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The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite Toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data

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Abstract

We present a comprehensive survey of genetic variation across the range of the narrowly distributed endemic Yosemite toad *Bufo canorus*, a declining amphibian restricted to the Sierra Nevada of California. Based on 322 bp of mitochondrial cytochrome *b* sequence data, we found limited support for the monophyly of *B. canorus* and its closely related congener *B. exsul* to the exclusion of the widespread western toad *B. boreas*. However, *B. exsul* was always phylogenetically nested within *B. canorus*, suggesting that the latter may not be monophyletic. SSCP (single-strand conformation polymorphism) analysis of 372 individual *B. canorus* from 28 localities in Yosemite and Kings Canyon National Parks revealed no shared haplotypes among these two regions and lead us to interpret these two parks as distinct management units for *B. canorus*. Within Yosemite, we found significant genetic substructure both at the level of major drainages and among breeding ponds. Kings Canyon samples show a different pattern, with substantial variation among breeding sites, but no substructure among drainages. Across the range of *B. canorus* as well as among Yosemite ponds, we found an isolation-by-distance pattern suggestive of a stepping stone model of migration. However, in Kings Canyon we found no hint of such a pattern, suggesting that movement patterns of toads may be quite different in these nearby parklands. Our data imply that management for *B. canorus* should focus at the individual pond level, and effective management may necessitate reintroductions if local extirpations occur. A brief review of other pond-breeding anurans suggests that highly structured populations are often the case, and thus that our results for *B. canorus* may be general for other species of frogs and toads.

Keywords: *Bufo canorus*, Bufonidae, conservation genetics, declining amphibian, mitochondrial DNA, SSCP

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Introduction

Concern over the widespread decline of amphibian species has grown during the last decade (Blaustein & Wake 1990; Pechmann & Wilbur 1994; Wake 1998), particularly for species from western North America (Fellers & Drost 1993; Bradford *et al*. 1994; Drost & Fellers 1996; Fisher & Shaffer 1996). Of special concern are narrowly distributed species with small natural ranges, which include many species of plethodontid salamander and several species of bufonid toad (Stebbins 1951; Stebbins & Cohen 1995). Narrowly distributed species are at a greater risk of extinction than more widely distributed taxa because catastrophic environmental change may simultaneously affect all individuals of a spatially restricted species (Lande 1988). Their extinction risk may be further heightened if narrowly distributed species consist of a genetically homogeneous, panmictic population. For

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example, if gene flow is occurring throughout a species' entire range, then there may be an enhanced risk of decline due to transmittable diseases (Berger *et al.* 1998; Lips 1998). However, if narrowly distributed species are comprised of multiple, differentiated lineages (evolutionary significant units [ESUs] or management units [MUs], Moritz 1994) with little or no gene flow occurring among populations, then the extinction risk will be lower for the species in general, but individual subpopulations may be highly susceptible to loss (Driscoll 1998). In either case, a knowledge of population substructure is critical for developing management strategies for insuring the long-term persistence of narrowly distributed species.

Throughout western North America, most species of pond-breeding amphibians appear to be declining over some or all of their historical ranges (Stebbins & Cohen 1995; Drost & Fellers 1996; Fisher & Shaffer 1996). One of the central foci of these declines is the Sierra Nevada of California, USA, where catastrophic declines have been documented in nearly all of the anuran species examined to date (Kagarise Sherman & Morton 1993; Jennings & Hayes 1994; Drost & Fellers 1996). Among these taxa, the Yosemite toad, *Bufo canorus*, is the most narrowly distributed Sierran endemic, and appears to be showing a pattern of range-wide decline. Originally known from an approximately 240×60 km region of the central Sierra Nevada from 1950 to 3444 m elevation (Karlstrom 1973; Kagarise Sherman & Morton 1993), *B. canorus* was an abundant toad within its limited range at least until the early 1970s. However, during intensive field surveys from the early 1980s and continuing into the 1990s, precipitous declines in most populations were documented by three different field research teams (Martin 1991 cited in Stebbins & Cohen 1995; Kagarise Sherman & Morton 1993; Drost & Fellers 1996), raising serious concerns about the long-term viability of the species. These declines have been viewed as particularly disturbing as much of the range of *B. canorus*, including the regions that have been intensively resurveyed, is contained in relatively remote regions of Yosemite and Kings Canyon National Parks, where habitat destruction is not a concern. Fortunately, while virtually all known populations of *B. canorus* have shown dramatic declines, the species is still distributed over most of its original range, and many populations still have active breeding and recruitment (Kagarise Sherman & Morton 1993; Drost & Fellers 1996). Thus, the Yosemite toad represents a situation where population samples can still be obtained from most breeding sites, and molecular population genetic data can be utilized to identify population substructure that existed before declines became a serious concern.

With this in mind, we initiated a study of population substructure in *B. canorus*. Our primary goal was to assemble population-level samples from across the range of the species for molecular analysis. We collected a large set of samples and screened populations for variation in a 160-bp fragment of the mitochondrial cytochrome *b* gene using single-strand conformation polymorphism analyses (SSCPs) (Orita *et al.* 1989). We also collected 322 bp of sequence data for about 50 individuals of *B. canorus* and two of its close congeners, *B. exsul* and *B. boreas*. We used these data to quantify population substructure in *B. canorus* (at spatial scales ranging from adjacent ponds a few km apart to the range of the species), to identify potential ESUs and MUs within the range of this narrowly distributed species, to examine species boundaries between *B. canorus* and its close congeners, and to make specific recommendations on management strategies for this extremely sensitive, declining amphibian.

Materials and methods

Specimens

We collected a total of 427 tadpoles (generally < 1 cm total length) from 28 natural breeding sites from across the range of *Bufo canorus* in Yosemite and Kings Canyon National Parks, plus one sample (six tadpoles) of the related species *B. boreas* from Yosemite as an outgroup population. Localities are mapped in Fig. 1 and listed in Appendix I. Tadpoles were either frozen immediately in liquid nitrogen or preserved in the field in 95% ethanol.

Molecular methods

DNA was extracted with standard chloroform–phenol methods (Hillis *et al.* 1996). We PCR-amplified two different fragments of the cytochrome *b* gene (cyt-*b*) for different components of this study. For the population genetics component of this study, our data consisted of a 199-bp fragment that was amplified, screened for variation using SSCP, and sequenced to confirm the identity of all variants. For this fragment, we used the primer pair cytb-10 (5′-CGGCGGAGAAGGTTCTAA-3′) and cytb-6 (5′-GCGCCTCATTCTTCTTTATCT-3′), which yielded 160 bp of DNA internal to the two primers (199 bp total). SSCP (Orita *et al.* 1989) screening followed standard procedures described in Hiss *et al.* (1994). We used two different sets of SSCP running conditions during the course of this study. In the first, we used large format (33×41 cm plates) sequencing gel rigs. Samples were PCR amplified (annealing temperatures 50 °C), and 3 μL of PCR product and 1.8 μL of loading buffer (95% formamide, 0.25% bromphenol blue and 0.25% xylene cyanol) were heat denatured at 95 °C for 10 min, and then plunged immediately into ice-cold water. The samples were loaded onto a nondenaturing polyacrylamide gel consisting of 15 mL MDE gel (AT Biochem), 3.8 mL of 10× TBE (0.45 M Tris, 0.45 M boric acid, 10 mM EDTA), 240 μL

