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Mitochondrial DNA evolution in the *Anaxyrus boreas* species group

Anna M. Goebel [a,b,*], Tom A. Ranker [c,1], Paul Stephen Corn [d], Richard G. Olmstead [e,2]

[a] University of Colorado Museum of Natural History, 265 UCB, University of Colorado, Boulder, CO 80309-0265, USA
[b] Florida Gulf Coast University, Department of Biological Sciences, Fort Myers, FL 33965, USA
[c] University of Colorado Museum of Natural History, Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309, USA
[d] USGS Northern Rocky Mountain Science Center, Aldo Leopold Wilderness Research Institute, 790 E. Beckwith Avenue, Missoula, MT 59801, USA
[e] E.P.O. Biology Department, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

The *Anaxyrus boreas* species group currently comprises four species in western North America including the broadly distributed *A. boreas*, and three localized species, *Anaxyrus nelsoni*, *Anaxyrus exsul* and *Anaxyrus canorus*. Phylogenetic analyses of the mtDNA 12S rDNA, cytochrome oxidase I, control region, and restriction sites data, identified three major haplotype clades. The Northwest clade (NW) includes both subspecies of *A. boreas* and divergent minor clades in the middle Rocky Mountains, coastal, and central regions of the west and Pacific Northwest. The Southwest (SW) clade includes *A. exsul*, *A. nelsoni*, and minor clades in southern California. *Anaxyrus canorus*, previously identified as paraphyletic, has populations in both the NW and SW major clades. The Eastern major clade (E) includes three divergent lineages from southern Utah, the southern Rocky Mountains, and north of the Great Basin at the border of Utah and Nevada. These results identify new genetic variation in the eastern portion of the toad's range and are consistent with previous regional studies from the west coast. Low levels of control region sequence divergence between major clades (2.2–4.7% uncorrected pair-wise distances) are consistent with Pleistocene divergence and suggest that the phylogeographic history of the group was heavily influenced by dynamic Pleistocene glacial and climatic changes, and especially pluvial changes, in western North America. Results reported here may impact conservation plans in that the current taxonomy does not reflect the diversity in the group.

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1. Introduction

Historical classifications of toads (Amphibia: Bufonidae) recognized species groups based on morphological similarity. Blair (1972b,c) identified at least 37 species groups in the genus *Bufo* (Laurenti, 1768) from the approximately 200 species recognized at that time and placed the North American toads into seven species groups (*boreas*, *punctatus*, *retiformis*, *debilis*, *quercicus*, *cognatus*, *americanus*). Collectively these groups comprise the Nearctic toads, genus *Anaxyrus* (Tschudi, 1845; Frost et al., 2006a). Relationships within groups are less clear than group identity, and cryptic speciation has long been recognized as a problem in toads (Blair, 1972b). More recently, mitochondrial DNA has been used to identify relationships within those groups and all studies have identified highly divergent toad lineages not recognized by taxonomy (Graybeal, 1993; Shaffer et al., 2000; Stephens, 2001; Masta et al., 2002; Smith and Green, 2004; Jaeger et al., 2005).

The *boreas* species group, as currently recognized (Stebbins, 2003; Frost, 2007), is comprised of two subspecies broadly distributed across North America and three species with localized distributions (Fig. 1). *Anaxyrus boreas* (Baird and Girard, 1852) is found from the east slope of the Rocky Mountains to the Pacific Ocean and from northern Baja California to Alaska and the Yukon. The subspecies *A. b. boreas* (Baird and Girard, 1852) occupies most of this range, but *A. b. halophilus* (Baird and Girard, 1853) occurs on the West Coast from northern California to Baja California. The subspecies are thought to be sympatric in northern California

* Corresponding author. Current address: Florida Gulf Coast University, Department of Biological Sciences, 10501 FGCU Boulevard South, Fort Myers, FL 33965, USA. Fax: +1 239 590 7200.
E-mail addresses: agoebel@fgcu.edu (A.M. Goebel), ranker@hawaii.edu (T.A. Ranker), scorn@usgs.gov (P.S. Corn), olmstead@u.washington.edu (R.G. Olmstead).
[1] Present address: University of Hawai'i at Manoa, Department of Botany, 3190 Maile Way, St. John 101, Honolulu, HI 96822, USA.
[2] Present address: Department of Biology, Box 355325, University of Washington, Seattle, WA 98195, USA.

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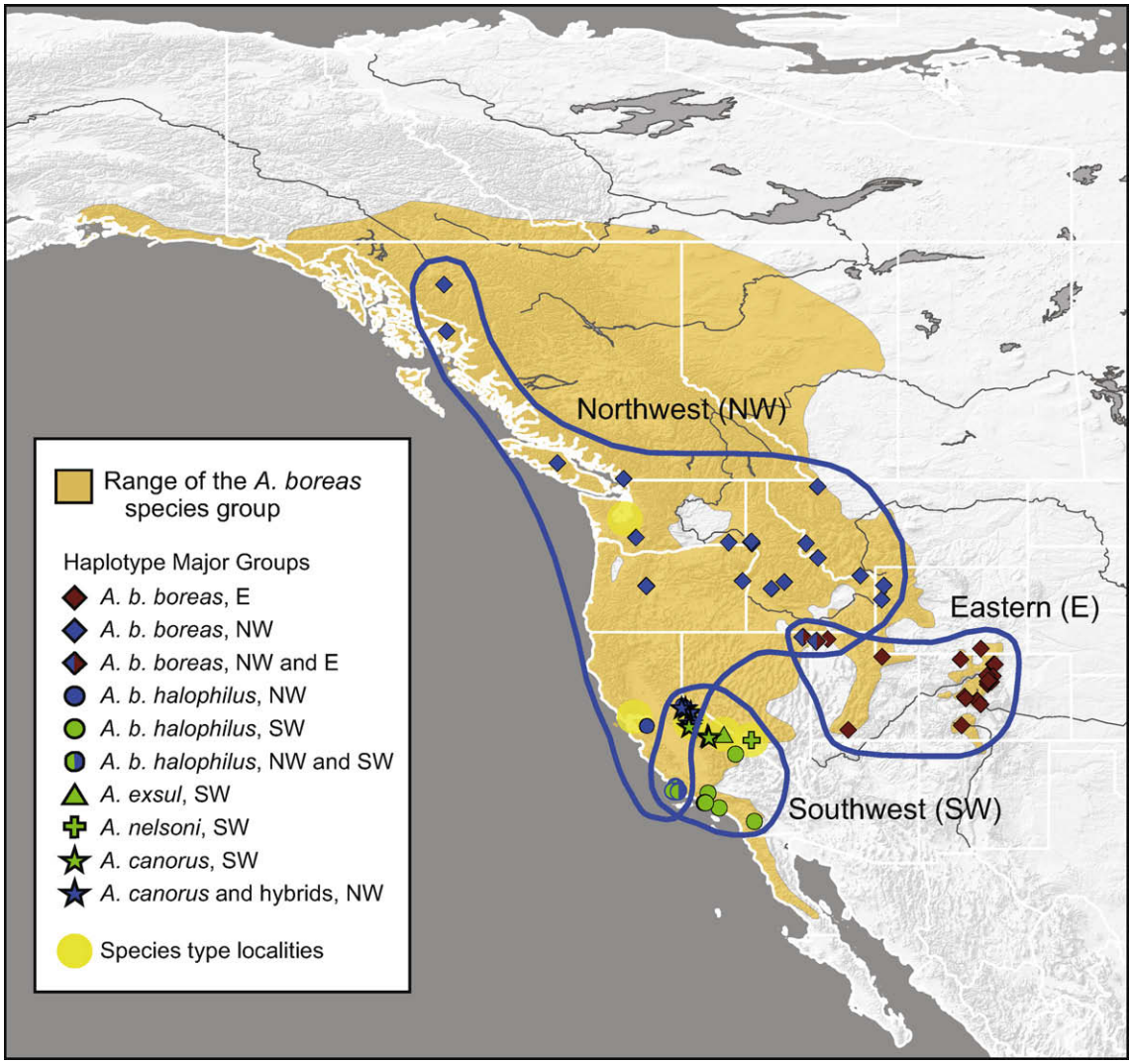

Fig. 1. Distribution of the *boreas* group and localities of specimens examined. Current taxon identities are indicated by symbol shapes (e.g., *Anaxyrus b. boreas*-diamond) as provided in the Key. The major mtDNA haplotype clades (NW-northwest, SW-southwest, E-eastern) are indicated by color/shade of symbol and their distributions are encircled. Populations enclosed by multiple circles contain representatives of multiple haplotype clades. Type localities are identified by large yellow or light circles. The range map was compiled using a number of published (Baxter and Stone, 1980; Committee on the Status of Endangered Wildlife in Canada, 2002; Environment Yukon, 2005; Green and Gregory, 2007; Grismer, 2002; Hammerson, 1999; Stebbins, 2003; Thompson et al., 2004) or online (http://www.alaskaherps.info/; http://imnh.isu.edu/digitalatlas; http://www.wdfw.wa.gov/wlm/gap/dataprod.htm) sources and expert advice.

(Camp, 1917a; Stebbins, 2003). The other three species are considered Pleistocene relicts (Myers, 1942; Karlstrom, 1958, 1962). *Anaxyrus exsul* (Myers, 1942) occurs only in Deep Springs Valley of east central California (Fellers, 2005). *Anaxyrus nelsoni* (Stejneger, 1893) is currently known only in the Amargosa River drainage of southwestern Nevada (Altig and Dodd, 1987; Goebel et al., 2005). *Anaxyrus canorus* (Camp, 1916) is narrowly distributed at high elevations in the Sierra Nevada and is sympatric with *A. boreas* at the northern end of its distribution (Karlstrom, 1962; Morton and Sokoloski, 1978; Davidson and Fellers, 2005).

Morphological characters that distinguish some *boreas* group taxa are striking (e.g., the black coloration of *A. exsul* contrasts brown color typical of toads), but morphological variation within the group is limited (Karlstrom, 1962; Myers, 1942). Schuierer (1963), Burger and Bragg (1946), and Karlstrom (1962), noted that specimens in Colorado and/or Alaska were morphologically different (e.g., smaller size, smoother skin, more pronounced warts) from toads in the more coastal northwest, but Karlstrom (1962) found these same characters to vary with age, sex and elevation and did not consider them diagnostic. Other unusual forms were noted in Montana, and Alberta (Black, 1970, 1971; Schueler, 1982). Sanders and Cross (1963), noted chromosomal differences between *A. b. boreas* in Colorado and *A. b. halophilus* in California but early chromosomal data are difficult to interpret due to the limited techniques available at the time. However, these comments suggest the possibility of cryptic speciation.

All previous molecular phylogenetic analyses that include members of the *boreas* group were either regional studies that examined a small portion of the toad's range on the west coast and western Nevada (Feder, 1973; Graybeal, 1993; Shaffer et al., 2000; Stephens, 2001; Simandle, 2006; Simandle et al., 2006) or were phylogenetic analyses of deeper relationships among toads and frogs that included few specimens of the *boreas* group (Maxson et al., 1981; Graybeal, 1997; Macey et al., 1998; Darst and Cannatella, 2004; Pauly et al., 2004; Goebel, 1996, 2005; Pramuk, 2006; Frost et al., 2006a). Molecular analyses of the group are further complicated because the species are recently diverged and quite distant from potential outgroups (Pauly et al., 2004; Pramuk, 2006; Frost et al., 2006a) making rooting by outgroups difficult (Wheeler, 1990; Huelsenbeck et al., 2002). Non-molecular phylo-

genetic studies that included specimens of the group similarly focused only on deeper relationships of bufonids (Karlstrom, 1962; Tihen, 1962; Schuierer, 1963; Blair, 1963, 1964, 1972b; Bogart, 1972; Sanders and Cross, 1963; Graybeal, 1997).

