	G-2015-811	TC - A	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Smith Lake, Fremont Co., WY	42.807763° N -109.1176359° W	NAD 27		1061FrWY	G-2015-815	TC - A	Sender: Charlotte Snoberger Observers: Cory Abrams, Cara Thompson (WGFD)
Middle Creek, Fremont Co., WY	42.813004° N -109.1258797° W	NAD 27		1062FrWY	G-2015-817	TC - A	Sender: Charlotte Snoberger Observers: Cory Abrams, Cara Thompson (WGFD)
Pacific Creek, Teton Co., WY	43.974335° N -110.3671387° W	NAD 27	1	1064TeWY*	G-2015-832	TC - A	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Pacific Creek, Teton Co., WY	43.978382° N -110.3486177° W	NAD 27	1	1065TeWY*	G-2015-834	TC - M	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
Enos Lake, Teton Co., WY	43.992307° N -110.2512149° W	NAD 27		1066TeWY	G-2015-835	TC - M	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
North Fork Popo Agie River, Fremont Co., WY	42.762741° N -109.1101518° W	NAD 27	1	1067FrWY*	G-2015-838	TC - J	Sender: Charlotte Snoberger Observers: David Brady, Cory Abrams (WGFD)
LaBarge Creek, Bridger-Teton National Forest, Lincoln Co., WY	42.475737° N -110.6172775° W	NAD 83	1	1070LiWY*	2014-26	TC - A	Sender: Wendy Estes-Zumpf
Upper LaBarge Creek, Bridger-Teton National Forest, Lincoln Co., WY	42.481398° N -110.6269765° W	NAD 83		1071LiWY	2014-28	TC - A	Sender: Wendy Estes-Zumpf
Hams Fork, Bridger-Teton National Forest, Lincoln Co., WY	42.207305° N -110.7371842° W	NAD 83	1	1072LiWY*	2014-31	TC - A	Sender: Wendy Estes-Zumpf
LaBarge Creek,Bridger-Teton National Forest, Lincoln Co., WY	42.485671° N -110.6663014° W	NAD 83	1	1073SuWY*	2014-34	TC - A	Sender: Wendy Estes-Zumpf
Hams Fork, Bridger-Teton National Forest, Lincoln Co., WY	42.207299° N -110.7344585° W	NAD 83	1	1074LiWY*	2014-45	TC - J	Sender: Wendy Estes-Zumpf
Bridger-Teton National Forest, Lincoln Co., WY	42.478387° N -110.6021144° W	NAD 83		1075LiWY	2014-50	TC - A	Sender: Wendy Estes-Zumpf
Indian Trail Ponds,,Bridger-Teton National Forest, Sublette Co., WY	42.914886° N -110.4295086° W	NAD 83		1087SuWY	BT-1403.10C	TC - A	Sender: Wendy Estes-Zumpf
Indian Trail Ponds, Bridger-Teton National Forest, Sublette Co., WY	42.915012° N -110.4295197° W	NAD83		1088SuWY	BT-1403.1C	TC - A	Sender: Wendy Estes-Zumpf
Indian Trail Ponds, Bridger-Teton National Forest, Sublette Co., WY	42.914607° N -110.4296092° W	NAD83		1089SWY	BT-1403.2D	TC - A	Sender: Wendy Estes-Zumpf
Indian Trail Ponds, Bridger-Teton National Forest, Sublette Co., WY	42.915826° N -110.4301861° W	NAD83		1090SuWY	BT-1403.3D	TC - A	Sender: Wendy Estes-Zumpf
North Horse Creek, Bridger-Teton National Forest, Sublette Co., WY	42.927664° N -110.5112615° W	NAD83	1	1091SuWY*	BT-1403.7E	TC - A	Sender: Wendy Estes-Zumpf