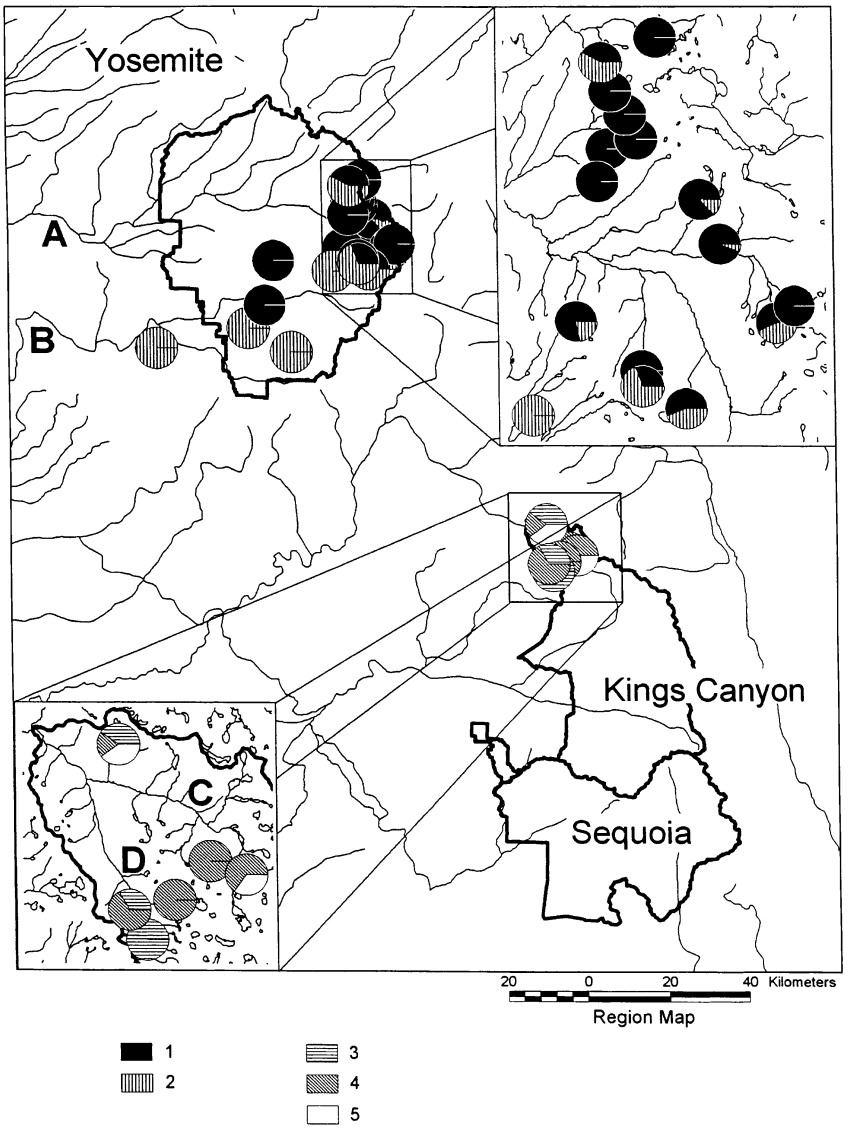

Fig. 1 Locality map for 28 *Bufo canorus* collection localities. Shaded circles show the allele frequencies of haplotypes 1–5; heavy black lines show the borders of Yosemite, Kings Canyon, and Sequoia National Parks. Major rivers referred to in the text are labelled as: A, Tuolumne River; B, Merced River; C, Evolution Creek; D, Goddard Creek.

of 10% APS, 24 µL of TEMED and 41.4 mL of deionized water. Electrophoresis was performed at a constant power of 8 W at room temperature for 14 h with 0.6× TBE as the gel buffer. Bands were visualized with standard silver staining (Hiss *et al*. 1994). In the second set of gel conditions, we used smaller-format (18 × 16 cm) 0.75-mm thick gels. Eight microlitres of PCR product and approximately 2 µL of loading buffer were heat denatured at 95 °C for 2 min and cooled on ice. The samples were loaded onto a gel consisting of 6.5% polyacrylamide, 3% glycerol, 3% 10× TBE, 0.2% TEMED, and 0.05% ammonium persulphate. The gels were run at 350 V for 4 h at room temperature with 0.6× TBE as the gel buffer, stained with ethidium bromide, visualized under UV, photographed, and scored from the photographs. When we began using the second set of conditions, we confirmed that both sets of electrophoresis conditions yielded identical results by examining known standards.

Genetic surveys using SSCP analysis should include a strategy for assessing false positives (distinct SSCP but the same DNA sequence) and false negatives (same SSCP, different DNA sequence), as both can commonly occur (Chakravarty *et al*. 1996). We used a PCR sample from a single individual as a reference standard at the beginning, middle, and end of each gel. Samples that differed from the standard in terms of band mobility or number were re-run on a common gel to further identify distinct SSCPs. Each distinct SSCP was then examined by bidirectional DNA sequence analysis of two or more PCR samples that were amplified from different individuals. Finally, with a knowledge of the relationship between SSCP and DNA sequence, SSCP haplotypes were assigned to samples on gels. This procedure led to sequencing many individuals that were identical, but it gave us confidence that our final scores were accurate.

We used the cyt-*b* primers MVZ 28 (5′-CGAGGC[C/G]-CC[T/C]GCAAT[A/G]ATAA-3′) and MVZ 43 (5′-GAGTC-TGCCT[A/T]AT[T/C]GC[C/T]CA[A/G]AT-3′) (Graybeal 1993) to obtain the complete DNA sequence of SSCP fragments. These *Bufo*-specific primers are external to cytb-6 and cytb-10, and amplify an approximately 450 bp fragment of cyt-*b* that allowed us to obtain a fully confirmed sequence for the cytb-6–cytb10 fragment without cloning, as well as providing additional nucleotide data on the subset of animals that we sequenced. We sequenced all individuals in both directions, and only fully confirmed portions of sequences were used for analysis. We conducted all sequencing with an ABI 377 automated sequencer. All sequences were confirmed for complementarity (using Sequencher v 3.0) and aligned with CLUSTAL W (Thompson *et al*. 1994).