Our goal was to provide a broader molecular analysis of the *boreas* group. By examining mtDNA of all taxa and toads from across the distribution, we hoped to put the regional studies into a larger context and to examine diversity within the whole group. We specifically wanted to include specimens from the eastern portion of the range as these were not included in previous analyses. Toads from the Southern Rocky Mountain Population (SRMP: Colorado and a few localities in south central Wyoming and northern New Mexico) were of special concern due to declines that probably began in the 1970's (Corn, 2003; Muths and Nanjappa, 2005). The SRMP is listed as endangered by the State of Colorado (Hammerson, 1999), but was removed as a candidate species for listing by the US Endangered Species Act in 2006 in part due to a lack of genetic distinction (Thompson, 2005). The combination of potential morphological divergence of the SRMP from the rest of the group (Schuierer, 1963; Burger and Bragg, 1946; Karlstrom, 1962), a disjunct distribution (Fig. 1), and recent declines, suggested a need for a phylogenetic analysis that included toads from the SRMP in Colorado. To identify relationships among more divergent lineages, we analyzed slowly evolving genes (12S ribosomal DNA and a portion of cytochrome oxidase I) and rapidly evolving DNA data (the control region and restriction sites of the whole mtDNA) with parsimony and Bayesian analyses.

2. Materials and methods

2.1. Data collection and alignment

Specimens (288 individuals from 58 sites, Table 1 and Fig. 1) were collected from all currently recognized taxa and throughout much of the range of the *boreas* group (Fig. 1). Specimens were chosen from localities where taxa exist in isolation whenever possible, because hybridization was suspected among some taxa (Karlstrom, 1962; Morton and Sokoloski, 1978; Mullally and Powell, 1958). All taxon identities were determined by collectors using morphology (hybrids were determined by intermediate morphological characteristics) and range maps (Stebbins, 2003). Thirteen species of *Anaxyrus* with varying levels of divergence from the *boreas* group were included as outgroups along with species of *Ollotis* (Frost et al., 2006b) and *Chaunus* (also called *Rhinella*, Chaparro et al., 2007) as further outgroups (Graybeal, 1997; Pramuk et al., 2001; Pauly et al., 2004; Pramuk, 2006; Frost et al., 2006a). Locality information, voucher identity, number of samples from each locality, restrictions site haplotype numbers and GenBank accession numbers for sequences, are in Table 1. Total DNA was extracted from tissue using standard phenol extraction and proteinase K digestion (Maniatis et al., 1982) or with either the DNeasy Tissue or QIAamp DNA Blood Mini Kits (Qiagen Inc., Valencia CA). Restriction site polymorphisms of the whole mtDNA molecule were identified using standard techniques (Southern, 1975; Maniatis et al., 1982; Koetsier et al., 1993). Genomic DNA was cut with 16 six-base cutting restriction enzymes (ApaI, BamHI, BglI, BglII, ClaI, Csp45I, DraI, EcoRI, EcoRV, KpnI, NheI, PstI, PvuII, SmaI, StuI and XhoI). After digestion, fragments were separated by size with agarose gel electrophoresis, transferred to nylon membranes, and probed with four fragments comprising the complete mtDNA of *Chaunus marinus* (syn. *Bufo marinus*). Restriction sites were mapped (Goebel, 1996) using double digests and serial probing with the four mtDNA fragments.

Sequences of the control region (CR) cytochrome oxidase I (COI), and 12S ribosomal DNA (12S) were determined with amplification and sequencing methods described by Goebel et al. (1999). The 12S was amplified using four primers (12SA-L, Kocher et al., 1989; tRNAphe-L, 12SF-H, tRNAval-H, Goebel et al., 1999). COI sequences were obtained using two primers (CO1e-H, Palumbi et al., 1991; CO1af-L, Goebel et al., 1999) and CR sequences were determined using six primers (CytbA-L, ControlJ-L, ControlK-H, ControlO-H, ControlP-H; Wrev-L, Goebel et al., 1999). The primer ControlP2-H (5′-CATAGATTCASTTCCGTCAGATGCC-3′) was located six bases internal to ControlP-H and was used for sequencing because it provided superior data compared to the terminal amplification primer ControlP-H. For outgroups, 537 bp of the 3′ end of the control region (CR_{537}) were obtained using a combination of four primers (Wrev-L, Control J-L, ControlB-H, ControlP-H; Goebel et al., 1999). Sequences of both strands were obtained for all 12S and COI sequences and at least one accession of all unique CR sequences.

Data were collected in a hierarchical fashion. Restriction sites (RS) were collected initially from all specimens available before 1995 and 31 haplotypes were identified. An 882 bp fragment of CR (CR_{882}) was obtained for all unique RS haplotypes in each *population* (collection site or set of geographically close sites) even when the same RS haplotype occurred in multiple populations. Sequences were also obtained for most *A. exsul*, *A. nelsoni* and *A. canorus* available. Sequences from 12S and COI were obtained from the more divergent haplotypes initially identified with RS and CR and from at least two accessions of all named taxa. For samples added after all RS data were collected the CR_{537} fragment was sequenced first. Then the additional 355 bp (the full CR_{882}) fragment was obtained from all unique CR_{537} haplotypes in each population. Sequence data assisted in refining restriction site maps. After identification of insertions, deletions, and repeated regions in the CR, restriction sites that mapped close to the repeated regions were re-scored or excluded from the analysis if they could not be identified with confidence in all samples.

Sequences were aligned manually. Within the *boreas* group, gaps due to insertions/deletions occurred as single bases with only a few exceptions. A 7-bp gap was found in the 5′ end of CR_{882} in samples from two geographically close sites (Teton Co., WY and Beaverhead Co., MT). The rarity of the deletion and its limited geographic distribution suggest it was a single evolutionary event and it was scored as a single gap. Several larger (163–173 bp) unique repeated regions and a common 21-bp repeated fragment were found within the 5′ end of CR_{882} also, and were excluded from analyses. Sequence alignments of 12S and CR_{537} partitions with outgroup taxa were more ambiguous due to multiple adjacent gaps and those sites were deleted from analyses (6 sites from 12S, 149 from CR_{537}). Only unique haplotypes were included in analyses. Alignments were deposited in TreeBase (Study accession number = S2194, Matrix accession number = M4155-M4161).

2.2. Data analysis

The four data partitions (12S, COI, CR, RS) were first assessed separately. Data for the control region were analyzed both for the larger CR_{882} fragment and the smaller CR_{537} fragment, because CR_{537} was obtained for many more specimens. The protein-coding gene COI was not partitioned further in analyses of the *boreas* group because there were no second position changes, only two first position changes, and no amino acid substations. In exploratory analyses of COI with outgroups data were partitioned further into first positions (11 variable positions) and third positions (there were no second position changes and no amino acid substitutions) but the additional partitioning did not affect rooting position or relationships within the *boreas* group, so COI data were not partitioned further in final analyses.

Table 1
Specimens examined: localities, voucher specimens, and DNA data.

Taxon locality(s)	Voucher	Locality code	Number of samples (n = 288)	RS haplotype (n = 194)	GenBank Accession Nos. for sequence data					
					CR_{882} (n = 117)		CR_{537} (n = 52)		COI (n = 50)	12S (n = 22)
Anaxyrus boreas boreas										
Kane Co., UT, 3 sites	USNMFT211044–8	KaUT	17	1 (9)	EF532065	EF532070	EF532068	EF532073	EF532015	EF531993
	USNMFT064347				EF532066		EF532069	EF532074	EF532016	EF531994
	USNMFT18024–9				EF532067		EF532071	EF532072	EF532017	
Box Elder Co., UT, Red Butte Canyon, Upper Rocky Pass Spring, Lynn Reservoir		BEUT	7	—	EF532075	EF532080	EF532078		EF532018	EF531995
					EF532076	EF532112	EF532079		EF532019	
					EF532077				EF532038	
Summit Co., UT, East Fork of Bear River	USNMFT211041	SuUT	1	2 (1)	EF532082				EF532020	EF531996
Elko Co., NV	AMG554	ElNV	3	—	EF532081	EF532101			EF532032	
					EF532100					
Larimer Co., CO, Rocky Mountain NP Lost Lake and Kettle Tarn	USNMFT064334	LaCO	23	3 (16)	EF532084	EF532094			EF532022	EF531997
	AMG138			4 (1), 5 (3)	EF532092				EF532028	
Gunnison Co., CO, near Crested Butte White Rock Basin and West Brush Creek		GuCO	3	3 (2), 4 (1)	EF532089				EF532026	EF531998
					EF532090				EF532027	
Summit Co., CO, near Montezuma	AMG027	SuCO	4	3 (4)	EF532086				EF532024	
Chaffee Co., CO, Brown Creek, Collegiate Peaks Cpgd Denny Creek and Hartenstein Lake	USNMFT064330	ChCO	27	3 (19)	EF532085				EF532023	
				4 (8)	EF532088				EF532025	
Albany Co., WY; SW Medicine Bow NP		AlWY	2	3 (2)	EF532083				EF532021	
Route Co., CO; First Creek		RoCO	2	3 (2)	EF532091					
Clear Creek Co., CO; Henderson Region, Georgetown, Bethyl Creek		CCCO	20	3 (7)	EF532095	EF532098			EF532030	
				4 (7), 5 (6)	EF532097	EF532099			EF532031	
Boulder Co., CO Indian Peaks Wilderness	USNMFT211037	BoCO	4	3 (2), 5 (2)	EF532093	EF532096			EF532029	
Mineral Co., CO, Cliff Creek	AMG544A	MiCO	1	—	EF532087					
Deschutes Co., OR, near Three Creeks Lake	USNMFT211042	DeOR	10	6 (4), 10 (1)	EF532102	EF532127			EF532036	EF532006
				11 (1), 12 (1)	EF532108	EF532136			EF532044	EF532007
				17 (1), 21 (1)	EF532109	EF532138			EF532048	
				22 (1)	EF532110				EF532049	
Surrey, British Columbia, Latimer Lake	MVZ178495,178498, 178500,501	SuBC	4	6 (2), 8 (2)	EF532103	EF532107			EF532033	
					EF532106				EF532035	
Vancouver Isle, British Columbia	AMG355	VaBC	3	7 (3)	EF532104	EF532105			EF532034	EF531999
Columbia Co., WA, N. Fork Touchet River	MJA:AMG112	CoWA	1	13 (1)	EF532116			EF532040	EF532001	
Skamania Co., WA, Mt. St. Helens	MSB 92531-92538	SkWA	8	—	EF532146		EF532148	EF532152	EF532052	
					EF532147		EF532150	EF532153		
					EF532149		EF532151			
Glacier Co., MT, Glacier NP	USNMFT211007–9	GlMT	4	13 (1)	EF532180		EF532181			
					EF532117		EF532182			
Ravali Co., MT, Kramis Pond	BSFS18016-18023	RaMT	8	—	EF532183	EF532187	EF532185		EF532055	
					EF532184	EF532189	EF532188			
					EF532186		EF532190			
Beaverhead Co., MT, Red Rocks NWR and Twin Lakes, Beaverhead NF	AMG033	BeMT	9	13 (5)	EF532124				EF532039	
				15 (4)	EF532113	EF532119			EF532041	
Teton Co., WY, Yellowstone NP, and Jackson Hole	USNMFT211036	TeWY	8	13 (4)	EF532118	EF532123			EF532042	EF532002
				14 (1)	EF532120	EF532125			EF532043	
				15 (2)	EF532121	EF532126				
				16 (1)	EF532122					
Nez Perce Co., ID, Mud Bog Meadows, China Creek, and Benton Meadows	USNMFT064339	NPID	5	9 (5)	EF532111	EF532115			EF532037	EF532000
					EF532114					
Washington Co., ID, Grouse Creek	AMG541	WaID	8	—	EF532154	EF532158	EF532155	EF532159	EF532053	
					EF532156	EF532160	EF532157	EF532161		