Indian Trail Ponds, Bridger-Teton National Forest, Sublette Co., WY	42.916859° N -110.4314508° W	NAD83		1093SuWY	BT-1403.9C	TC - A	Sender: Wendy Estes-Zumpf
Blackrock Oxbow, Bridger-Teton National Forest, Teton Co., WY	43.83463° N -110.3518004° W	NAD83		1094TeWY	BTNZ-1401-A2	TC - A	Sender: Wendy Estes-Zumpf
Buffalo Fork, Bridger-Teton National Forest, Teton Co., WY	43.846311° N -110.3367699° W	NAD83		1097TeWY	BTNZ-1401-G5	TC - J	Sender: Wendy Estes-Zumpf
North Fork Spread Creek, Bridger-Teton National Forest, Teton Co., WY	43.734885° N -110.2088326° W	NAD83		1098TeWY	BTNZ-1402-D10	TC - A	Sender: Wendy Estes-Zumpf
North Fork Spread Creek, Bridger-Teton National Forest, Teton Co., WY	43.736269° N -110.209696° W	NAD83		1100TeWY	BTNZ-1402-G2	TC - J	Sender: Wendy Estes-Zumpf
Shoal Falls, Bridger-Teton National Forest, Teton Co., WY	43.33939° N -110.4076492° W	NAD83		1101TeWY	BTNZ-1402-G5	TC - A	Sender: Wendy Estes-Zumpf
Buffalo Fork, Bridger-Teton National Forest, Teton Co., WY	43.844846° N -110.3388139° W	NAD83		1102TeWY	BTNZ-1402-H1	TC - J	Sender: Wendy Estes-Zumpf
North Buffalo Fork, Bridger-Teton National Forest, Teton Co., WY	43.894493° N -110.4076492° W	NAD83		1109TeWY	UW2014-537	T	Sender: Wendy Estes-Zumpf
Hams Fork, Bridger-Teton National Forest, Lincoln Co., WY	42.209006° N -110.7365472° W	NAD83		1110LiWY	2014-32	TC - J	Sender: Wendy Estes-Zumpf
HBR-Pond 1 Hochback/ RIM 2, Bridger-Teton National Forest, Sublette Co., WY	43.133228° N -110.2212678° W	NAD83		1205SuWY	BT-1407-7A	TC	Sender: Wendy Estes-Zumpf Observers: L. Hoyle, T. Erickson
HBR Pond 2, Bridger-Teton National Forest, Sublette Co., WY	43.133186° N -110.2230511° W	NAD83		1212SuWY	BT-1407-5J	TC	Sender: Wendy Estes-Zumpf Observers: L. Hoyle, T. Erickson
HBR-Pond 1 Hochback/ RIM 2, Bridger-Teton National Forest, Sublette Co., WY	43.133142° N -110.2218836° W	NAD83		1214SuWY	BT-1407-7J	TC	Sender: Wendy Estes-Zumpf Observers: L. Hoyle, T. Erickson
RIM 2, Bridger-Teton National Forest, Sublette Co., WY	43.140811° N -110.2375877° W	NAD83		1215SuWY	BT-1407-8B	TC	Sender: Wendy Estes-Zumpf Observers: L. Hoyle, T. Erickson
BT3 3 North Horse Creek, Bridger-Teton National Forest, Sublette Co., WY	42.928546° N -110.5109727° W	NAD83		1217SuWY	BT-1407-9C	TC - J	Sender: Wendy Estes-Zumpf Observers: J. Behrens, T. Erickson
Big Meadow Rocky Mountain National Park, Grand Co., CO	40.317868° N -105.8045734° W	unknown		1242GrCO 1243GrCO	Hcor 16 Hcor 18	TC, TI	Sender: Harry Crockett Observer: Harry Crockett
East Vail, Rocky Mountain National Park, Eagle Co., CO	39.643238° N -106.3177173° W	unknown		1246EaCo	Hcor 23	TC, TI	Sender: Harry Crockett Observer: Harry Crockett
Buzzard Creek, Rocky Mountain National Park, Mesa Co., CO	39.194146° N -107.6219667° W	unknown		1247MeCO	Hcor 27	TC, TI	Sender: Harry Crockett Observer: Harry Crockett
Triangle Pass, Rocky Mountain National Park, Gunnison Co., CO	38.998007° N -106.9358263° W	unknown		1251GuCO	Hcor 37	TC, TI -	Sender: Harry Crockett Observer: Harry Crockett
Fourmile Creek, Rocky Mountain National Park, Chaffee Co., CO	38.969559° N -106.143229° W	unknown		1254ChCO	Hcor 38	TC, TI	Sender: Harry Crockett Observer: Harry Crockett
Samples included in previous analyses (Goebel 2000, Goebel et al., 2009)							
Darwin Canyon, Inyo Co., CA	36.34067° N -117.49346° W	NAD 27	1	012InCA*			D.A.Good
Corral Hollow Road, Alameda Co., CA	37.64122° N -121.59057° W	NAD 27	1	019AmCA*			R.L.Seib

Agua Blanca Creek at 1145Æ 0.3 mi W junction Piru Creek, 9.0 air mi NNE of Piru, Ventura Co., CA	34.54149° N -118.76675° W	NAD 27	1	023VeCAh*			S.Sweet
Lost Lake, Indian Peaks Wilderness, near Eldora, Boulder Co., CO	39.9507° N -105.61772° W	unknown	1	100BoCO*		TL	Collector: Steve Corn
S.E. Entrance of Yellowstone National Park, Teton Co., WY	44.13412° N -110.6635° W	unknown	1	106TeWY*		M, TL	Collector: Steve Corn
Santa Monica Mts, AOR Yerba Buena Rd., 5.3 mi E jct Cotharin Rd. SE of Thousand Oaks, Ventura Co., CA	34.10727° N -118.90266° W	NAD 27	1	121VeCA*		J	W.B.Jennings
Ballard Canyon Rd. 3.2 mi S. Los Olivos, Santa Barbara Co., CA	34.63817° N -120.13983° W	NAD 27	1	135SBCAh*		J	S. Sweet
Ballard Canyon Rd. 4.2 mi S. Los Olivos, Santa Barbara Co., CA	34.62488° N -120.14714° W	NAD 27	1	136SbCAh*		J	S. Sweet
Lost Lake, Rocky Mountain National Park, Larimer Co., CO	40.50783° N -105.5994° W	unknown	1	138LaCO*		A	Collectors: Steve Corn/AMG/Mike Jennings
Kettle Tarn, Rocky Mountain National Park, Larmier Co., CO	40.49777° N -105.5994° W	Unknown	1	154LaCO*		B	Collectors: Steve Corn/AMG/Mike Adams
Crystal Springs, Amargosa River Valley, Nye Co. NV	36.98456° N -116.75972° W	unknown	2	167NyNVOn* 168 NyNVOn*		J - T	?
Lower Whiterock Basin, Gunnison Co., CO	38.99987° N -106.9364° W		1	231GuCO*		T	Collector: Steve Corn/AMG
Triangle Pass, White Rock Basin off Copper Creek, 2km from Gothic, Gunnison Co., CO	38.99987° N -106.9364° W		1	233GuCO*		T	P.S.Corn, A.M.Goebel
2 mi N of three Creeks Lake, Deschutes Co., OR	44.13171° N -121.6278° W		1	246DeOR*		J	Collectors: A.Bloustein, S.Walls, J.Kiesecker, B.Edmund
1 mi NW Three Creeks Lake, Deschutes Co., OR	44.10777° N -121.63474° W		3	251DeOR* 253DeOR* 254DeOR*		M	Collectors: A.Bloustein, S.Walls, J.Kiesecker, B.Edmund
Far side Lost Lake, Rocky Mountain National Park, Larimer Co., CO	40.50783° N -105.5994° W		1	257LaCO*		M, TL	Steve Corn
SW Butte, Twin Lakes, Beaverhead National Forest, Beaverhead Co., MT	45.41195° N -113.69529° W	NAD 27	1	261BeMT*		A	C.Carey
China Creek, Craig Mt., Nez Perce Co., ID	46.06734° N -116.75398° W		1	272NPID*		B - A	Collector: Frances Cassirer
Little Indian Valley, Eldorado National Forest, Alpine Co., CA	38.59433° N -119.93094° W	NAD 27	1	288AlCAh*		T	D.L.Martin
Stanislaus N. E. Tryon Meadow, Alpine Co., CA	38.50823° N -119.79459° W	NAD 27	1	293AlCAc*			D.L.Martin
Kane Co., UT	37.47215° N -112.30697° W		2	295KaUT* 297KaUT*		B - A	Steve Corn?
0.95 mi, below Clear Creek, Off HWY 40, Clear Creek Co., CO	39.70336° N -105.78854° W		1	321CCCO*		B - A	Collectors: Steve Corn/AMG
Buckhorn Spring, 0.8 mi S of Deep Springs College, Deep Springs Valley, Inyo Co., CA	37.2628° N -118.0337° W	NAD 27	2	326InCAe* 327InCAe*	MVZ 142946 MVZ 142947	LI	?
Sonora Pass, meadows below Levitt, W. side, Mono Co., CA	38.32522° N -119.55123° W	NAD 27	3	328MoCAc* 329MoCAc* 330MoCAc*	MVZ 164900 MVZ 164901 MVZ 164902	LI	?
East Fork of Bear River, Summit Co., UT	40.85147° N -110.74263° W	NAD 27	1	332SuUT*	USNM211041	LI	D. Ross
Vancouver Isle, BC	49.77615° N -125.72923° W		3	354VaBC* 355VaBC* 356VaBC*		T	Collector: Heather Way