Analysis

We used both phylogenetic and population genetic methods to analyse our results. For phylogeny reconstruction, we conducted parsimony analyses using PAUP* on 322 bp of aligned sequence for the 15 unique sequences among the 51 individuals that we sequenced or retrieved from GenBank. These included our own sequence data (11 unique *B. canorus* and one unique *B. boreas* sequence) as well as the homologous cyt-*b* sequence for *B. exsul* (one sequence), *B. boreas* (one unique sequence) and *B. canorus* (one unique sequence) reported by Graybeal (1993). For the analysis of population variation we used the analysis of variance procedure for sequence data (AMOVA) proposed by Excoffier *et al*. (1992), and implemented in either AMOVA or ARLEQUIN (both distributed by L. Excoffier). Using this procedure, we partitioned the total sequence variation into among- and within-subdivision components. Following Excoffier *et al*. (1992), we calculated ϕ statistics from these variance components at three levels: ϕ_{ST} is the correlation of random haplotypes within populations relative to the species as a whole (summarizing the among-pond variance) ϕ_{CT} is the correlation of random haplotypes within a group of populations relative to the species as a whole (summarizing the among-region variance), and ϕ_{SC} is the correlation of random haplotypes within populations relative to that found within its region. We considered each tadpole as an independent genotype, although it is possible that individuals from the same sibship were included in our samples. (If this were an extreme problem, which we doubt, it could inflate our interpretation of among-site genetic variance.) We included sequence divergence as well as haplotype frequency information for the 160-bp SSCP fragment to take full advantage of the added resolution that sequence divergence provides (Chenoweth *et al*. 1998). To analyse the relationship between geographical distance and genetic divergence among populations, we followed the Chenoweth *et al*. (1998) modification of Slatkin's (1993) isolation-by-distance approach. Using least-squares regression, we plotted effective female gene flow against geographical distance between all pairs of sites, where gene flow is estimated as

$$N_e m_f = 1/2[\phi_{ST} - 1]$$

and geographical distance is great circle distance between localities. We deleted all comparisons involving ϕ_{ST} values of zero or one because they lead to values of $N_e m_f$ that are either mathematically or biologically undefined. Under this model, a slope of −1.0 implies that the populations are consistent with female isolation-by-distance maintained by an equilibrium between genetic drift and dispersal. As the slope increases (i.e. approaches 0), this interpretation becomes less likely, and several nonequilibrium situations may be consistent with the data (Slatkin 1993).

Table 1 SSCP haplotype frequencies for 28 *Bufo canorus* populations and one *B. boreas* outgroup population. Population localities are described in Appendix I. Haplotypes are numbered, with their frequency within populations in parentheses

Population (no.)	Species	*N*	Haplotype (frequency)
Y483 (1)	*canorus*	4	1 (1.00)
S202 (2)	*canorus*	5	3 (.40), 4 (.20), 5 (.40)
S230 (3)	*canorus*	5	4 (1.00)
S532 (4)	*canorus*	20	4 (.65), 5 (.35)
S579 (5)	*canorus*	4	4 (1.00)
S584 (6)	*canorus*	10	3 (1.00)
S626 (7)	*canorus*	3	3 (.33), 4 (.67)
Y004 (8)	*boreas*	5	6 (1.00)
Y167 (10)	*canorus*	16	1 (.94), 2 (.06)
Y172 (11)	*canorus*	10	1 (1.00)
Y173 (12)	*canorus*	9	2 (1.00)
Y176 (13)	*canorus*	9	1 (.78), 2 (.22)
Y259 (14)	*canorus*	24	2 (1.00)
Y269 (15)	*canorus*	23	1 (.87), 2 (.13)
Y274 (16)	*canorus*	21	1 (.57), 2 (.43)
Y278 (17)	*canorus*	5	1 (.80), 2 (.20)
Y290 (18)	*canorus*	22	1 (1.00)
Y311 (19)	*canorus*	22	1 (1.00)
Y316 (20)	*canorus*	18	1 (1.00)
Y317 (21)	*canorus*	25	1 (1.00)
Y325 (22)	*canorus*	24	1 (1.00)
Y328 (23)	*canorus*	24	1 (1.00)
Y336 (24)	*canorus*	5	2 (1.00)
Y337 (25)	*canorus*	18	1 (.56), 2 (.44)
Y341 (26)	*canorus*	5	1 (1.00)
Y344 (27)	*canorus*	16	1 (.31), 2 (.69)
Y462 (28)	*canorus*	4	2 (1.00)
Y490 (29)	*canorus*	8	1 (1.00)
Y498 (30)	*canorus*	12	1 (.42), 2 (.58)

Y, Yosemite population; S, Sequoia/Kings Canyon (for these data, all from Kings Canyon) population.
N, number of haplotypes (individuals) sampled.

Table 2 Pairwise genetic distances between all *Bufo canorus*, *B. boreas*, and *B. exsul* haplotypes. Upper triangular matrix: percent sequence divergence for 322 bp of cytochrome *b* (top entry) followed by percent sequence divergence for 160 bp SSCP fragment (lower entry). Lower triangle matrix: absolute number of nucleotide differences for 322 bp of cytochrome *b* (upper entry) followed by absolute number of nucleotide differences for 160 bp SSCP fragment (lower entry)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. 172–3 (1)	—	0.006	0.003	0.031	0.028	0.034	0.040	0.043	0.037	0.031	0.034	0.034	0.028	0.025	0.034
		0.006	0.000	0.025	0.025	0.038	0.044	0.044	0.044	0.031	0.031	0.025	0.025	0.019	0.031
2. 337–12 (1)	2	—	0.003	0.019	0.016	0.022	0.028	0.031	0.025	0.019	0.022	0.028	0.025	0.022	0.031
	1		0.006	0.006	0.006	0.019	0.025	0.025	0.025	0.013	0.013	0.019	0.025	0.019	0.019
3. 278–4 (1)*	1	1	—	0.028	0.025	0.031	0.037	0.040	0.034	0.028	0.031	0.031	0.025	0.022	0.037
	0	1		0.025	0.025	0.038	0.044	0.044	0.044	0.031	0.031	0.025	0.025	0.019	0.031
4. 337–14 (2)	10	6	9	—	0.003	0.009	0.016	0.019	0.012	0.006	0.009	0.037	0.022	0.019	0.022
	4	1	4		0.000	0.013	0.019	0.019	0.019	0.006	0.006	0.025	0.025	0.019	0.019
5. 337–15 (2)†	9	5	8	1	—	0.006	0.012	0.016	0.009	0.003	0.006	0.037	0.019	0.016	0.019
	4	1	4	0		0.013	0.019	0.019	0.019	0.006	0.006	0.025	0.025	0.019	0.019
6. 202–4 (3)‡	11	7	10	3	2	—	0.012	0.016	0.009	0.003	0.006	0.043	0.019	0.016	0.019
	6	3	6	2	2		0.019	0.019	0.019	0.006	0.006	0.038	0.025	0.019	0.019
7. 532–4 (4)§	13	9	12	5	4	4	—	0.003	0.003	0.009	0.012	0.050	0.025	0.022	0.025
	7	4	7	3	3	3		0.000	0.000	0.013	0.013	0.044	0.031	0.025	0.025
8. 532–17 (4)	14	10	13	6	5	5	1	—	0.006	0.012	0.016	0.053	0.028	0.025	0.028
	7	4	7	3	3	3	0		0.000	0.013	0.013	0.044	0.031	0.025	0.025
9. 626–1 (4)¶	12	8	11	4	3	3	1	2	—	0.006	0.009	0.047	0.022	0.019	0.022
	7	4	7	3	3	3	0	0		0.013	0.013	0.044	0.031	0.025	0.025
10. 202–2 (5)	10	6	9	2	1	1	3	4	2	—	0.003	0.040	0.016	0.012	0.016
	5	2	5	1	1	1	2	2	2		0.000	0.031	0.019	0.013	0.013
11. 532–18 (5)**	11	7	10	3	2	2	4	5	3	1	—	0.037	0.019	0.016	0.019
	5	2	5	1	1	1	2	2	2	0		0.031	0.019	0.013	0.013
12. FC-12481	11	9	10	12	12	14	16	17	15	13	12	—	0.037	0.034	0.056
	4	3	4	4	4	6	7	7	7	5	5		0.038	0.031	0.044
13. FC-12659 (boreas)††	9	8	8	7	6	6	8	9	7	5	6	12	—	0.003	0.025
	4	4	4	4	4	4	5	5	5	3	3	6		0.006	0.019
14. B. boreas‡‡	8	7	7	6	5	5	7	8	6	4	5	11	1	—	0.022
	3	3	3	3	3	3	4	4	4	2	2	5	1		0.013
15. FC-12555 (exsul)	11	10	12	7	6	6	8	9	7	5	6	18	8	7	—
	5	3	5	3	3	3	4	4	4	2	2	7	3	2	