Boise Co., ID, Missouri Mines	AMG532	BoID	9	—	EF532163	EF532167	EF532162	EF532170	EF532054	
					EF532164		EF532165	EF532169		
					EF532166		EF532168			
Boise Co., ID. Bull Trout Lake	AMG554	BoID	9	—	EF532172		EF532171	EF532176		
					EF532174		EF532173	EF532177		
					EF532178		EF532175	EF532179		
Alaska, Chickamon Rivers	AMG633	ChAK,	19	—	EF532193		EF532191	EF532203	EF532056	
					EF532196		EF532192	EF532204		
					EF532197		EF532194	EF532205		
							EF532195	EF532206		
							EF532198	EF532207		
							FF532199	EF532208		
							EF532200	EF532209		
							EF532201	EF532202		
British Columbia, Little Tahltan River		LTBC	2				EF532210	EF532211		
Anaxyrus boreas halophilus										
Inyo Co., CA, Darwin Canyon	MVZ178484–9	InCA	6	27 (2), 28 (2)	EF532218	EF532220			EF532061	EF532012
				29 (2)	EF532219	EF532221				
Mariposa Co., CA, Yosemite NP	Shaffer et al. (2000)[c]	MaCA	1		EF532230					
Santa Clara Co., CA	DM:AMG294	SCCA	1	22 (1)			EF532137			
Ventura Co., CA, Piru and Santa Monica Mts	UCSB29622-29623	VeCA	5	29 (4), 30 (1)	EF532224				EF532063	EF532013
Los Angeles Co., CA Santa Monica Mts and California State University	UCSB29624-29625	LACA	3	29 (3)	EF532222					
	ROM21064									
Santa Barbara Co., Santa Maria and Lompac to Solvang	UCSB29619-29621	SBCA	16	21 (1)	EF532144	EF532223			EF532062	
	UCSB29626-29637			20 (13)	EF532145					
				25 (1), 31 (1)	EF532226					
Alpine Co, CA, Eldorado NF, Little Indian Valley	DM:AMG286	AlCA	5	6 (1), 19 (4)	EF532128	EF532129				
San Diego Co., CA, S. of Warner Springs		SDCA	6	29 (6)	EF532225	EF532227			EF532064	EF532014
Contra Costa Co., Corrall Hollow Road	MVZ186282–8	CCCA	7	23 (6)	EF532139	EF532142			EF532050	
				24 (1)	EF532140	EF532143			EF532051	
					F532141					
Anaxyrus exsul										
Inyo Co, CA, Buckhorn Spring	MVZ142943–142947	InCA	5	26 (5)	EF532212	EF532214			EF532057	EF532008
					EF532213	EF532215			EF532058	EF532009
Anaxyrus nelsoni										
Nye Co., NV, Crystal Springs	KH:AMG167-8	NyNV	2	27 (2)	EF532216				EF532059	EF532010
					EF532217				EF532060	EF532011
Anaxyrus canorus										
Mono Co., CA, Sonora Pass	MVZ164900–02	MoCA	3	18 (3)	EF532130	EF532132			EF532045	EF532003
					EF532131				EF532046	EF532004
Alpine Co., Co., CA, Tryon Medow	DM:AMG293	AlCA	2	19 (1), 20 (1)	EF532133				EF532047	EF532005
Mariposa Co., CA, Yosemite NP	Shaffer et al. (2002)[a]	MaCA	2	—	EF532228	EF532232				
Fresno Co., Kings Canyon NP	Shaffer et al. (2002)[b]	FrCA	3	—	EF532229	EF532233				
					EF532231					
A. canorus X A. boreas										
Alpine Co., CA, Wheeler Lake	DM:AMG291-2	AlCA	2	6 (1), 19 (1)	EF532134	EF532135				
Outgroups										
Anaxyrus hemiophrys(Manitoba, Canada)	DMG4337	–	1	–	–		EF532270		EF532252	EF532234
Anaxyrus americanus (Ontario, Canada)	ROM21661	–	1	–	–		EF532271		EF532253	EF532235
Anaxyrus houstonensis (Texas, USA)	AHPFS3095	–	1	–	–		EF532272		EF532254	EF532236
Anaxyrus woodhousii (Colorado, USA)	AMG-1	–	1	–	–		EF532273		EF532255	EF532237

(*continued on next page*)

Table 1 (*continued*)

Taxon locality(s)	Voucher	Locality code	Number of samples (n = 288)	RS haplotype (n = 194)	GenBank Accession Nos. for sequence data			
					CR_{882} (n = 117)	CR_{537} (n = 52)	CO1 (n = 50)	12S (n = 22)
Anaxyrus terrestris (Mississippi, USA)	AHPFS3200	–	1	–	–	EF532274	EF532256	EF532238
Anaxyrus microscaphus (Nevada, USA)	EG:AMG288	–	1	–	–	EF532275	EF532257	EF532239
Anaxyrus californicus (California, CA)	EG:AMG292	–	1	–	–	EF532276	EF532258	EF532240
Anaxyrus cognatus (Nebraska, USA)	HS:AMG83	–	1	–	–	EF532277	EF532259	EF532241
Anaxyrus speciosus (Nebraska, USA)	HS:AMG84	–	1	–	–	EF532278	EF532260	EF532242
Anaxyrus quercicus (South Carolina, USA)	DMH93–56	–	1	–	–	EF532281	EF532263	EF532245
Anaxyrus debilis (Arizona, USA)	ASDM88275	–	1	–	–	EF532279	EF532261	EF532243
Anaxyrus retiformis (Arizona, USA)	ASDM90116	–	1	–	–	EF532280	EF532262	EF532244
Anaxyrus punctatus (Arizona, USA)	ASDM93107	–	1	–	–	EF532282	EF532264	EF532246
Chaunus[d] *marinus* (Peru)	USNM206332	–	1	–	–	EF532285	EF532267	EF532249
Chaunus[d] *marinus* (Mexico)	AMG33 (purchased)	–	1	–	–	EF532286	EF532268	EF532250
Chaunus[d] *beebei* (Trinidad)	USNM286990	–	1	–	–	EF532287	EF532269	EF532251
Ollotis mazatlanensis (Mexico)	ASDM90125	–	1	–	–	EF532283	EF532265	EF532247
Ollotis alvarius (Arizona, USA)	ASDM90124	–	1	–	–	EF532284	EF532266	EF532248

Locality codes are on tree terminals. The identity of haplotypes from restriction site (RS) data only (1–31) is followed in parentheses by the number of individuals in each locality with that RS haplotype. GenBank accession numbers are provided for each DNA fragment (CR, COI, 12S). Abbreviations for voucher placements are: AHPFS, University of Texas, Austin TX; ASDM, Arizona Sonora Desert Museum, Tucson, AZ; BSFS, Biological Survey Field Series (Stephen corn); MSB, New Mexico Museum of Southwestern Biology; MVZ, Museum of Vertebrate Zoology, Berkely CA; RM, Redpath Museum, McGill University; ROM, Royal Ontario Museum, Ontario, Canada, AZ; UCSB, University of California, Santa Barbara; USNM, United States National Museum collection in Washington DC, USA; USNMFH, United States National Museum Field Herp (these specimens to be deposited at UCM, University of Colorado Museum, Boulder CO); and collections by AMG, Anna M. Goebel; DMG, David M. Green; DM, David Martin; DMH, David M. Hillis; EG, Eric Gergus; HS, Hobart Smith; KH, Karin Hoff; MJA, M.J. Adams. Adult and juvenile animals from Colorado were not killed for vouchers, when tadpoles or toadlets were collected a single animal was kept as a voucher for each locality. Many samples from Colorado were blood tissue only and do not have vouchers, *Anaxyrus boreas boreas* is listed as Endangered by the State of Colorado and many historic vouchers exist.

[a] Tissue from samples Y173–3, Y172–3 published in Shaffer et al. (2000).

[b] Tissue from samples S202–4, S202–2, S230–5 published in Shaffer et al. (2000).

[c] Tissue from sample Y004–4 published in Shaffer et al. (2000).

The data partitions were combined in two ways so that only samples with all data were included in analyses. The first combination included 22 samples for which four partitions (12S/COI/CR_{882}/RS) were available. The second combination included 44 samples from three partitions (COI/CR_{882}/RS). By excluding 12S from this combination, the number of samples was doubled and few informative sites were excluded. Because RS data were collected from the whole mtDNA, overlap between RS and sequence data was identified at ten restriction sites (DNA Strider 1.01, Christian Marck). In phylogenetic analyses with multiple partitions restriction sites, found within sequenced regions, were excluded.

In order to compare the utility of the partitions, 22 samples with all data types were examined with independent analyses for each partition (trees not shown). Utility was first assessed by estimating the number of unique haplotypes and variable characters in each partition, because variable markers are critical for examining differentiation within and among closely related populations. A second measure of utility, the proportion of parsimony informative characters per total length of alignment, was also calculated. A larger number of parsimony informative characters does not necessarily result in greater resolution, or support for clades, but we include it as a first measure to estimate efficiency of data collection.

Maximum parsimony (MP) methods were used to generate phylogenetic hypotheses using PAUP* (Swofford, 2002). Parsimony analyses were conducted on each partition and on the two combined datasets. Two MP analyses were conducted per partition (RS, CO1, CR_{882}, CR_{537}, 12S), one with all characters weighted equally and a second with all characters weighted on the re-scaled consistency index (RCI). Transversions and transitions were treated equally and gaps were weighted equally with substitutions (Ogden and Rosenberg, 2007). Heuristic searches were performed using tree bisection-reconnection (TBR) branch-swapping and using the steepest descent option. One million random addition sequence replicate searches were performed for all analyses. Due to the large number of trees in RS analyses, only three trees were saved per replicate. Nodal support for all parsimony analyses was assessed using non-parametric bootstrapping (Felsenstein, 1985), which was computed from 10^4 replicates using a heuristic search, TBR branch-swapping and saving 100 random addition sequence replicates per replicate.