Red Butte Canyon, Grouse Creek Mtn, Box Elder Co., UT	41.658066° N -113.8523519° W		2	400BEUT* 401BEUT*		B	Collectors: D.A. Ross, T. Hansen
Upper Rocky Pass spring, Grouse Creek Mtns., Box Elder Co., UT	41.53961° N -113.75295° W		3	404BEUT* 405BEUT* 407BEUT*		M	Collectors: D.A. Ross, T. Hansen
Stock Pond, Walton Gulch, Curtis Ridge, Cache Co., UT	41.6092818° N -111.4961243° W Estimated**		1	408CaUT*		B	Collector: D.A.Ross
Mt. St. Helens, Obscurity Lake, Skamania Co., WA	46.3335° N -122.1076° W		4	410SkWA* 412SkWA* 413SkWA* 415SkWA*		B - A	Collector: Steve Corn
Fern Creek, Mineral Co., CO	37.6803° N -107.07042° W		1	422MiCO*		B - A	Collector: John Goettle
500 m w of Pine Bar Camp, Tincup Creek, Caribou Co., ID	42.726467° N -111.662032° W		2	441CaID* 444CaID*			Collector: Stephen Burton, (given to AMG by C. Peterson)
Trout Creek, Mineral Co., CO	37.64742° N -107.08961° W		1	467MiCO*		M	Collector: Terry Ireland
Kane Co., UT	37.463176° N -112.3183053° W		2	491KaUT* 493KaUT*			Collector: Steve Corn
Kramis Pond (next to campground at Como Lake), Ravalli Co., MT	46.0678° N -114.2444° W		1	496RaMT*		A	Collector: Steve Corn
Missouri Mine, Boise Co., ID	43.9982° N		1	530BoID*		M	Collector: Ed Wessman
	-115.8551° W						
Grouse Creek, Washington Co., ID	44.34249° N -117.18717° W		4	538WaID* 539WaID* 540WaID* 543WaID*		M	Collector: Ed Wessman
Rock Springs, Rock Creek, Thousand Springs Creek, Bonneville Basin, Elko Co., NV	41.7233° N -114.3786° W		1	549ElNV*		TO	Collector: Peter Hovingh
S. End of Bull Trout Lake, Boise Co., ID	44.29588° N -115.252° W		1	557BoID*		M	Collector: Ed Wessman
Chickamon River, Chickamon Co., Alaska	55.8635° N -130.8481° W		1	564ChAK*			Collector: Keith Pahlke
Logan Pass, Glacier National Park, Glacier Co., MT	48.68069° N -113.71087° W		1	586GlMT*	USNM-21107		Collectors: Steve Corn/S. K. Megan
3 mi S Warner Springs, San Diego Co., CA	33.23668° N -116.63009° W	NAD 27	3	602SDCAh* 605SDCAh* 607SDCAh*			E.Gergus
Upper Rocky Passpond, Box Elder Co., UT	41.529391° N -113.76495° W		2	644BEUT* 645BEUT*			?
Mud Basin Spring, Box Elder Co., UT	41.522628° N -113.7790905° W		2	647BEUT* 650BEUT*		T	?
Triangle Pass, Gunnison Co., CO	38.99987° N -106.9364° W		1	704GuCO*		A	Collectors: Nick Gallowich, Jessica Divens
Pole Creek, Grand Co., CO	39.9829° N -105.90313° W		1	757GrCO*		B	?
Trout Creek, Larimer Co., CO	40.95975° N -105.66196° W		1	816LaCO*		T	?
West Trout Creek, Hinsdale Co., CO	37.64087° N -107.1376° W		1	884HiCO*			?
Muddy Pass Creek, Jackson Co., CO	40.38829° N -106.59116° W		1	891JaCO*		TO	?
North Fork Morrison Creek, Routt Co., CO	40.17488° N -106.68398° W		1	895RoCO*		T	?
Torso Creek, Routt Co., CO	40.85485° N -106.71958° W		1	896RoCO*		T	?
Collegiate Peaks Campground, Chaffee Co., CO	38.8115° N -106.31505° W		1	917ChCO*		T	?`

Appendix 3. Network analyses of mtDNA haplotypes. Network analyses were similar to those described by Switzer et al (2009).