*Individuals 337–18, 344–19, 176–3, 311–1, 344–15, 269–11, 334–6, 317–15, 328–3, 274–3, 317–1, 337–17, 317–9 and FC-12484 are identical to this sequence for the 322 bp fragment.
†Individuals 274–1, 337–6, 344–11, 344–13, 344–16, 278–5, 176–4, 173–3 are identical to this sequence for the 322 bp fragment.
‡Individual 584–5 is identical to this sequence for the 322 bp fragment.
§Individuals 532–14 and 230–5 are identical to this sequence for the 322 bp fragment.
¶Individuals 579–3 and FC-12417 are identical to this sequence for the 322 bp fragment.
**Individuals 532–2 and 532–12 are identical to this sequence for the 322 bp fragment.
††Individuals FC-12499, FC-11668 and AG43 (Graybeal, 1993) are identical to this sequence for the 322 bp fragment.
‡‡Individual FC-12660 is identical to this sequence for the 322 bp fragment.

Results

SSCP accuracy and haplotype frequencies

We successfully amplified and genotyped 372 specimens of *Bufo canorus* plus an additional five *B. boreas* outgroup samples. Based on SSCP scores alone, we identified six putative haplotypes from a total of 29 populations (Table 1). Because SSCP can lead to both false positives and false, we obtained confirmed sequences for 43 individuals, or about 11% of our total sample. This included two representatives (when available) of all potential SSCP haplotypes, plus individuals that showed 'extra' ghost bands that sometimes appear on the SSCP gels. To examine the potential for false negatives, we reasoned that the greatest likelihood of variation existed among populations, and so we sequenced one representative of each haplotype class from most of the populations where it occurred.

In general, band mobilities on SSCP gels correctly identified actual DNA sequence variation. We sequenced a total of 18 individuals of haplotype 1 (from 11 different populations), 11 individuals of haplotype 2 (7 populations), two individuals of haplotype 3 (2 populations), seven individuals of haplotype 4 (4 populations), four individuals of haplotype 5 (2 populations), and one individual of haplotype 6. Of these, we found a single individual (337–12) that was misscored as haplotype 1, from which it differed by a single nucleotide substitution (Table 2). In all of the remaining cases, individuals that

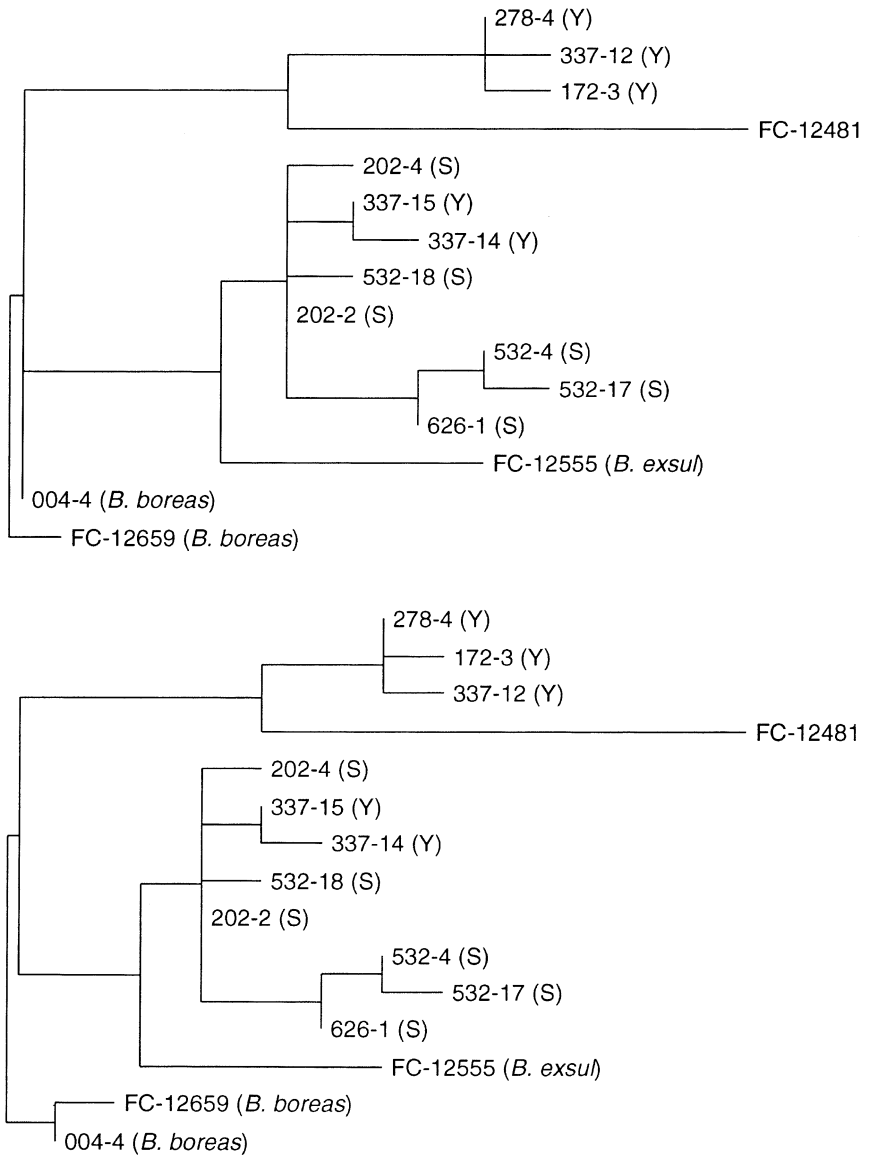

Fig. 2 Two equally parsimonious trees for 15 unique haplotypes of *Bufo canorus*, *B. boreas*, and *B. exsul*. Population number (Table 1), followed by the individual from that population and its source area, is shown for sequences produced for this study (i.e. 202–4 (S) is individual 4 from population 202, which is a Kings Canyon sample). FC-12481 is a *B. canorus* from Sonora Pass, Mono Co, CA; FC-12555 is a *B. exsul* from Inyo Co, CA; FC-12659 is a *B. boreas* from Mendocino Co, CA (all from Graybeal 1993). 004–4 is a *B. boreas* from the Yosemite Valley. Of 322 bp of data, 26 were variable, and 15 were parsimony informative. CI = 0.82, CI excluding uninformative characters = 0.71, rescaled CI = 0.69.

were scored as identical at the SSCP level had identical sequences. (Some of these individuals were different in the extra nucleotides that we sequenced outside of the cytb-6–cytb-10 region, but not in the SSCP fragment.) We did find that subtle variation, particularly in the form of extra, faint bands, led us to occasionally score individuals as different at the SSCP level when they were actually the same sequence. However, these instances were rare and were identified by sequencing all such individuals.