Bayesian methods were also used to generate phylogenetic hypotheses for all partitions of the data, and the two combined datasets. Appropriate models for sequence evolution were explored (Modeltest 3.7, Posada and Crandall, 1998; Mr. Modeltest 2.2, Nylander, 2004). However, there was uncertainty surrounding model choice for ingroup analyses because different models were chosen with hierarchical likelihood ratio tests (hLRT), the Akaike Information Criterion (AIC and AICc; Akaike, 1974), and Bayesian Information Criterion (BIC and BICc, Schwarz, 1978), especially for the smaller data sets. Models for final analyses were based on the model chosen by AIC. However, exploratory analyses using the alternate models were examined for conflict in topology and variation in support levels. For the RS data a single substitution rate (nst = 1) and a proportion of invariant sites (rates = propinv) was used. The presence of invariant sites (coding = noabsence) allowed the data set to have some cutting sites in all samples. For analyses of combined data sets, the models chosen for individual partitions were used and the partitions were unlinked. The relative rates were also unlinked by setting the rate prior to 'variable'.

Bayesian analyses were conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003; Huelsenbeck and Ronquist, 2005). For both exploratory and final analyses two simultaneous runs were conducted from random starting trees using four Markov chains (one cold, three heated, temperature of 0.2). In shorter exploratory analyses, five million generations were run and trees

Table 2
Data description for data partitions and combinations of partitions for the *boreas* group

Data description	Single partitions					Partition combinations	
	12S	COI	CR_{882}	CR_{537}	RS	All data: 12S/COI/CR_{882}/RS	Three partitions COI/CR_{882}/RS
Number samples	22	50	117	169	194	22	44
Length of alignment	890	394	882	537	60 sites[a]	2226	1336
Number unique haplotypes	15	18	59	45	31	19	40
Number variable characters[b]	25	27	115	100	30	154	141
Number parsimony informative characters[b]	18	19	91	76	22	111	106
Consistency index	0.90	0.80	0.69	0.66	0.68	0.78	0.73
Rescaled consistency index	0.78	0.72	0.61	0.58	0.58	0.69	0.68
Range of uncorrected *p*-distances (no gaps)	1.1–0.0	3.3–0.3	4.7–0.1	6.0–0.2	–	2.6–0.0	3.8–0.0

[a] The 60 restriction sites represent 360 bp; restriction enzymes recognized six bases at each cutting site.
[b] A character consists of a DNA base or restriction site.

were sampled every 400 generations. The program Tracer 1.4 (Rambaut and Drummond, 2007) was used to assess stationarity by examining plots of all parameter values against generation, convergence was assessed by comparing the values across four runs (two exploratory and two final). Analyses suggested that both stationarity and convergence were achieved for all individual and combined partitions of the data very early (within 500,000 generations all ingroup analyses and one million generations for analyses with outgroups). In the exploratory analyses we set a very conservative burnin of four million generations on the two runs which yielded 5000 trees total. In final analyses 20 million generations were run, trees were sampled every 1000 generations and burnin was set at five million generations yielding 30,000 trees total. These trees were used to create a majority rule consensus tree (FigTree, Rambaut, 2008) as shown in figures.

Two approaches were used to identify a root within the *boreas* group. Outgroup rooting was conducted with Bayesian methods described for final analyses above, on a combined 12S/COI/CR data set and 13 outgroup taxa. Bayesian methods described above were also used to root with a molecular clock (as in Steele and Storfer, 2006) except that the prior probability distribution on branch lengths was set to a coalescence clock model, which forces a root on the tree. Ingroup rooting with a molecular clock was conducted on the larger CR data set and the two combined data sets.

3. Results

3.1. Identification of major clades and minor groups

A combined analysis of all data partitions (2226 characters, Table 2) from 22 specimens identifies three major haplotype groups (Fig. 2). Rooting with molecular clock methods identifies the three major groups as clades (but see outgroup rooting below). We refer to the three lineages as Northwest (NW), Eastern (E) and Southwest (SW); the clade names correspond to their respective geographic regions (Fig. 1). The three major clades correspond only in part with their taxonomic identities. The NW major clade corresponds roughly with the species *A. boreas* and includes the type localities for both subspecies, *A. b. boreas* (Columbia River and Puget Sound: Baird and Girard, 1852) and *A. b. halophilus* (Solano Co., CA; Baird and Girard, 1853), which is near San Francisco. The SW major clade includes *A. exsul*, *A. nelsoni* and some *A. canorus*, which are identified from their type localities and are discussed below. The SW clade also includes some divergent lineages in southern California currently considered *A. b. halophilus*. The eastern clade (E) is not differentiated taxonomically, but is currently part of *A. b. boreas*, and sister to the NW clade.

Due to the more intensive sampling ($N = 117$, Table 2) analyses of CR_{882} identifies all major clades as well as minor groups (Fig. 3), and refines geographic distributions (Figs. 1 and 4). Rooting with a clock model identifies the major groups as clades, but fails to resolve their sister relationships. Minor groups within the SW major clade (Figs. 3 and 4) include a weakly supported *A. nelsoni* (SW-*nelsoni*), a divergent and strongly supported group from nearby Darwin Canyon, Inyo Co, CA (SW-Darwin Canyon), and a strongly supported, but less divergent group of toads from the southernmost distribution of the *boreas* group in California (SW-SCA). Four genetically divergent but geographically close specimens of *A.*

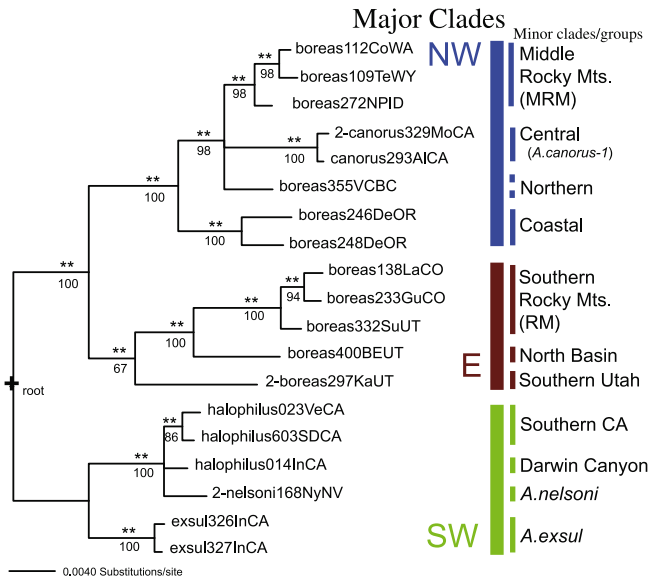

Fig. 2. Major haplotype clades: Bayesian majority rule consensus tree from analyses of 22 samples with all data types combined (2226 aligned sites, Table 2). The terminals are identified first by taxon names: *boreas* (*Anaxyrus b. boreas*), halophilus (*A. b. halophilus*), nelsoni (*A. nelsoni*), exsul (*A. exsul*) and canorus (*A. canorus*). The numbers after the species name are unique identification numbers for individual specimens (AMG numbers). Abbreviations for localities (as in Table 1) follow the AMG number. When multiple specimens had identical haplotypes, the number of specimens with that haplotype precedes the species name and all localities for that haplotype are identified. Bayesian posterior probability values are above the branches and are indicted by double asterisks (**) for values 97–100, numeric values are provided for lower support values. Numbers below the branches are bootstrap values above 50 based on RCI-weighted parsimony analyses of the same data set. The major haplotype clades (NW-northwest, SW-southwest, E-eastern) are identified by thick bars to the right of the tree. The minor haplotype clades (identified by thin bars) or assemblages (identified by dotted lines) are identified in greater detail in the analysis of the control region (Fig. 3). The tree shown was based on the HKY + I evolutionary models chosen for each partition and the partitions were unlinked. Additional results (exploratory analyses not shown) including majority rule trees based on clock models and strict consensus trees of RCI-weighted parsimony analyses, did not conflict with, and varied little in support values, to the tree shown. The analysis shown was not rooted, but the position of the root (symbol +) was inferred from an independent analysis using a coalescence clock model.

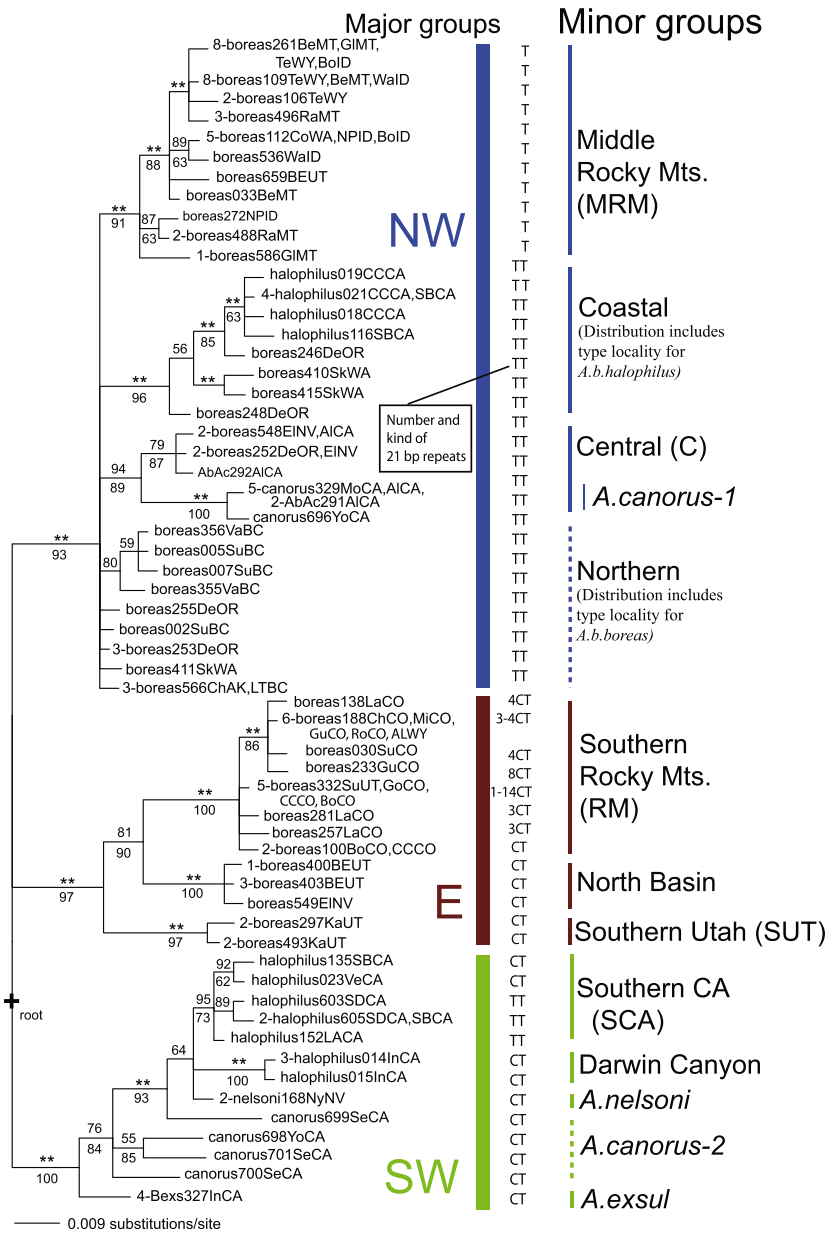

Fig. 3. Major and minor groups: Bayesian majority rule consensus tree from analyses of the large fragment of the control region (CR_{882}; 117 samples, Table 2). Terminals, Bayesian support values, bootstrap values and the root were identified as in Fig. 2 with one exception: the taxon designation AbAc represents a hybrid of *A. boreas* and *A. canorus*. The minor group names *A. canorus-1* and *A. canorus-2* follow the conventions of Shaffer et al. (2000). Thick bars identify the major haplotype clades (NW, SW, E, Fig. 1). Thin bars identify the minor haplotype groups (Fig. 4). Note that two minor groups, NW-northern in the NW major group and *A. canorus*-2 in the SW major group, are not clades and are identified by dotted lines. Majority rule tree shown was based on the HKY + I model. Additional exploratory analyses (not shown) based on HKY + Γ, HKY + I + Γ were identical to or consistent in topology with the analysis above and varied little in support values. The analysis shown was not rooted, but the position of the root (symbol +) was inferred from an independent analysis using a coalescence clock model. Notations to the right of the thick bars identify the kind and number of a 21 bp repeated fragment (Table 4). The letters "T" and "C" each refer to the specific sequence of the repeated fragments present (C = GTACA TATTA TGAAT GCA**C**G A; T = GTACA TATTA TGAAT GCA**T**G A). The number preceding the "C" refers to the number of copies of the "C" fragment if more than one copy was found, and a range is provided for terminals representing haplotypes from multiple specimens that were identical except for the number of repeated copies.