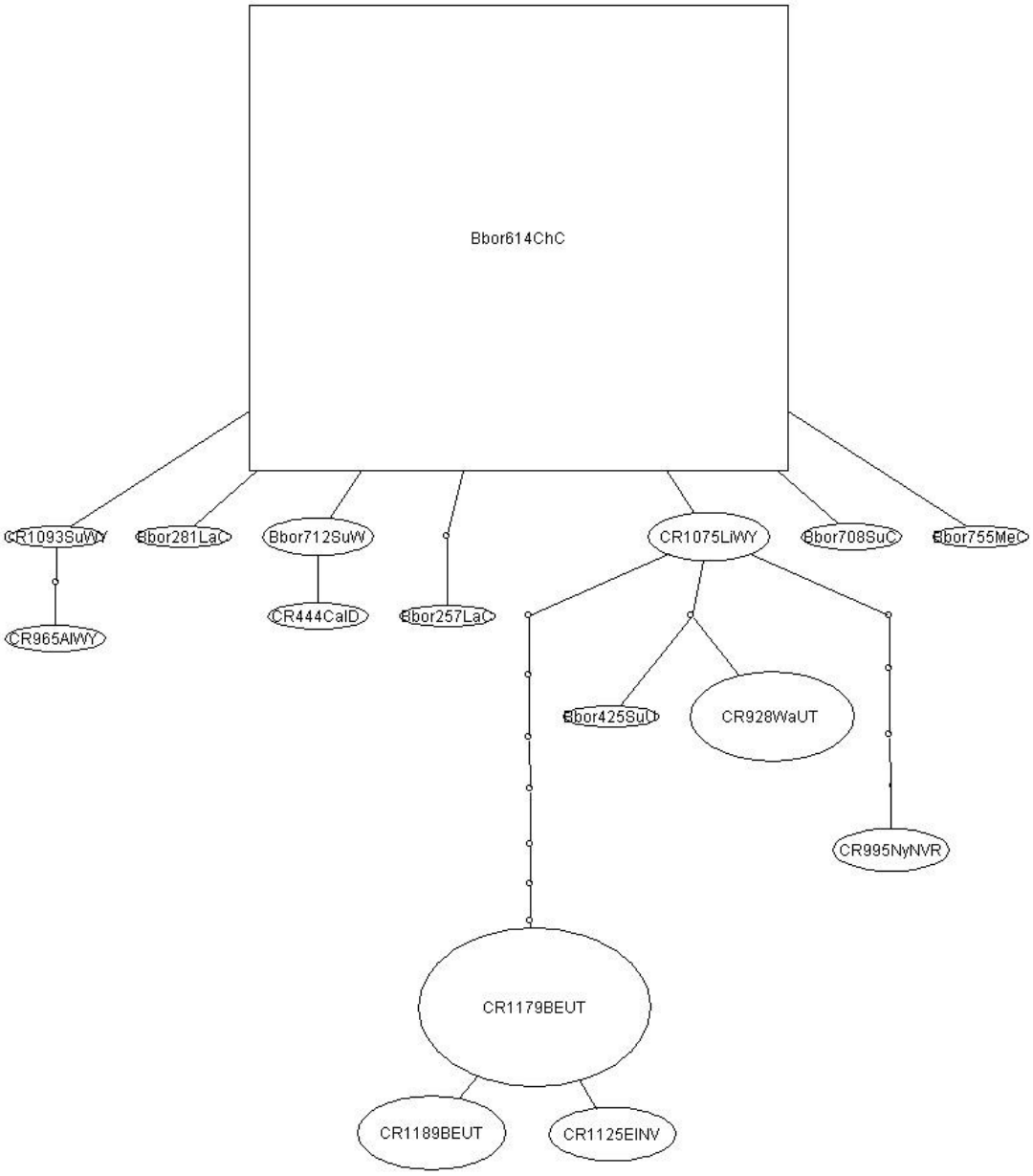


Figure A. Haplotype network of the Eastern mtDNA clade. Most samples in the clade are comprised of a single haplotype identical to sample Bbor614from Chaffee Co., CO, as depicted here. The two divergent lineages include the samples found in Box Elder Co., UT (samples CR 1179 and identical haplotypes) and a sample in northeast Nevada (sample CR1125ElNV from Elko Co., NV). All samples from Railroad Valley were identical and are seen here as sample

CR995NyNVR (N=5). Samples from Piute and Wayne Cos, UT are represented by sample CR928WAUT. This is similar to Switzer's group 3-1.

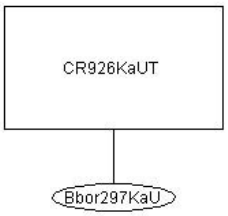


Figure B. Haplotype network of the Kane Co., UT mtDNA clade. Only two haplotypes were found, one sample differed by a single base pair. Switzer found a single haplotype in three samples (network BO).

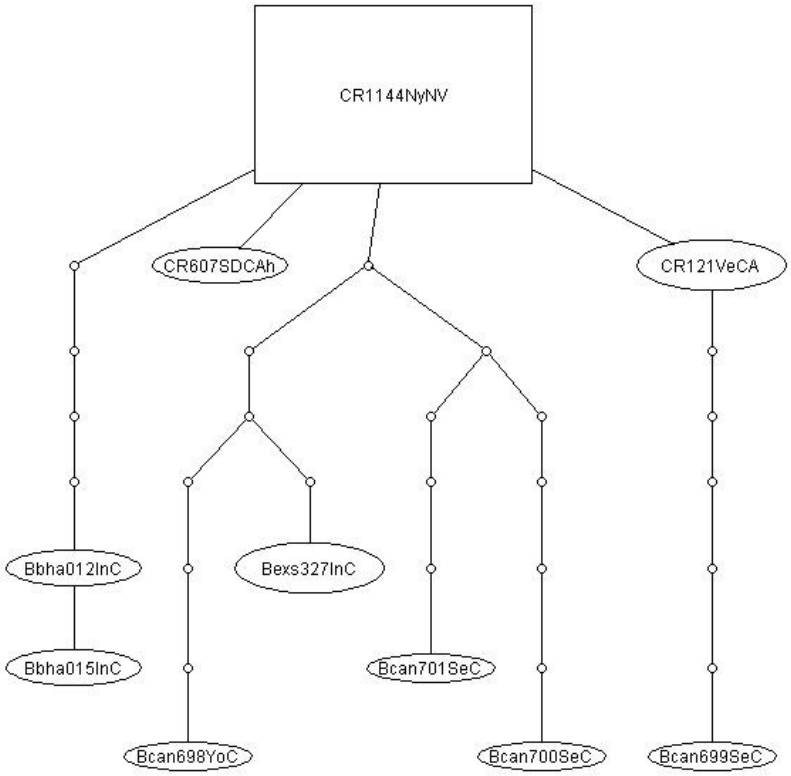


Figure C. Haplotype network of the SW mtDNA clade. This is similar to Switzer et al (2009) network 2-10 and includes *A. exsul*, and *A. ne*lsoni as well as samples from southern California and Nevada. Analyses here also include samples from *A. canorus* south not analyzed by Switzer.