Haplotype frequencies for all populations are presented in Table 1 and mapped in Fig. 1 (SSCP haplotypes only). Three patterns immediately emerge from these data. First, *B. canorus* is distinct from a nearby population of *B. boreas* and thus there are no signs of introgression between these species. We can be relatively certain that the one *B. boreas* sequence is not present in *B. canorus*, although our restricted sampling of *B. boreas* makes it impossible for us to generalize to other California populations of this widespread species. Second, *B. canorus* from Kings Canyon are completely differentiated from Yosemite populations, with no shared haplotypes between the two regions. This is the case both for the SSCP haplotypes in Table 1, and for our data set of 42 haplotypes for 322 bp of sequence (see below). Haplotypes 1 and 2 are found exclusively in Yosemite, whereas haplotypes 3, 4 and 5 are restricted to our Kings Canyon samples. Third, local populations within Yosemite and Kings Canyon can be highly differentiated, to the point of being fixed for alternative haplotypes among adjacent ponds (Fig. 1).

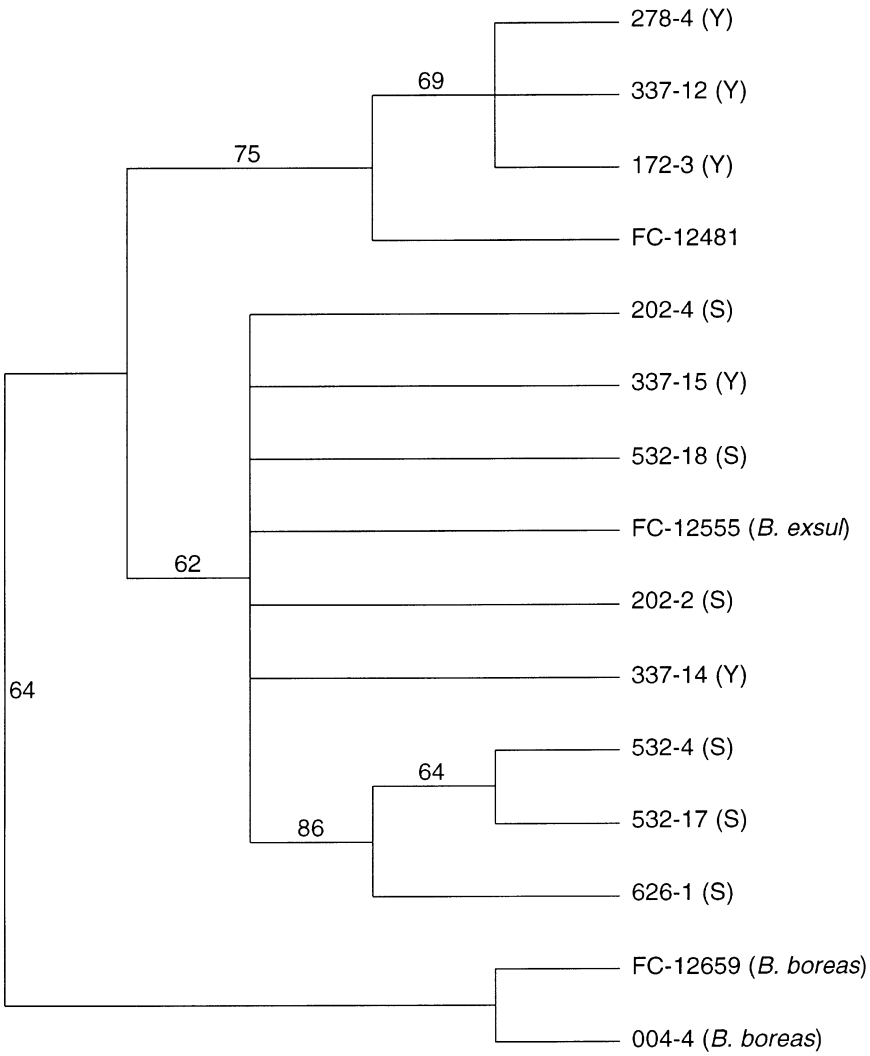

Fig. 3 Consensus bootstrap tree for 15 unique haplotypes of *Bufo canorus*, *B. boreas*, and *B. exsul*. Population numbers as in Fig. 2. Bootstrap proportions for 1000 pseudoreplicate samples (heuristic search) are above each branch; only values greater than 50% are shown on the tree.

Gene genealogy and geography in B. canorus

Our gene genealogy analysis is based on 322 bp of cyt-*b* sequence data and includes our own data and published sequences (from Graybeal 1993; identified in our paper by their FC accession numbers). The 12 unique *B. canorus* haplotypes that we identified (11 in our sample, plus FC-12481) varied by one to 17 nucleotide substitutions, or 0.3–5.3% sequence divergence (Table 2). When combined with additional sequences for *B. boreas* and *B. exsul*, several features emerge from the resulting gene genealogy (Figs 2, 3). At the species level, these data are consistent with the reciprocal monophyly of both *B. boreas* and *B. canorus* with respect to each other, but not to *B. exsul*. Both in terms of the two most parsimonious trees (Fig. 2) and the bootstrap consensus tree (Fig. 3), *B. boreas* may be either a monophyletic sister group to *B. canorus*, or is paraphyletic with respect to *B. canorus.* In addition, individual *B. boreas* from Alameda Co, CA, Mendocino Co, CA, San Diego Co, CA, and Lake Co, OR were identical to the two sequences reported here for this 322-bp fragment (Table 2), suggesting that California and Oregon populations of *B. boreas* may be monophyletic with respect to *B. canorus*. However, our data are not consistent with the monophyly of *B. exsul* with respect to *B. canorus*, and suggest that *B. exsul* is nested within *B. canorus*.

Within *B. canorus*, the sequence data provide some insights into the relationships of Yosemite and Kings Canyon samples, although bootstrap levels are relatively low (Fig. 3). For the 322-bp fragment of cyt-*b*, we found two clades with reasonable bootstrap support levels, one northern (Yosemite plus FC-12481 from Sonora Pass, bootstrap proportion = 75%) and one southern (Kings Canyon plus Lake Mary, Mono Co, CA [FC-12417], bootstrap proportion = 86%). In addition, a large set of mixed haplotypes from both regions plus the single *B. exsul* sample have uncertain phylogenetic affinities (Fig. 3).