canorus form an additional paraphyletic assemblage within the SW group (called *A. canorus*-2 in discussions below). The mtDNA of *A. canorus* was previously found to be paraphyletic or polyphyletic (Graybeal, 1993; Shaffer et al., 2000; Stephens, 2001; Pauly et al., 2004) and analyses here show *A. canorus* to be paraphyletic and place the two previously recognized and highly divergent groups (*A. canorus*-1 and *A. canorus*-2) within the NW and SW major clades, respectively. The last SW minor haplotype group, *A. exsul*, is sister to all other toads in the SW major group. The Eastern (E) major clade consists of three strongly supported haplotype groups (Figs. 3 and 4), including toads at the northern end of the Great Basin (E-NBasin) at the border of Utah and Nevada, toads in the southern Rocky Mountains of Colorado including the Uinta Mts. of Utah (E-RM), and a third group of toads from southern Utah (E-SUT) that is sister to all other toads in the E major clade.

The NW major haplotype clade covers the largest geographic region and is comprised of three generally less divergent monophyletic groups and one non-monophyletic assemblage. The NW-middle Rocky Mountain (NW-MRM) group consists of toads from western Washington, Idaho, Montana, and northwestern Wyoming. A second group consists of toads along the western coast (NW-Coastal Fig. 3 and 4) from Washington, Oregon, and into

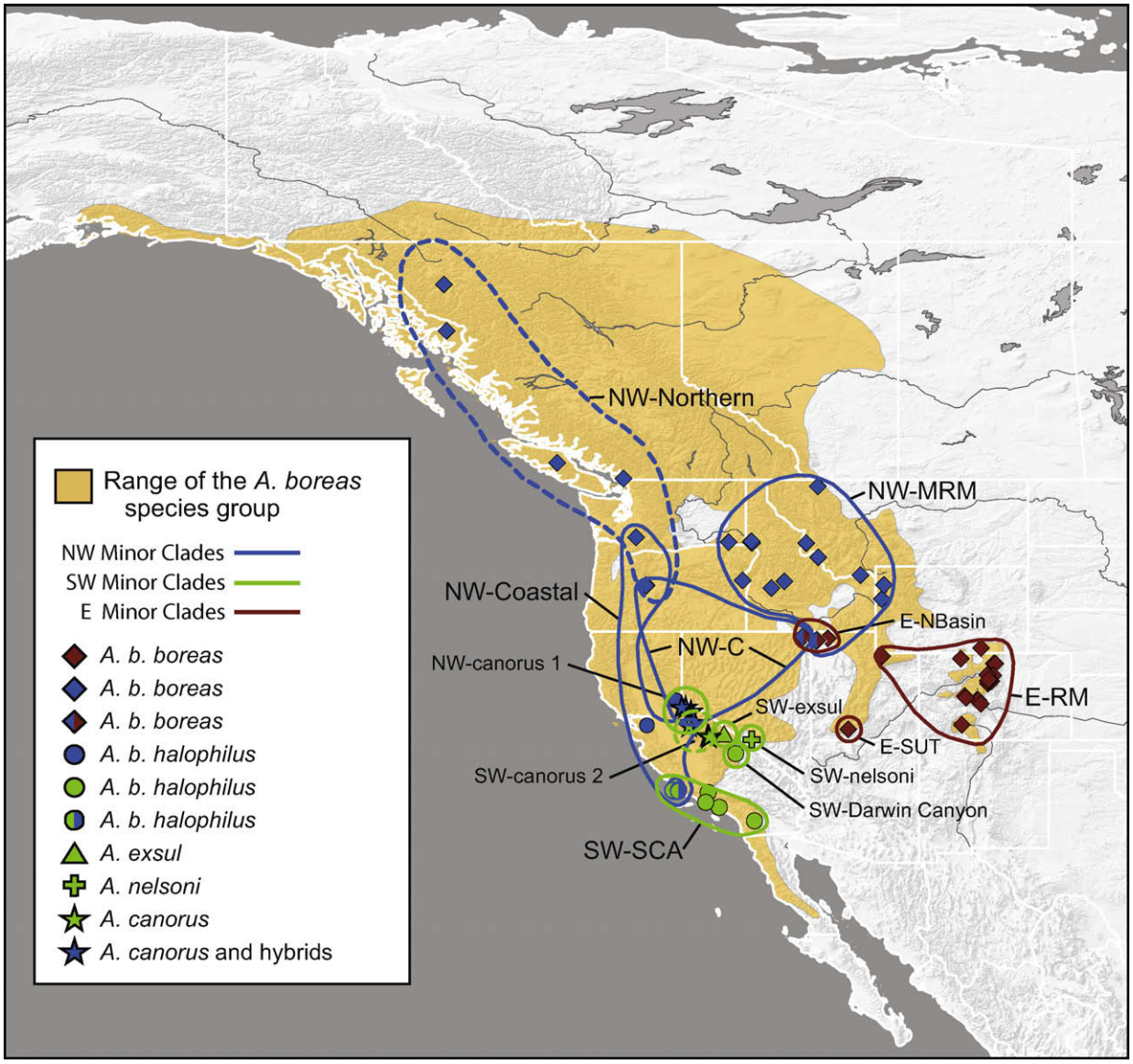

Fig. 4. Sample localities and distributions of minor haplotype groups. Specimens analyzed, species identities and map information are as in Fig. 1. Circles indicate geographic distributions of minor mtDNA haplotype groups. Groups drawn with solid lines are clades, groups drawn with dashed lines are non-monophyletic assemblages (NW-northern and *A. canorus*-2). Sites with multiple divergent mtDNA lineages are within overlapping circles.

California, as far south as Santa Barbara and east into the Sierra Nevada. This clade includes the type locality of *A. b. halophilus*. The NW-coastal clade corresponds to the northern distribution of the subspecies *A. b. halophilus* (Stebbins, 2003), although it extends further north, into Washington State. A third minor group, NW-central (NW-C), consists of toads from the central region of western US (northeastern Nevada, central Oregon, and the Sierra Nevada of California) and includes toads identified as *A. boreas* as well as *A. canorus*, and known hybrids of *A. b. boreas* × *A. canorus* (within *A. canorus*-1). All localities sampled from the NW-central clade share haplotypes with other NW clades (Deschutes Co., OR) or other major clades (E in northwest Nevada, SW at the northern end of the Sierra Nevada). A fourth group (NW-northern) is a non-monophyletic assemblage of toads in the northern coastal regions of North America from Oregon north into Canada and Alaska and the type locality for *A. b. boreas* (vicinity of Puget Sound; Schmidt, 1953) is found within the distribution of this group. The Bayesian majority rule tree using a coalescent molecular clock (not shown) identified NW-northern as monophyletic but this was not strongly supported (posterior probability 83%).

3.2. Data partitions

Bayesian analyses of RS, COI and 12S (Fig. 5A–C) although less resolved, are consistent with the major and minor groups discovered with CR_{882} (Fig. 3) and combinations of partitions (Figs. 2 and 5D) with a single exception. The COI data partition identifies the E group as polyphyletic, with the E-Southern Utah group sister to the NW clade rather sister to the rest of the E clade, although this placement is not strongly supported. Identical results for this placement were obtained in exploratory analyses using all models chosen for the COI partition (GTR + I + Γ, GTR + I, GTR + Γ, HKY + I + Γ, HKY + I, HKY + Γ) and placement as sister to the NW clade was supported in some analyses with posterior probability values up to 91%.

12S provided little data (Table 2) but results were consistent with other partitions and combinations of partitions. The majority rule consensus tree of 12S based on the HKY + I model chosen by the Akaike weights (Fig. 5C) was less resolved than exploratory analyses (not shown) with more complex models using a gamma distribution for across-site rate variation (HKY + I + Γ, one of the