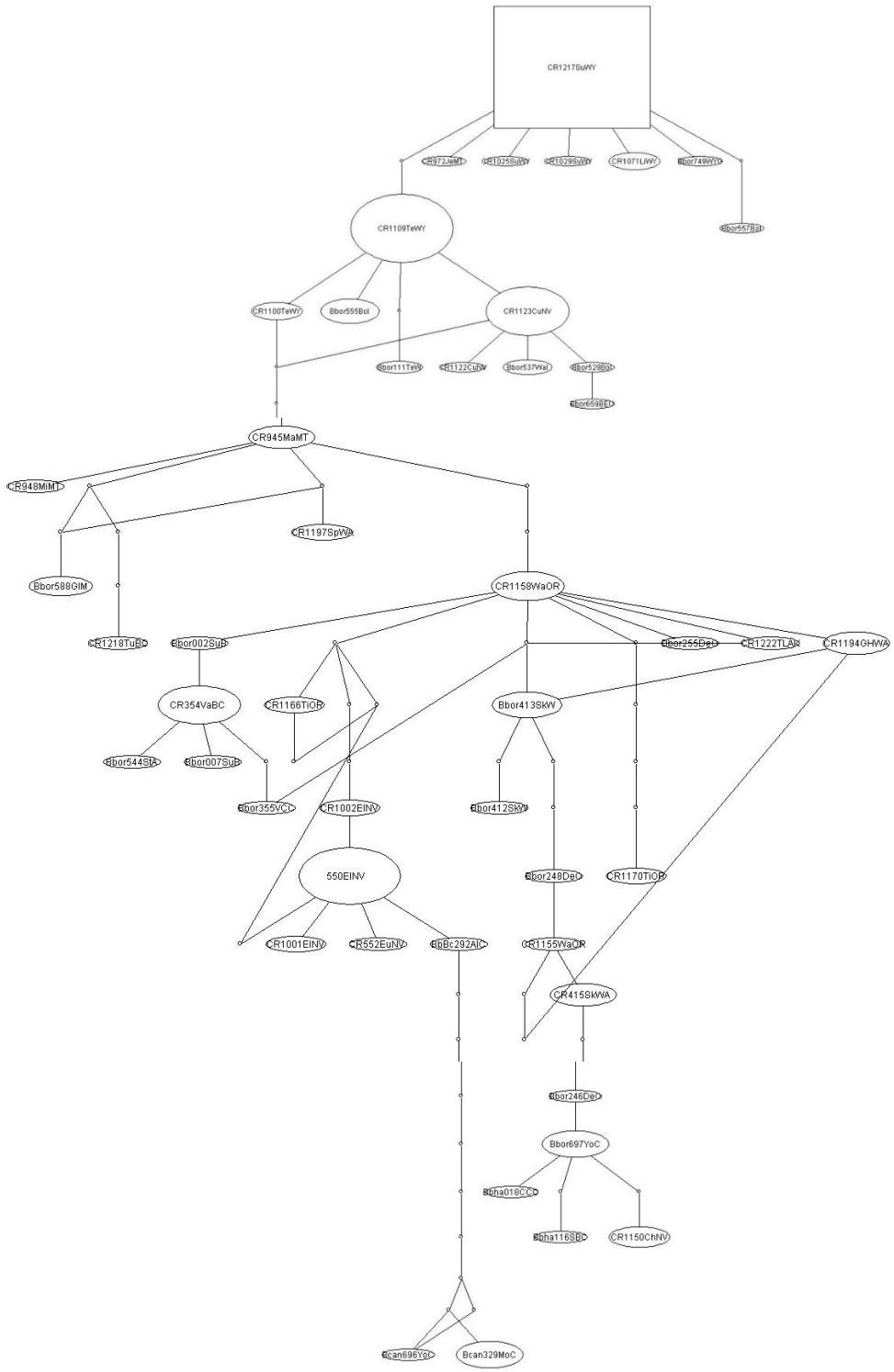


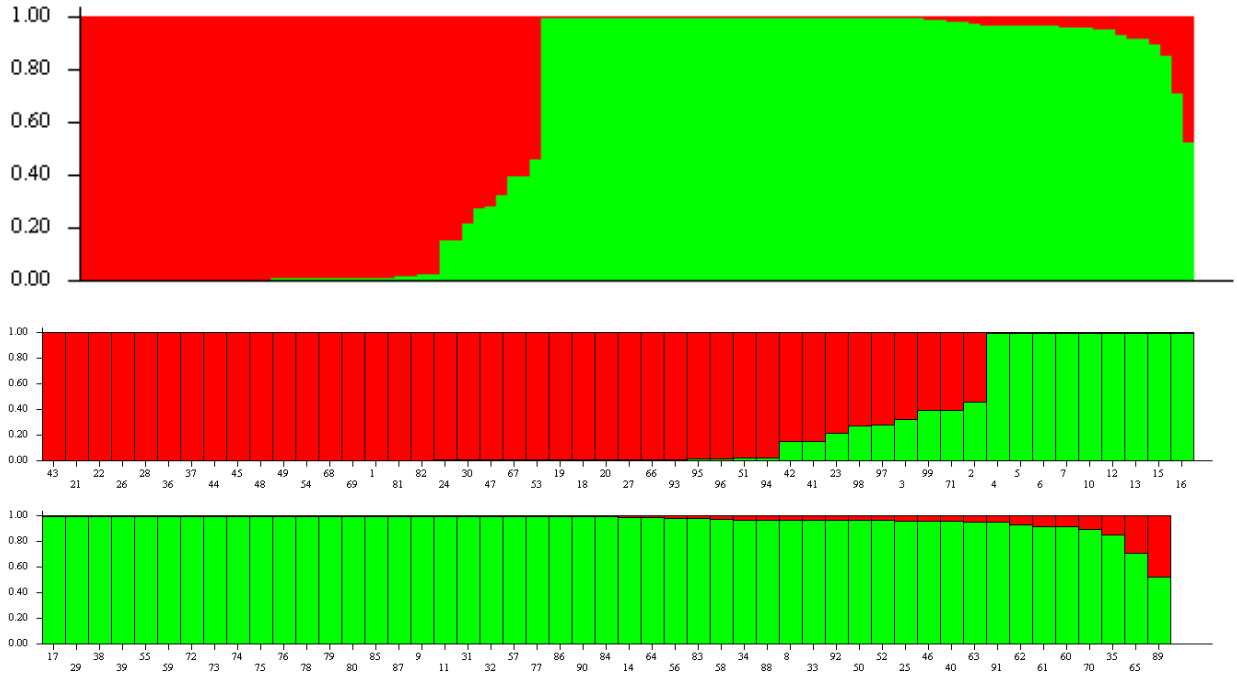
Figure D. Haplotype network of the NW mtDNA clade based on the small fragment of the