Table 3 Population subdivision for *Bufo canorus* at different spatial scales. All analyses are sequence-based. ϕ statistics follow Excoffier *et al*. (1992), with the percent of the variation at a given level in parentheses. N/A values cannot be calculated for that level of analysis

Variation	*N*	ϕ_{CT} (%)	ϕ_{SC} (%)	ϕ_{ST} (%)
Among all ponds	28	N/A	N/A	0.76*** (76)
Kings Canyon vs. Yosemite	28	0.70*** (70)	0.63*** (19)	0.89*** (11)
Yosemite populations from:				
Tuolumne vs. Merced	20	0.40*** (40)	0.46*** (28)	0.68*** (32)
Cathedral	4	N/A	N/A	0.19*** (19)
Cathedral	3	N/A	N/A	0.22* (22)
Kings Canyon populations from:				
Goddard vs. Evolution	6	0.001 (0.1)	0.59*** (59)	0.59*** (41)
Goddard vs. Evolution	5	0.03 (3)	0.65*** (63)	0.66*** (34)

*$P = 0.078$; ***$P < 0.002$ level.
N, number of populations.

AMOVA results

Regional differentiation (ϕ_{CT}) at different spatial scales. We analysed population substructure at several spatial scales to quantify levels of variation in the 160-bp SSCP fragment of cyt-*b*: (i) across the range of *B. canorus* regardless of topographic features of the landscape; (ii) between Kings Canyon and Yosemite National Parks; (iii) between major drainages within Kings Canyon and within Yosemite; and (iv) at a very localized level within a single, small range (the Cathedral Range) in Yosemite.

We detected considerable variation among regions at most of the spatial scales examined (Table 3). At the largest regional level (Kings Canyon vs. Yosemite, for all 28 populations) Kings Canyon and Yosemite populations are clearly differentiated, with no shared haplotypes between these two regions (Table 2), and approximately 70% of the total molecular variance in our data set was attributable to variation among these two areas. Within Yosemite, populations which fall along the two major river drainages (the Tuolumne and Merced rivers) are also significantly different, and variance among these two regions accounts for about 40% of the total variation in Yosemite (Table 3).

This pattern of strong regional variation among drainages in Yosemite was not found in Kings Canyon. Here, our six breeding sites were distributed among two major drainages, three along Goddard Canyon on the upper San Joaquin River, and two (or three) along Evolution Creek, a major tributary of the San Joaquin. Population 2, although only about 2 km from Evolution Creek, is at the edge of its watershed, and as such is questionably within the Evolution drainage. We analysed the Kings Canyon data including (six populations) and not including (five populations) Population 2, and came to the same general conclusions (Table 3). In both analyses, variation among watersheds accounted for a negligible fraction of the total among-pond variation (0.1% or 2.6% for six or five populations, respectively).

Variation among ponds (ϕ_{ST}). ϕ_{ST} (analogous to F_{ST} for diploid nuclear loci) quantifies levels of variation among ponds (without respect to major subdivisions) in different parts of the range of *B. canorus*. For all levels analysed, among-pond ϕ_{ST} values were statistically significant and accounted for 19–76% of the total genetic variation found (Table 3). Levels of ϕ_{ST} were approximately similar across 20 Yosemite populations (0.68) and six Kings Canyon populations (0.59), indicating that among-pond variation is consistently high across the range of the species. Even at the finest spatial scale in our analysis, we found significant variation among four ponds in the Cathedral range separated by a total of about 7.5 linear (airline) km of habitat ($\phi_{ST} = 0.19$, $P \leq 0.002$). Only in the case of the three closest ponds from the Cathedral range, separated by a total of about 3 linear km, was the observed among-pond variance not statistically significant ($P = 0.078$). However, given that this is based on only three ponds, and that the variance attributable to among-pond differentiation is quite high (22%) this marginal significance level may reflect a low level of statistical power rather than a true lack of among-pond variation.

Isolation by distance in B. canorus. We examined isolation by distance at three spatial scales. Across all *B. canorus* populations (Fig. 4) and across all Yosemite populations (Fig. 5), isolation by distance was strongly negative, suggesting a reasonable fit to an equilibrium isolation-by-distance model. For the entire set of 28 populations (Fig. 4), the slope of the regression between female-effective migration and distance was −0.98, with an $R^2 = 0.40$. This level dropped across 20 Yosemite populations (slope = −0.60,

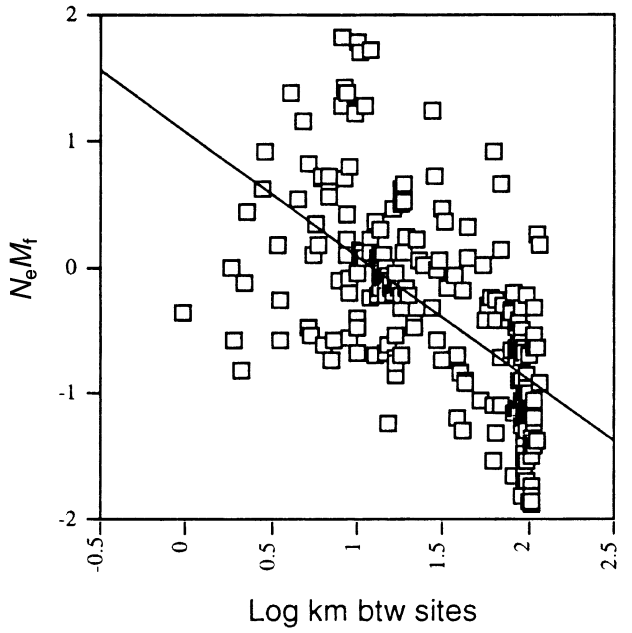

Fig. 4 Isolation-by-distance plot for all populations of *Bufo canorus* based on SSCP haplotype sequence data. For the least squares regression line, $y = -0.98x + 1.05$.

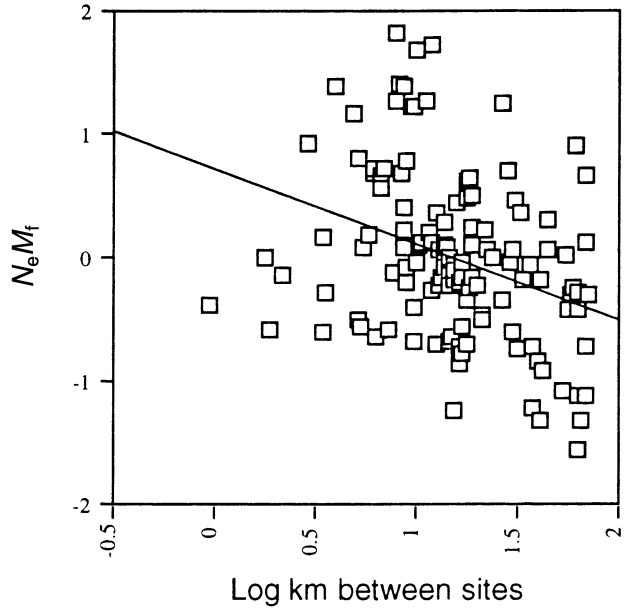

Fig. 5 Isolation-by-distance plot for Yosemite populations of *Bufo canorus* based on SSCP haplotype sequence data. For the least squares regression line, $y = -0.60x + 0.71$.