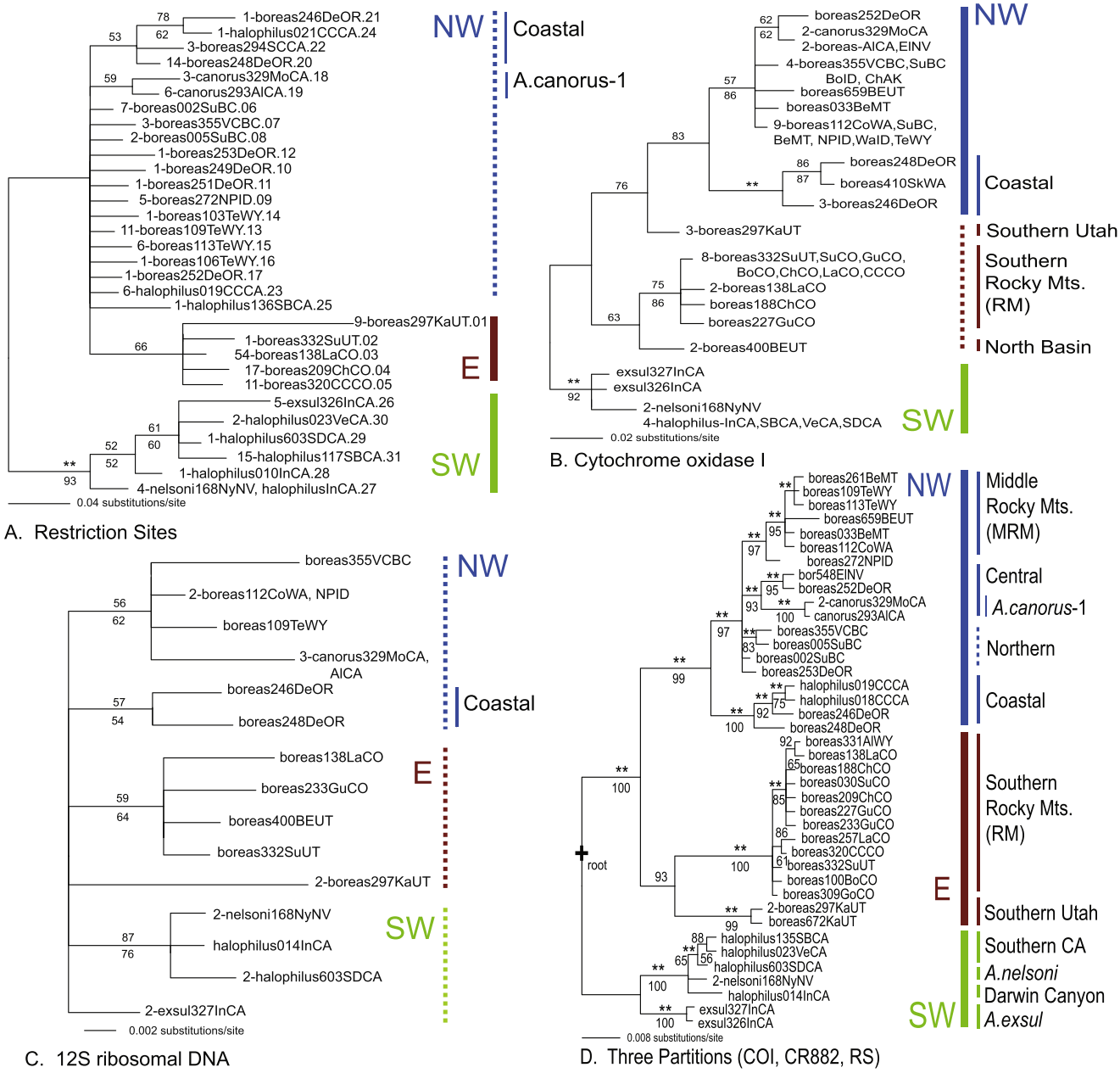

Fig. 5. Bayesian majority rule consensus trees from three analyses of individual partitions (RS, COI and 12S) and a combined analysis (COI/CR_{882}/RS). Terminals, Bayesian support values, bootstrap values, and roots were shown as in Fig. 2. Thick bars to the right of trees identify the major haplotype clades (NW, SW, E; Fig. 1). Minor groups (Fig. 4) are identified as clades (thin bars) or assemblages (dotted lines). (A) Bayesian majority rule consensus tree of restriction sites. The last number of each OTU is the haplotype number based on RS only identified in Table 1. (B) Bayesian majority rule consensus tree of cytochrome oxidase I (HKY + I model). (C) Bayesian majority rule consensus tree of 12S ribosomal DNA (HKY + I model). (D) Bayesian majority rule consensus tree of three partitions, COI/CR_{882}/RS, combined (HKY + I models for sequence data, partitions unlinked). The analysis shown is not rooted, but the position of the root (symbol **+**) was inferred from an independent analysis using a coalescence clock.

models chosen by hLRT, and GTR + I + Γ, used with outgroups), and with analyses using a molecular clock. Although analyses with more complex models identified the major clades E, NW, SW and some minor clades (as found in partition combinations) support was still low, but higher than for less complex models (Lemmon and Moriarity, 2004). The combined analyses of three partitions excluding 12S (COI/CR_{882}/RS, Fig. 5D) identified all major groups and all minor groups for which the larger data set were available (samples from E-north Basin and from *A. canorus*-2 were missing some data). Thus, in analyses of the *boreas* group only, excluding the 12S (loss of 890 bp) resulted in the loss of only a few variable and parsimony informative characters (Table 2), but due to the hierarchical sampling strategy, doubled the number of samples that could be analyzed without missing data.

Within CR_{882}, the number of copies and the sequence of a 21-bp repeated region showed a phylogenetic pattern (Fig. 3, Table 4). The repeat varied in number from a single copy to more than 14 copies although the exact number of copies was not identified in samples with large numbers of repeats (greater than 14) due to poor sequence data common in long highly repeated regions. Within the *boreas* group, the sequence of the repeat varied at site 19, where specimens had either a "C" or a "T" (Table 4). Some phylogenetic patterns can be seen in both the number and sequence of copies although the patterns were not always fixed among clades (Fig. 3). The sequence of the repeated fragment seems to be relatively conserved; additional variation was found at one site in a single specimen of the *boreas* group and the homologous fragment could be found in all outgroups (Table 4). The number varied with-

Table 3
Data description and tree information of analyses of 22 samples in the *boreas* group with all data types

Data partition or combination:	12S	COI	CR_{882}	CR_{537}	RS
Length of alignment in base pairs	890	394	882	537	60 sites[a]
Number unique haplotypes	15	13	17	14	14
Efficiency: % haplotypes per bp	1.6%	**3.3%**	1.9%	2.6%	**3.9%**
Number variable characters	25	23	**79**	54	24
Efficiency: % of variable characters per bp	2.8%	5.8%	9.0%	**10.1%**	6.7%
Number parsimony informative characters	18	19	**64**	44	19
Efficiency: % PI characters per base pair	2.0%	4.8%	7.2%	**8.2%**	5.3%
Range of uncorrected *p*-distances (no gaps)	1.1–0.0	3.0–0.0	4.5–0.0	6.0–0.0	–
Length of most parsimonious tree	29	29	104	73	30

Numbers discussed in the text are in bold and underlined.
[a] The 60 restriction sites represent 360 base pairs.

in the *boreas* group, but high numbers of copies were found only in the "C" copy and in the E-southern Rocky Mountains, except for one specimen from Contra Costa Co., CA, that had eight or more copies of "T". Only a single copy was found in outgroups. Although neither the number of copies nor the sequence variation were included in the analyses of the whole group, both seem to show some phylogenetic information that might be useful in examining regional variation.

The utility of partitions varied in a comparison of 22 samples with all data (Table 3). The shortest fragments (RS and COI) were the most efficient in identifying the largest number of unique haplotypes per base pair of sequence obtained (3.9% and 3.3% respectively). The larger CR_{882} fragment identified the greatest total number of variable (79) and parsimony informative characters (64), but the smaller CR_{537} fragment was the most efficient in identifying the greatest proportion of variable (10.1%) and parsimony informative characters (8.2%) per length of sequence obtained. Comparing only three efficiency parameters (% haplotypes/bp, variable characters/bp, and parsimony informative characters/bp), CR_{537} was the most efficient in identifying variable and parsimony informative characters per length of sequence obtained. Analyses of CR_{537} (not shown) included only 61% of the larger CR_{882} fragment. This resulted in the loss of 14 unique mtDNA haplotypes, however, all relationships were identical to analyses with CR_{882} data and all major and all but one minor group was resolved (some haplotypes from the southern California were identical to *A. nelsoni* haplotypes).

3.3. Rooting

Bayesian analysis conducted with a coalescence clock identifies the SW group as sister to a NW/E clade (Figs. 2, 3 and 5D). Bayesian analyses with outgroups (Fig. 6) strongly supports the monophyly of the *boreas* species group, the monophyly of both the E and NW major clades, and the monophyly of a combined E/NW clade. However, the majority rule tree identifies the SW group as paraphyletic, and *A. exsul* as sister to the NW/E clade. This root placement is not strongly supported, but suggests that at least portions of the SW, if not the entire SW, may be ancestral in the species complex. With the exception of root placement, all relationships within the *boreas* species group identified from analyses with outgroups, are consistent with analysis of ingroups only. Strongly supported relationships among the taxa used as outgroups were consistent with strongly supported results from previous analyses of mitochondrial genes (Frost et al., 2006a; Pauly et al., 2004; Graybeal, 1997; Pramuk et al., 2001; Pramuk, 2006). In exploratory analyses the root position was affected by outgroups chosen; rooting with single species within the Nearctic clade resulted in various weakly supported placements of the root (analyses not shown). However, rooting with multiple divergent species in the Nearctic clade, rooting with species in the *Ollotis* or *Chaunus* genera, or a combination of Nearctic and *Ollotis*/*Chaunus* always resulted in a root placement between *A. exsul* and the rest of SW.

4. Discussion

4.1. Discovered mtDNA Clades

The phylogenetic pattern of mtDNA indicates that the species *A. boreas*, as recognized by Stebbins (2003), is not monophyletic (Figs. 2, 3 and 5D). *Anaxyrus boreas* is either paraphyletic, with multiple localized species (*A. exsul*, *A. nelsoni*, *A. canorus* and perhaps other undescribed taxa) derived from within *A. boreas*, or *A. boreas* is polyphyletic and comprises only portions of three major mtDNA clades, NW, SW and E (Fig. 3). The subspecies *A. b. boreas* occurs in both the NW and E major clades, and A. *b. halophilus*, in the SW and NW. We suggest that *A. boreas* comprises a widespread clade corresponding only to the NW major clade whose distribution includes the type locality (mouth of the Columbia River, Baird and Girard, 1852). Although taxon rank (species or subspecific evolutionary units) is not clear based solely on mtDNA, the NW haplotype groups and assemblages comprise a set of monophyletic units. *Anaxyrus boreas boreas* is best represented by the NW-northern assemblage, because the type locality occurs within its distribution (vicinity of Puget Sound; Baird and Girard, 1852). *Anaxyrus boreas halophilus* is best represented by NW-coastal, because this mtDNA haplotype is the only one that occurs in the vicinity of the type (Benicia, Solano Co., CA; Baird and Girard, 1853). The distribution of the mitochondrial NW-coastal clade and *A. b. halophilus* (Stebbins, 2003) differ somewhat at the northern and southern edges: the NW-coastal clade occurs a little farther north (into Washington State), but not as far south as the previously described *A. b. halophilus* (NW-coastal occurs only down to Santa Barbara, CA). Although we included few samples from central California, the sole distribution of the NW-Coastal haplotype in this region is supported by more extensive sampling by Stephens (2001), who identified the "central CA *boreas*" clade with a similar distribution to our NW-Coastal clade, and a similar relationship to the northern *A. canorus* and *A. boreas*. Our results are also consistent with the geographic distributions of clades/groups of Graybeal (1993) and Feder (1973), who examined mitochondrial cytochrome b sequences and allozymes, respectively. Careful morphological studies of the whole group, especially of the type specimens, are clearly needed in light of the mtDNA evidence because genetic analyses have previously identified unrecognized morphological differentiation (e.g., Shaffer et al., 2004; Vredenburg et al., 2007). More extensive analyses of nuclear data (e.g., genes examined in Feder, 1973; Maxson et al., 1981; Graybeal, 1997; Simandle, 2006; Pramuk, 2006; Frost et al., 2006a) and finer sampling would be valuable to determine taxonomic status.