control region is comprised of a number of highly interbranched and unresolved networks.
Appendix 4. Data from STRUCTURE

Identification of K from Structure Harvester:

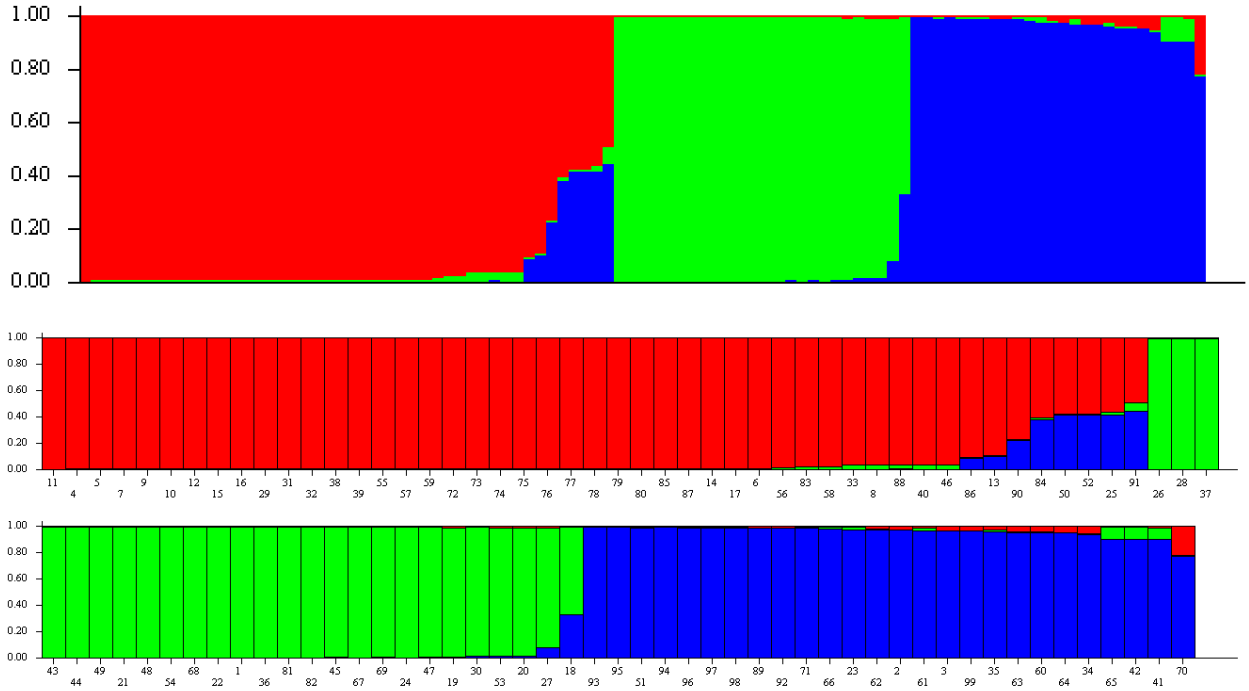
# K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	3	-2688.7667	0.4933	NA	NA	NA
2	3	-2156.7000	3.1480	532.066667	255.833333	81.268137
3	3	-1880.4667	5.6146	276.233333	169.600000	30.207152
4	3	-1773.8333	29.3539	106.633333	26.266667	0.894826
5	3	-1693.4667	27.8443	80.366667	64.266667	2.308075
6	3	-1548.8333	4.0526	144.633333	119.533333	29.495678
7	3	-1523.7333	18.1935	25.100000	15.933333	0.875771
8	3	-1514.5667	13.2580	9.166667	19.300000	1.455730
9	3	-1524.7000	40.2068	-10.133333	20.400000	0.507376
10	3	-1514.4333	50.9885	10.266667	NA	NA