$R^2 = 0.11$. However, in Kings Canyon (Fig. 6), there was no hint of any effect of geographical isolation on levels of gene flow among populations (for all six populations, slope = 0.02, $R^2 = 0.00$).

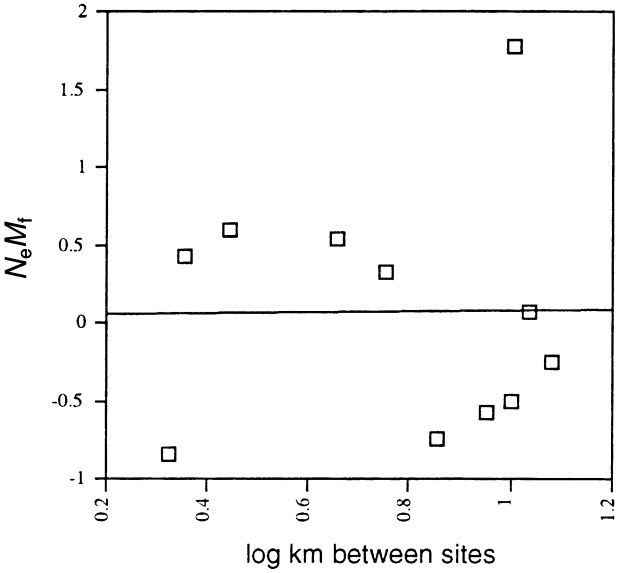

Fig. 6 Isolation-by-distance plot for Kings Canyon populations of *Bufo canorus* based on SSCP haplotype sequence data. For the least squares regression line, $y = 0.02x + 0.06$.

Discussion

Recent field work indicates that *Bufo canorus* has declined dramatically in the last 20 years throughout its range. Based on field surveys at two areas of Yosemite (Kagarise Sherman & Morton 1993; Drost & Fellers 1996), and one across the entire range of the species (Martin 1991; summarized in Stebbins & Cohen 1995), it appears that approximately 50% of the historic sites occupied by *B. canorus* are currently unoccupied (Jennings & Hayes 1994). Currently occupied localities also appear to have greatly reduced population sizes, with an approximate ninefold decrease documented over 20 years at one well-studied breeding site (Kagarise Sherman & Morton 1993). Given these declines, we consider two questions for which genetic data can inform us of the population biology, and thus the management of *B. canorus*. First, are there geographically defined units within *B. canorus* that can help guide management decisions? Second, are local breeding sites (ponds) sufficiently distinct to warrant management at the pond level, or is the ecologically appropriate management unit a drainage or larger landscape unit? Finally, we briefly discuss the broader management implications of these data for narrowly distributed amphibian species, and for pond-breeding amphibians in general.

Species limits, management units, and evolutionarily significant units in B. canorus

A key issue in the management of *B. canorus* is whether

or not it is a 'good' species. Under a phylogenetic or genealogical species concept (Baum 1992; Baum & Shaw 1995), this question reduces to whether *B. canorus* is monophyletic with respect to its close congeners *B. boreas* and *B. exsul*. When we combined our data with the published sequences for the homologous region of cyt-*b* in *B. canorus*, *B. exsul*, *B. boreas* across a wide geographical range (Graybeal 1993), we found two equally parsimonious trees (Fig. 2). In both cases, including the bootstrap consensus tree (Fig. 3), *B. canorus* is reconstructed as paraphyletic with respect to *B. exsul*, and the *B. canorus* + *B. exsul* group may or may not be monophyletic with respect to *B. boreas* (Figs 2, 3). As discussed by Graybeal (1993), some of the resolution of this problem centres on the proper rooting of the *boreas* group tree: we have used two haplotypes of *B. boreas* as an outgroup to *canorus* and *exsul*, which may or may not be appropriate. Given the limited mitochondrial DNA (mtDNA) variation that we have to work with, the fact that our data are at least consistent with a monophyletic *B. boreas*, and the morphological, colour pattern, and ecological differences that characterize all three taxa (Stebbins 1951), we see no compelling evidence to suggest a change in the taxonomic status of these species based on the currently available molecular evidence. However, if the results presented here hold up with greater sampling of *B. boreas*, it suggests that *B. exsul* be subsumed into a monophyletic *B. canorus*. Additional sequence data should help resolve this issue, and these data are currently being collected (A. Goebel, personal communication).

Whatever the status of *B. canorus* with respect to *B. boreas*, our AMOVA results suggest that two separate management units exist within *B. canorus*. The possibility that more than one entity may exist within *B. canorus* was initially suggested by Stebbins (1951). He noted that the light colour of the parotoid gland is sharply contrasting with the dark background colour of the head in toads from near Devil's Postpile in the San Joaquin river drainage east of Yosemite National Park, whereas those from Tuolumne Meadows (Tuolumne River drainage) to the north and east in Yosemite National Park lack this contrasting colour pattern. Because these observations were based on only two populations, Stebbins (1951) simply noted that 'The desirability of further study of geographical variation in the species is evident' (p. 248). Unfortunately, we lack samples from the vicinity of Devils Postpile, because it falls in the region between our southernmost Yosemite sample and our northernmost Kings Canyon animals. However, FC-12417 is from Lake Mary (Inyo Co, CA), which is a few kilometres east of Devils Postpile. This individual is identical in its mtDNA sequence to our haplotype 4 from Kings Canyon (Table 2), and is thus differentiated from our Yosemite material. Thus, there may well be two groups within *B. canorus* based on both morphological and molecular data, with a potential contact zone in far eastern Madera Co, CA.

Fine scale differentiation among B. canorus *populations*

Given the strong differentiation between Yosemite and Kings Canyon populations, we focus our discussion of fine-scale differentiation within, rather than between, these two regions. Interestingly, population differentiation follows a rather different pattern in these two areas. In Yosemite, a large and significant fraction of the population variation is among geographically defined regions and among ponds, regardless of the scale of analysis (Table 3). This is clearly the case among ponds in the Tuolumne and Merced river drainages (39.8% of the total variance is among these two areas), and by this genetic criterion, these two regions should probably be considered as separate management units. In addition, variation among ponds is a consistent source of genetic variation in Yosemite, regardless of the geographical scale of analysis. Finally, across all of the Yosemite populations, there is evidence of an isolation-by-distance pattern of genetic divergence (Fig. 5), with a slope of −0.67 implying a stepping stone-like model of isolation (Slatkin & Maddison 1990). Taken together, these results are consistent with the interpretation that *B. canorus* breeding ponds are relatively isolated in the Yosemite region, with occasional migration between nearby breeding sites.

The results from Kings Canyon are quite different and suggest a different population substructure in this nearby park. Although the AMOVA results confirm that there is considerable differentiation among ponds (Table 3, Goddard Canyon vs. Evolution creeks), there is no indication of any genetic variation among these two major drainages. In addition, there is no hint of increased genetic isolation with geographical distance among ponds (Fig. 6). Such a pattern suggests that Kings Canyon populations are not in equilibrium between migration and genetic drift. A lack of isolation by distance may be caused by many factors, including a complete lack of gene flow or a recent range expansion from within Kings Canyon (Slatkin 1993). We have no reason to favour either of these explanations at the present time, although additional data from more variable segments of the mitochondrial genome may help resolve this question.