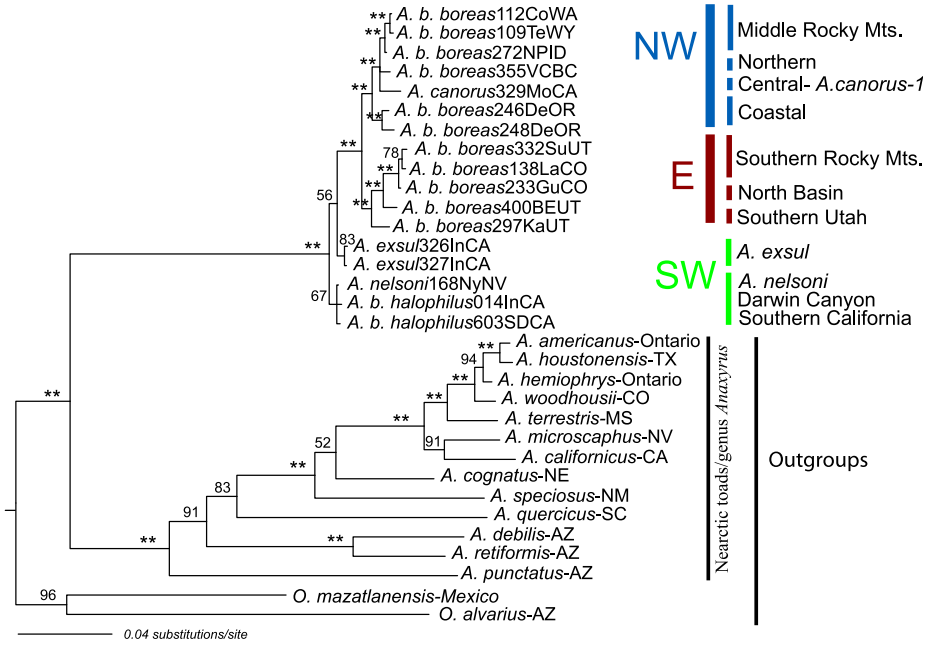

Fig. 6. Bayesian majority rule consensus tree of the *boreas* species group rooted with outgroups. Analysis based on 1671 bp of sequence data (Table 2) including the 12S (894 aligned sites), COI (394 aligned sites) and CR (379 aligned sites) DNA partitions. Analysis includes 35 unique haplotypes (from 40 samples) although some of the more divergent outgroups included in the analysis (Table 1) were removed from the figure so that the topology and branch lengths within the *boreas* group could be seen more clearly. Terminals, Bayesian posterior probabilities and clades are identified as in Fig. 2. Analysis is based on unlinked partitions and the GTR + I + Γ model for all partitions of the data.

Table 4
Sequence alignment of a 21 bp repeated region

Species groups	Number samples	Sequence of all 5′ copies variable site: 19	Sequence of final 3′ copy variable sites: 17, 19
boreas species group:			
Common forms:			
E-(all), SW(n = 16)	35	GTA CAT ATT ATG AAT GCA **C**GA	GTA CAT ATT ATG AAT GCA **T**GA
NW(n = 38), SW(n = 4)	42	GTA CAT ATT ATG AAT GCA **T**GA	GTA CAT ATT ATG AAT GCA **T**GA
NW-MRM	29		GTA CAT ATT ATG AAT GCA **T**GA
Unique sequence:			
NW-MRM (AMG586)	1	GTA CAT ATT ATG AAT GCA **T**GA	GTA CAT ATT ATG AAT G**T**A **T**GA
Outgroups:			
americanus species group	5		GTA CAT ATT AT**T** AAT G**T**A **TW**A
A. microscaphus	1		GTA CAT ATT AT**T** AAT G**T**A **TV**S
A. punctatus	1		GTA CAT ATT AT**T** AAT GCA **TA**G
O. mazatlanensis, *O. alvarius*	2		GTA CAT ATT ATG **Y**AT GCA **T**GA
C. marinus	2		GTA CAT ATT ATG **Y**AT GCA **C**GA

The number of copies and the sequence of the repeat fragment varied within and among major groups (Fig. 3).
When sequence variation was found in outgroups the variation was identified with standard abbreviations: W = A/T, V = A/C/G, S = C/G, Y = C/T.

We suggest the SW major clade corresponds to a suite of new and previously described species or assemblages (Figs. 3 and 4). These include *A. exsul*, *A. nelsoni*, several lineages from southern CA including Darwin Canyon (currently regarded as *A. b. halophilus*) and the assemblage *A. canorus-2* (discussed below). *Anaxyrus exsul* occurs in only four isolated desert springs in the Deep Springs Valley, between the Inyo and White Mountains of California (Fellers, 2005; Simandle, 2006), the type locality. The small population size and relatively long time of geographic isolation (Hubbs and Miller, 1948) are consistent with the monophyly and high divergence found in mtDNA here. *Anaxyrus nelsoni* is currently known only from several desert springs and the Amargosa River within the Oasis Valley, NV (Altig and Dodd, 1987; Goebel et al., 2005; Simandle, 2006) and specimens for analyses here were collected from the type locality at Crystal Springs. The mtDNA of two *A. nelsoni* were sister, but were not highly differentiated from mtDNAs in southern California. This lack of divergence suggests a close relationship to previously unrecognized lineages of the SW clade. A broad distribution of close relatives is further supported both by allozyme data (Feder, 1973), which identified populations in Owens Valley and Darwin Canyon that shared alleles (in low frequency) with *A. nelsoni* and *A. exsul* and by the wider distribution of *A. nelsoni*, suggested in early studies (Stejneger, 1893; Linsdale, 1940; Wright and Wright, 1949; Karlstrom, 1962). In contrast, results from Pauly et al. (2004) suggest that some *A. nelsoni* mtDNA haplotypes are nested within our NW group (one specimen of *A. nelsoni* was more closely related to *A. boreas* of Alaska and *A. canorus-1*, than to *A. exsul* and toads from southern CA). It is possible that like *A. canorus*, mtDNAs of *A. nelsoni* may contain haplotypes of both the NW and SW mtDNA major clades. The clade, SW-Darwin Canyon, has a divergent haplotype but is not recognized taxonomically. The minor clade with the largest distribution, SW-southern CA is found only in southern California. This clade is consistent with the "southern *boreas*" clade of Stephens (2001) in its distribution and

relationship to the southern *A. canorus*. Similarly, Graybeal (1993) found *A. boreas* from San Diego to be sister to the southern *A. canorus*, and both were closely related to *A. exsul*.

The eastern mtDNA clade comprises three divergent groups. Southern Utah (E-SUT), is a disjunct population discovered in 1994 (Ross et al., 1995). The group E-north Basin is similarly divergent, but haplotypes from the NW-central and NW-middle Rocky Mountains also occur in the region. The E-Rocky Mountain clade was discovered largely from the geographically disjunct region in Colorado and southeastern Wyoming (the Southern Rocky Mountain Population, SRMP), but a single haplotype from this clade was also discovered in the Uinta Mts. of Utah. The SRMP, listed as endangered in Colorado, is disjunct from all other toads (Fig. 1): the Red Desert and dry plains in southwest and central Wyoming serve as effective barriers between toads in northwest Wyoming and southeast Wyoming, and toads in Colorado are separated from those in Utah by at least 200 km and the dry intermountain basin of the Green River. The complete geographic isolation of the toads in the SRMP suggest that the closely related haplotype in the Uinta Mountains, Utah, is due to incomplete lineage sorting, commonly found in recently isolated groups.

Previous studies (Graybeal, 1993; Shaffer et al., 2000; Stephens, 2001; Pauly et al., 2004), found *A. canorus* to be polyphyletic or paraphyletic with *A. canorus*-2. The regional studies by Graybeal (1993) and Shaffer et al. (2000) identified *A. exsul* as a sister taxon to the southern lineage, *A. canorus-2*, corroborating a placement within the SW major clade. Data presented here identifies *A. canorus-2* as a paraphyletic assemblage, as was found by Stephens (2001). *Anaxyrus canorus-1* was found in this study to be within the widely distributed NW major clade (monophyletic with toads from northern and central CA as well as southern OR) and this is also consistent with Stephens (2001). The derivation of *A. canorus* from within *A. b. boreas* was suggested by both Stebbins (1951) and Karlstrom (1962) based on morphological similarities, and this is consistent with finding the *A. canorus*-1 lineage within the NW major clade. At this point *A. canorus* appears to be either multiple entities or derived from multiple divergent mtDNA lineages.

Results here are remarkably consistent with the very first molecular phylogeographic analysis of the group (Feder, 1973) based on allozyme data. UPGMA dendrograms, based on distances between populations, showed *A. exsul* to be most genetically similar to *A. nelsoni*, and an *A. exsul*/*A. nelsoni* group to be most similar to a *A. b. boreas*/*A. b. halophilus* group. Feder examined *A. b. boreas* only from Washington near the type locality (our NW-Northern group), and her *A. b. halophilus* were collected from within the distribution of our NW-Coastal clade; thus her results from nuclear DNA are similar to those found with mtDNA. Feder did not sample *A. boreas* from southern California (SW-CA clade) so it is still unclear whether nuclear DNA will identify a SW-southern California clade found with mtDNA. In contrast to our study, Feder found *A. canorus* to be sister to all other specimens in the group. This finding may reflect the difficulty of rooting a group of close taxa with distant outgroups, or is a result due to sampling a paraphyletic *A. canorus* from both the SW and NW lineages.

4.2. Sympatry, hybridization and introgression among mtDNA lineages

Introgression of mtDNA is of concern because it precludes accurate identification of organismic lineages with mtDNA analyses. Hybridization is of special concern among toads because both close and divergent species interbreed where they are sympatric (or in captivity; Blair, 1972a), and F1 specimens develop. This unusual level of hybridization in toads may occur because of external fertilization and the "trial and error" method of mate recognition by males in this species group (Karlstrom, 1962). Within the *boreas* group, *A. boreas* hybridizes with *A. hemiophrys* in Alberta (Stebbins, 2003), with *A. microscaphus* in southwestern Utah (Blair, 1955), and with *A. punctatus* in California, despite differences in habitat preferences, species-specific male mating calls, and different timing of reproduction among species (Feder, 1979). In addition, hybridization among lineages of divergent species may not always be identified by morphology (Lamb and Avise, 1987); some F1 hybrid individuals between *A. boreas* and *A. punctatus* were not recognized without genetic data (Feder, 1979). If hybrids from taxa that are highly morphologically divergent cannot be identified in the F1, surely hybrids among morphologically similar lineages go unnoticed. However, the occurrence of hybrids is not always associated with introgression and does not always imply conspecificity (i.e., lack of speciation, Mebert, 2008; Nosil, 2008). All hybrids identified in this study (from morphology) were among closely related lineages and limited to the NW-central minor group. Hybrids of *A. boreas* and *A. canorus* were identified by collectors at the northern end of the range of *A. canorus* (Figs. 2 and 4). Hybridization studies produced F2 hybrids of *A. canorus* and *A. boreas* in the laboratory (Blair, 1972c), but the collection localities of these specimens were not identified by Blair so their correlation with mtDNA studies is not clear. Hybridization between *A. b. boreas* and *A. b. halophilus* in northern California was mentioned, but not described in any detail by Camp (1917a) and Storer (1925), but the large range of sympatry was identified with morphological intermediates (Stebbins, 1951). It is not likely that specific levels of mtDNA divergence indicate reproductive isolation (Hillis, 1988). However, genetic distances (uncorrected *p*-differences) among *A. americanus*, and *A. hemiophrys*, used as outgroups here, had lower levels of mtDNA divergence than those found among the major clades in the *boreas* group yet they are maintained by hybrid zones (Green, 1983) with limited introgression (Green and Pustowka, 1997). Yet regions of sympatry are of special concern because introgression is possible, but not necessarily occurring, where the toads have the opportunity to interbreed. Analyses of nuclear genes that assort independently are critical in these regions.