Representative Barplots. K=2 groups



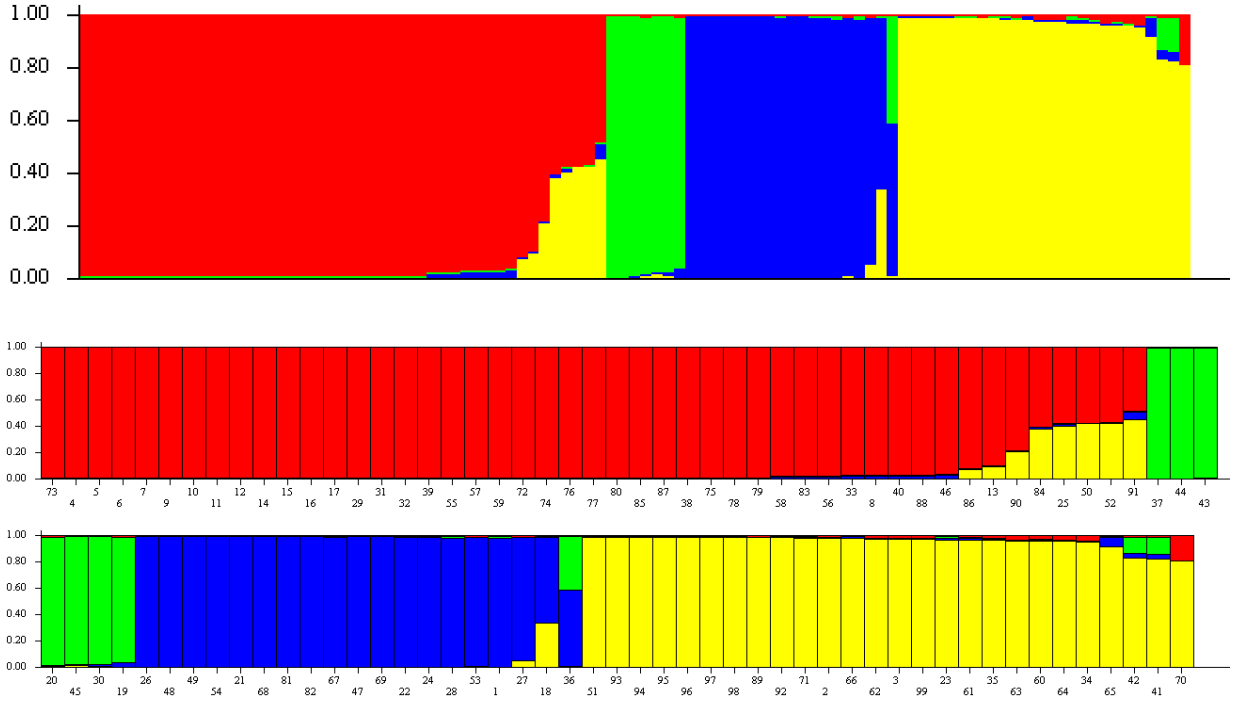
Red consists of the Northwest, Southwest, *A. nelsoni*, *A. canoris* and Hot Creek populations.
Mostly red but greed admixture – Lake Lahuntan, *A. exsul*, Railroad Valley groups,
Green consists of Eastern, and Middle Rocky Mountains with admixture identified as largely green with some red in the Middle Rocky Mountains.

Representative Barplots. K=3 groups.



Red-consists of samples in the Eastern group.
Green-consists of samples in Northwest, Southwest, *A. canorus*-northern, *A. nelsoni*, and Dixie Valley Populations.
Blue- consists of samples in the *A. exsul*, Lake Lahontan, Railroad Valley, and Middle Rocky Mountain groups.
Mixed red blue - are samples from Box Elder Co., UT and samples in NW Wyoming and SE Montana (haplotyes in both the E and MRM groups).

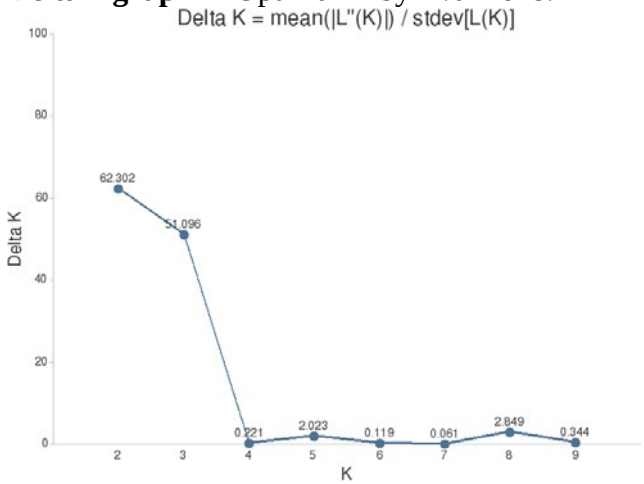
Representative Barplots. K=4 groups.



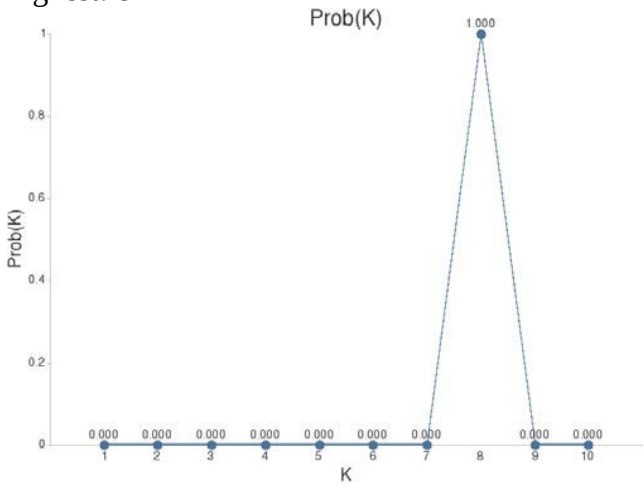
Red-consists of samples in the Eastern group.
Green – *A. canorus* and *A. nelsoni*
Blue – consists of samples in the Northwest and Southwest groups excluding *A. canorus* and *A. nelsoni*
Yellow - - consists of samples in the *A. exsul*, Lake Lahontan, Railroad Valley, and Middle Rocky Mountain groups.

Output of Clumpak for best K:

DeltaK graph Optimal K by Evanno is: 2



But, Probability By K graph Using median values of Ln(Pr Data) the k for which Pr(K=k) is highest: 8



Appendix C

This appendix contains the results of a genomic analysis of the Western Boreal Toad completed by Dr. Sara Oyler-McCance, USGS, provided via email, April 3, 2017.

Appendix 3. Genomic analyses by Dr. Sara Oyler-McCance, USGS.

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Results of genomic analysis (SNP data from RAD-seq) are largely congruent with analyses of sequence data of Goebel (Appendix 2). Samples here were included in analyses of sequence data with two exceptions; sequence data did not include three samples (sample 704 form Gunnison Co, CO, 586 from Glacier National Park, MT, and 1200StWA from Stevens Co., WA). Sequence data included more samples, but far fewer loci than genomic data which was based on about 8,000 loci. The same groups were recovered in sequence analyses (Goebel, Appendix 2, Figures 3 and 4) and the same individuals are found within the recovered groups, but relationships differ slightly among the major groups. Specifically, genomic analyses shown here identify the Lake Lahontan group as more closely related to the Eastern group. In contrast, in the sequence analyses (Appendix 2) the Middle Rocky Mountain cluster was more closely related to the Eastern clade. Similar relationships were also recovered from analyses of microsatellites (Switzer et al., 2009). The relationships among the four groups might vary depending on the identification of a clear root for the tree. But most importantly, both analyses identify the four groups providing evidence for their independent existence, even though the relationships among them might vary with further analyses. Note that analyses of genomic and sequence data are preliminary and analyses are still in progress.