Management implications for B. canorus

The population genetic data presented here, combined with what is known of the natural history and recent population declines in *B. canorus* (Kagarise Sherman & Morton 1993; Stebbins & Cohen 1995) imply several management strategies for this narrowly restricted, declining amphibian. First, *B. canorus* should continue to

be recognized as a species distinct from *B. boreas*, at least until additional molecular data become available. Second, Yosemite and Kings Canyon populations of *B. canorus* are sufficiently distinct genetically that they should be considered separate MUs, and managed as such (Moritz 1994; Moritz & Faith 1998). Finally, the high levels of among-pond variation suggest that on-site management for each breeding site is called for, and we should probably expect that extirpated populations will not naturally re-establish. This implies that reintroduction efforts may be required to re-establish extirpated populations, and that the stocks for such efforts should be from geographically proximate sites, especially in the case of Yosemite.

How general are these results for other anurans?

Given the widespread concern over anuran declines worldwide, an important consideration is the extent to which the pattern of highly substructured populations with restricted gene flow found in *B. canorus* is representative of other species. An examination of several recent papers suggests that anuran populations may generally show high levels of population substructure. A striking example comes from Driscoll's (1998) allozyme study of *Geocrinia vitellina* and *G. alba*, a pair of very narrowly distributed species of western Australian myobatrachid frogs. Although their ranges are tiny (encompassing a total of approximately 16 and 100 km^2, respectively), F_{ST}-values among conspecific populations were 0.30 and 0.44, indicating essentially zero realized gene flow among breeding sites, and leading Driscoll to recommend that on-site protection and human-mediated reintroduction programs are critical management tools.

This result appears to be general across a wide variety of habitat types and taxonomic groups. For example, Gascon and colleagues recently reported allozyme results for four species of anurans from the upper (Gascon *et al*. 1998) and one from the lower (Gascon *et al*. 1996) Amazon basin. In most cases, F_{ST} and/or Nei's *D*-values within species were high (F_{ST} = 0.37 for the one species reported, average D = 0.031 – 0.132 for the other four species), indicating restricted gene flow among lowland tropical populations. A series of studies of nearctic species shows the same pattern: the ranid *Rana temporaria* in the UK (allozyme data, F_{ST} among nearby urban sites is 0.388, among nearby rural sites is 0.145, Hitchings & Beebee 1997), the ranid *R. pipiens* in the southwestern USA (RAPD data, genetic distances among nearby breeding sites range from 0 to 0.2, Kimberling *et al*. 1996), and the bufonid *B. calamita* in the UK (microsatellite data, F_{ST} across a 200 by 400 km region ranged from 0.23 to 0.58, Rowe *et al*. 1998).

Based on this limited survey of anuran genetic substructure data, it appears that our results for *B. canorus* may apply to several other anuran taxa. Similar patterns have been known for a long time for salamanders (Shaffer & Breden 1989), suggesting that many amphibians may be characterized as genetically highly substructured. For species where this is the case, effective management will rely on two important pieces of information. First, we need to determine, on a case-by-case basis, the limits of the genetically defined management units within species, and be certain to protect the genetic integrity of these units. Second, as habitats become increasingly fragmented, managers and planners may need to include human-mediated reintroduction and dispersal among genetically similar sites into long-term management plans. Although this remains a controversial strategy (Dodd & Seigel 1991), carefully planned reintroductions can apparently work (as Rowe *et al*. 1998 discussed for *B. calamita* in Britain), and this may be the only viable option for maintaining adequate population numbers for some species.

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Appendix 1 Collecting localities for *Bufo canorus*

S202	Unnamed meadow 1.9 km NNW Evolution Meadow, Kings Canyon National Park, Tulare Co, California
S230	Eastern-most of McGee Lakes, Kings Canyon National Park, Tulare Co, California
S532	Meadow marsh area adjacent to SE end of Sapphire Lake, Kings Canyon National Park, Tulare Co, California
S579	Unmapped pond and meadow, 0.5 km S Mt. McGee, Kings Canyon National Park, Tulare Co, California
S584	Unnamed meadow adjacent to S. fork of San Joaquin river, 1.6 km NW Martha Lake, Kings Canyon National Park, Tulare Co, California
S626	Unnamed, unmapped meadow 400 m W of S fork San Joaquin river and 3.0 km NNW of Lake Confusion, Kings Canyon National Park, Tulare Co, California
Y004	Ahwahnee Meadow, Yosemite Valley, Yosemite National Park, Mariposa Co, California
Y167	Tioga road SW of Dana Meadows, 2.9 km SW Yosemite NP entrance station, Inyo National Forest, Mariposa Co, California
Y172	Porcupine Flat, Yosemite National Park, Tuolumne Co, California
Y173	Westfall Meadow, Yosemite National Park, Mariposa Co, California
Y176	Elizabeth Lake, Yosemite National Park, Tuolumne Co, California
Y259	Meadow along trail, 0.5 km S Merced Pass, Yosemite National Park, Madera Co, California
Y269	Meadow NE of western-most Gaylor Lake, 1.1 km SW of Granite Lakes, Yosemite National Park, Tuolumne Co, California
Y274	Spillway Lake, Yosemite National Park, Tuolumne Co, California
Y278	Marsh 0.5 km SW of Summit Lake/Mono Pass, Yosemite National Park, Tuolumne Co, California
Y290	Parker Pass Creek and Meadow, 2.1 km WNW Mono Pass, Yosemite National Park, Tuolumne Co, California
Y311	Western-most Young Lake, Yosemite National Park, Tuolumne Co, California
Y316	Meadow 1.3 km NE of western-most Young Lake, Yosemite National Park, Tuolumne Co, California
Y317	Ponds and meadow area, 0.9 km S Lake Roosevelt, Yosemite National Park, Tuolumne Co, California
Y325	Meadow S of pond 0.8 km W of Lake Roosevelt, Yosemite National Park, Tuolumne Co, California
Y328	Dingley Meadow, Yosemite National Park, Tuolumne Co, California
Y336	Marshy area adjacent to E side of Ireland Lake, Yosemite National Park, Tuolumne Co, California
Y337	Unnamed lake, 0.9 km NE of Ireland Lake, Yosemite National Park, Tuolumne Co, California
Y341	Meadow 0.8 km ENE of Evelyn Lake, Yosemite National Park, Tuolumne Co, California
Y344	East end of Evelyn Lake, Yosemite National Park, Tuolumne Co, California
Y462	Marshy area at Buena Vista Pass, 0.4 km E of Buena Vista Lake, Yosemite National Park, Madera Co, California
Y483	W side of Glacier Point road, 13 km W of Badger Pass road, Yosemite National Park, Tuolumne Co, California
Y490	meadow 0.3 km NE Upper McCabe Lake, Yosemite National Park, Tuolumne Co, California
Y498	Meadow 1.1 km WNW Sheep Peak, Yosemite National Park, Mariposa Co, California