4.3. Value of partitions

Due to the increased ease of sequencing, RS of the whole mtDNA are rarely used today in phylogenetic analyses and were thought to have a limited lifespan even when they were first collected (Felsenstein, 1992). But RS here provided two surprises. First, RS were most efficient at identifying the largest number of haplotypes per bp examined (Table 3), a characteristic that is very useful in identifying large numbers of individuals and in looking at very fine relationships (Avise et al., 1998; Waldman et al., 1992). A second surprise was the emergence of phylogenetic signal consistent with other sequence data, when RS were analyzed with Bayesian methods (Fig. 5A). Similar topologies among Bayesian analyses of data partitions suggest that RS data contain usable phylogenetic signal and, if available from past analyses, could be combined with sequence data rather than discarded. Similarly, 12S was one of the first DNA regions for which primers were developed (Palumbi et al., 1991) and was used commonly for vertebrate systematics. Despite the limited variability among close lineages (Tables 2 and 3), the gene can provide a tree topology consistent with larger data sets (Figs. 2 and 5C) especially with analyses using more complex models of evolution.

The control region provided a higher number of variable characters than ribosomal and protein-coding genes (Tables 2 and 3) as was found in previous studies (Liu et al., 2000; Fu et al., 2005). However, in some species the 5′ end of CR_{882} contains inserts or repeated regions that make amplification, sequencing, or alignment difficult (Goebel et al., 1999; Liu et al., 2000; Smith and Green, 2004; Stöck et al., 2006; this study) and was excluded in analyses

with outgroups in this study due to both the inability to amplify the fragment in some species and difficulty in aligning taxa from multiple divergent species groups. The smaller CR_{537} fragment, which excludes the 5′ end of the longer CR_{882}, still provided the greatest number of variable and parsimony informative characters per bp examined (Table 3) with only slightly less resolution than the longer CR_{882} fragment. COI has been proposed as a gene useful in barcoding (Herbert et al., 2003; Herbert and Gregory, 2005), which is a process to provide a unique genetic identity for divergent lineages. In this group COI identified divergent lineages (Fig. 5B), even this small fragment (394 bp) would function as a barcode. Although barcoding has many limitations (Meier et al., 2006), CR_{882} or CR_{537} might be useful among bufonids to assist in this process.

4.4. Rooting and estimating time of divergence

Lack of a definite root is not uncommon in intraspecific phylogenetic analyses due to the high similarity of haplotypes within species or species groups and the often distant outgroup haplotypes (Castelloe and Templeton, 1994; Wood et al., 2008). In this study, rooting methods with a molecular clock provided consistent results (SW was sister to a NW/E clade in analyses with larger combined data sets), whereas rooting with outgroups suggested the root was within the SW group. Absence of a clearly inferred root precludes identification of monophyletic groups, because monophyly depends on root position. However, all lines of evidence suggest that the E and NW groups are monophyletic and that the SW group is either sister to the E/NW clade, or sister to that clade plus *A. exsul*.

Estimates of divergence times can be made from mtDNA sequence similarities if a relatively constant rate of molecular evolution is assumed (e.g., Shaffer and McKnight, 1996; Macey et al., 1998; Masta et al., 2003). We estimated times of divergence from a rate of 1.644% bp changes per lineage, per million years as estimated by Stöck et al. (2006) for control region sequences in *Bufo virdis*. We recognize that our estimate is limited because *B. virdis* is quite distant from *A. boreas* (Frost et al., 2006a) and estimated rates change both among lineages and with the depth of evolution. In addition, dates based on single mtDNA genes (compared to 5–10 nuclear genes) have a high variance (Carstens and Knowles, 2007) and the rate of 1.644% did not include an estimate of error. In the *boreas* group, the largest uncorrected pair-wise sequence divergences of CR_{882}, varied between major lineages (E-SW: 2.846–4.684%, E-NW: 2.163–4.299%, S-NW: 2.278–4.303%) about twice as much as within major lineages (E: 0.0–2.253%, SW: 0.0–2.088%, NW: 0.0–2.507). Estimated from rate of 1.644%, the mtDNA of the major groups began diverging at least 1.425–0.658 Mya, and mtDNA began diverging within major groups at least 0.762–0.685 Mya (NW-0.762, SW-0.635, E-0.685 Mya). In general, the divergence of mtDNA predates isolation of populations into species (Arbogast et al., 2002). Acknowledging the substantial variance that might be associated with these estimates, it is reasonable to assume that the major clades began diverging from each other as long ago as the early to mid-Pleistocene, and minor groups began diverging after that. This is consistent previous hypotheses of Pleistocene divergence within the group (Myers, 1942; Karlstrom, 1958 and 1962; Blair, 1972c; Maxson et al., 1981).

4.5. MtDNA phylogeography and biogeographic history

Pleistocene glaciation has long been thought to affect the evolutionary history of species in western North American (Avise et al., 1998; Pielou, 1991; Hewitt, 1996, 2000), leaving two specific phylogeographic patterns in multiple species. First, low diversity in many species of the northern regions of North America are often explained by range expansions following retreating glaciers (e.g., Highton and Webster, 1976; Zink, 1996 (birds), Green et al., 1996; Hovingh, 1997; Lee-Yaw et al., 2008 (amphibians), Soltis et al., 1997(plants); Conroy and Cook, 2000 (rodent)). This pattern is best seen in the NW-northern group (Fig. 4) because the control regions of toads in Alaska were quite similar to those in Washington State (Fig. 3). The NW-Middle Rocky Mountain Group also has less genetic diversity compared to the E clade although the geographic distributions sampled here were similar in size. Second, refugia from Pleistocene glaciations resulted in shared phylogeographic distributions of species. The Klamath-Siskiyou Mountains, near the border of Oregon and California, remained unglaciated throughout the Pleistocene and still contain high biological diversity and endemism (e.g., Wake, 1997; Wilke and Duncan, 2004 (Slug); Mead et al., 2005; Steele and Storfer, 2006). This region could have served as a refugium for boreal toads in the NW group, and allowed the divergence of the NW-coastal minor group from the more northern NW-northern assemblage (Fig. 4). Other refugia in the Pacific Northwest have been proposed (e.g., Columbia River, McCusker et al., 2000 (fish); Miller et al., 2005; Wagner et al., 2005) and these too may have resulted in distinct northern and southern lineages of multiple species of plants, salamanders and newts (Soltis et al., 1997; Brunsfield et al., 2001; Steele and Storfer, 2006; Kuchta and Tan, 2005) and the distinct minor groups seen in *A. boreas*. A similar pattern of species with northern and southern populations is seen in the Sierra Nevada in frogs (Macey et al., 2001), salamanders (Moritz et al., 1992; Tan and Wake, 1995), and snakes (Rodrgíuez-Robles et al., 1999) as well as *A. canorus* (Shaffer et al., 2000; Stephens, 2001) which occurs in both the NW and SW clades. Explanations for other patterns of divergence are less clear. Divergence among minor groups further from the coast (between the NW-northern and NW-middle Rocky Mountains) echoes variation found in diverse organisms, including amphibians, mammals and trees (Carstens et al., 2005a,b). However, the vicariance between western and inland populations of tailed frogs (*Ascaphus*; Nielson et al., 2001, 2006), giant salamanders (*Dicamptodon*; Daugherty et al., 1983), and lungless salamanders (*Plethodon*; Howard et al., 1993) resulted from drying of the Columbia Plateau after the rise of the Cascade Mountains during the Pliocene. These amphibians are all associated with streams or seeps in forest habitats and inland and western species are distinctly allopatric. *Anaxyrus boreas* occupies a wider range of habitats, and is currently distributed across the Columbia Plateau between the middle Rocky Mountains and Cascades (Nussbaum et al., 1983). It is more likely that the phylogeography of *A. boreas* in this region more resembles that of voles (*Microtus richardsoni*) and willow (*Salix melanopsis*), which show evidence of post-Pleistocene dispersal (Carstens et al., 2005a).

Species that were highly water-dependent were also impacted heavily by the complex pluvial cycles in the Great Basin, that may have resulted in multiple range contractions and expansions (Mifflin and Wheat, 1979; Stokes, 1986; Green et al., 1996; Hovingh, 1997; Hewitt, 1996 and 2000; Masta et al., 2003). Present distributions of salamanders (*Ambystoma tigrinum*) and anurans (*Lithobates pipiens*, *Rana luteiventris*, *Anaxyrus woodhousii*, *A. punctatus* and *A. boreas*) are all consistent with fragmentation of populations in the Pliocene and Pleistocene within the Great Basin region. Flooding over large regions from glacial melting could have allowed great dispersal distances perhaps explaining nearly identical haplotypes of the NW-central group, found in northern California, north eastern Nevada and central Oregon. Wet periods may have allowed toads to enter regions that are now geographically isolated by dry deserts, such as eastern California (*A. exsul*), Nevada (*A. nelsoni*), and the Southern Rocky Mountains in Colorado (SRMP). Subsequent isolation may have allowed populations to diverge. The complexity of the divergence pattern may depend heavily on

factors that are difficult to ascertain now, such as the number of pluvial cycles, population sizes, and whether ancestral haplotypes were retained or lost (e.g., Masta et al., 2003).

4.6. Conservation implications

A rearrangement of the taxonomy of the *boreas* species group would profoundly influence the conservation of several species and lineages, some of which have undergone recent declines (Hammerson, 1999; Corn, 2003; Davidson and Fellers, 2005; Muths and Nanjappa, 2005), or exist in small numbers of isolated, vulnerable populations (Stephens, 2001; Fellers, 2005; Goebel et al., 2005; Simandle, 2006). *Anaxyrus canorus* is a current candidate for Federal listing (US Fish and Wildlife Service, 2002), but is paraphyletic, split between the NW and SW haplotype groups. Different taxonomic outcomes from additional research are possible (recognition as two distinct species or, conversely, combination with other minor groups). These two possibilities would have significant but likely opposite effects on decisions to list populations as threatened or endangered. Populations of *A. b. boreas* in southern Wyoming, Colorado and northern New Mexico (SRMP) were removed from the US Federal candidate species list, because their loss would not significantly affect the distribution of *A. b. boreas*, and they were not genetically distinct from populations in Utah (Thompson, 2005). Recognition of the eastern major group as one or more distinct species could result in reconsideration of that decision. The *boreas* species group has many highly divergent and isolated lineages at the southern edge of its distribution and especially surrounding the Great Basin region (similar to the *Rana luteiventris*, Bos and Sites, 2001). Recognizing this phylogeographic pattern may encourage wildlife agencies to proceed with caution when managing and protecting toads and/or other amphibians in and surrounding the Great Basin, as they may be composed of many cryptic lineages.

Although we are cautious about delimiting species here, we, like Wood et al. (2008), believe some, if not many divergent mtDNA lineages are species, and provide a better reflection of species diversity than the current taxonomy. Several previous names exist that might be appropriate for phylogeographic groups. Provo, UT is the type locality of *A. pictus* (Cope, 1875) which was later determined to be *A. boreas* (Cope, 1889). This name may be appropriate for clades in the eastern portion of the region, depending on their taxonomic status. Specimens from Provo were not examined here, and both the E-N Basin and E-Rocky Mountain haplotype clades occur close by. The La Brea Tar Pits (Camp, 1917b) are the type locality for *A. nestor* (currently a synonym of *A. b. halophilus*, Tihen, 1962). This name may be appropriate for potential species within the SW clade, and falls within the distribution of the SW-southern California haplotype clade.

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