Despite the difference in the relationships among the major groups, these preliminary analyses support the major conclusions drawn. These include the existence of an unnamed divergent group within the eastern portion of the toad's range previously described from mtDNA data as the Eastern group. Individuals from Kane Co., UT, despite having highly divergent nuclear DNAs are closely related to the toads in the SRMP. The geographic distribution of these toads includes Colorado, Utah, Wyoming, and southwest Montana (one sample 971GaMT).

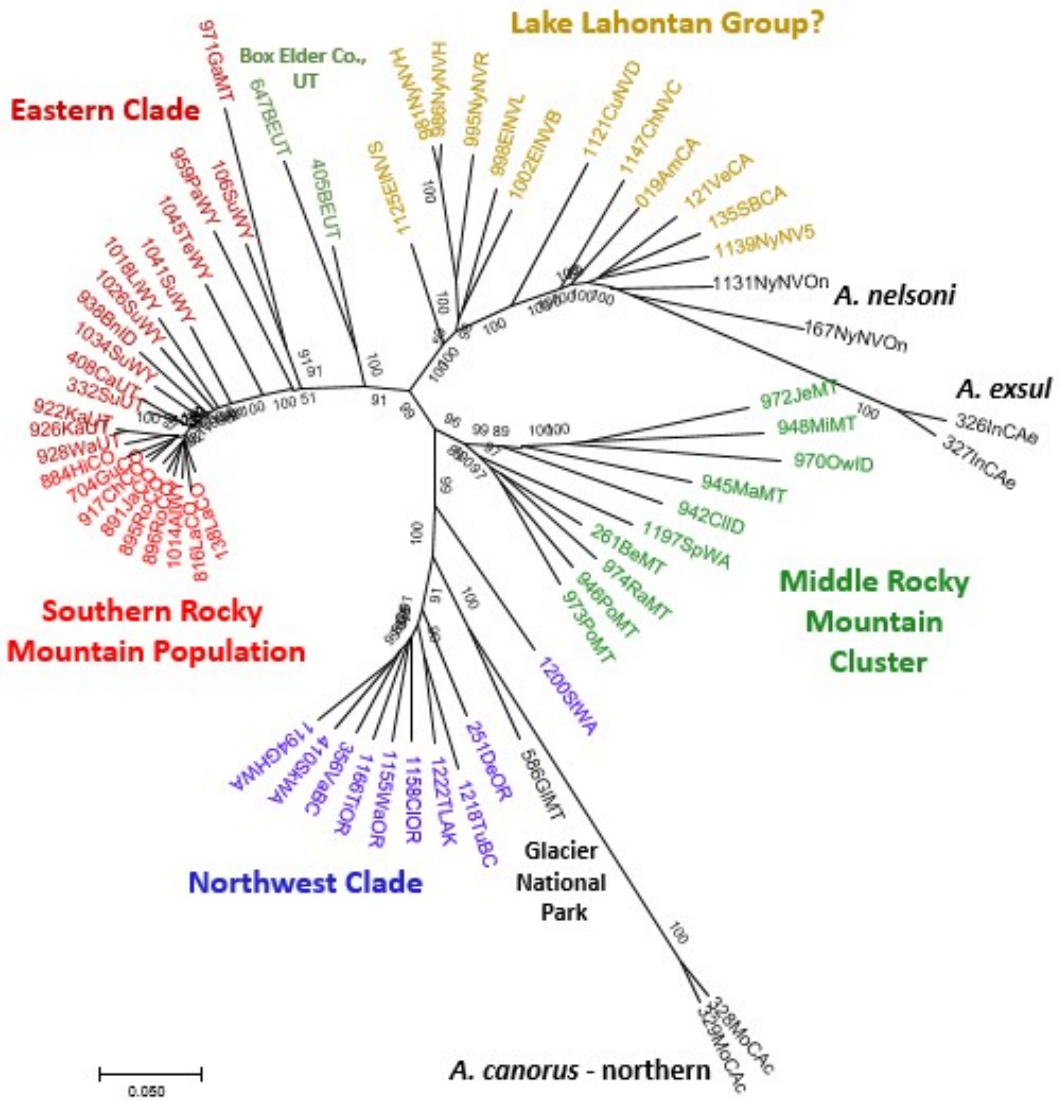

Figure 1. Phylogenetic analysis of *A. boreas* group based on genomic SNP analyses (based on RAD-seq). Samples are a subset of samples from Goebel (Appendix 2, Figures 3 and 4). The same groups are recovered in both analyses, and the same individuals are found within the recovered groups, but relationships differ slightly among the major groups. Specifically, genomic analyses show here identify the Lake Lahontan group as more closely related to the Eastern clade. In sequence analyses (Appendix 2) the Middle Rocky Mountain cluster was more closely related to the Eastern clade. *These data are preliminary or provisional and are subject to revision. They are being provided to meet the need for timely best science. The data have not received final approval by the U.S. Geological Survey (USGS) and are provided on the condition that neither the USGS nor the U.S. Government shall be held liable for any damages resulting from the authorized or unauthorized use of the data.*

Figure 2. Geographic distribution of the samples analyzed based on genomic SNP analyses (based on RAD-seq). The groups are the same as in Appendix 2 and are: Red- Eastern clade, Green – Middle Rocky Mountain group, Blue- Northwest group, Yellow- Lake Lahontan group, Black- named species in the group *(A. canorus*, *A nelsoni* and *A. exsul*) and a divergent lineage found at Glacier National Park (not included in sequence analyses of Goebel, Appendix 2). The samples in Box Elder Co., UT, are a brown green and are closely related to the Eastern and Lake Lahontan groups. Fewer samples from Box Elder county were analyzed with genomic analyses compared too analyses of sequence data, but their placement as possibly intergrades between divergent clades is still evident.