

Population genetic structure of the toad *Bufo woodhousii*: an empirical assessment of the effects of haplotype extinction on nested cladistic analysis

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Abstract

Nested cladistic analysis (NCA) is increasingly being used to infer historical population-level processes, including population fragmentation, range expansion and long-distance colonization. However, the effects on interpretation of NCA inferences of stochastic extinction of haplotypes due to genetic drift (lineage sorting), or of haplotype loss via localized biotic or climatic influences, have not been thoroughly explored. We provide empirical evidence suggesting that NCA may misinterpret population history when haplotypes or haplotype groups from one clade are replaced by those of another clade. We do so by using NCA to analyse mitochondrial sequences from the toad *Bufo woodhousii* from 45 locations spanning the Great Plains and southwestern USA. Portions of this region were glaciated and/or desertified in the late Pleistocene and early Holocene, and hence uninhabitable for plains-dwelling organisms. Although NCA inferences of isolation-by-distance and gradual range expansion in *B. woodhousii* are compatible with expectations based on climatic data and toad biology, NCA also detected several instances of long-distance movement. Such movement seems unlikely, given the low vagility of this species. We conclude that inferences of long-distance colonization likely result from extinction of haplotypes in intervening areas. We suggest using additional methods to look for congruent inferences, and amending the NCA inference key, to help avoid misinterpretations resulting from haplotype extinction.

Keywords: *Bufo woodhousii*, haplotype extinction, lineage sorting, mitochondrial DNA, nested clade analysis, phylogeography

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Introduction

Nested cladistic analysis (NCA) has been increasingly used with a wide variety of organisms for making inferences about both ongoing gene flow and historical processes that may have influenced population structure (e.g. Hammer *et al.* 1998; Durand *et al.* 1999; Matos & Schaal 2000; Carbone & Kohn 2001; Creer *et al.* 2001; Wilke & Pfenninger 2002). The method was developed by Templeton *et al.* (1995) to separate historical (unique) and recurrent processes by integrating information from gene genealogies, haplotype frequencies and geographical data.

Previous methods based on haplotype frequency and geography, such as F_{ST} -type measures, do not make use of the historical information contained in genealogies, and therefore are not able to separate ongoing processes from historical ones. Therefore, NCA should, in principle, be better able to make a wider array of inferences about population genetic history.

To implement NCA, a haplotype network is first estimated by parsimony, and the network is then 'nested' into a series of increasingly inclusive sets (called clades) following a series of rules (Templeton *et al.* 1987; Templeton & Sing 1993; Crandall 1996). This information on the genetic relatedness of nested groups of haplotypes is combined with information on the geographical locations in which these haplotypes are found, and permutation analyses test whether a statistical association exists between nested

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haplotypes and geography. If a statistical association is found, NCA then uses the information from nested haplotype group geographical distances to make inferences about population history and ongoing gene flow.

The inferences NCA makes are based upon joint analysis of the patterns of geographical association within and between nested haplotype groups, and assume that a significant association of geography and nested haplotypes is due to some form of restricted gene flow. The patterns of geography and haplotype relatedness that are expected when gene flow has been restricted have been explored by Neigel and others (Neigel *et al.* 1991; Neigel & Avise 1993), and this assumption seems valid. Likewise, the prediction that fragmentation will cause genetic differentiation among geographically isolated populations has also been well-justified (reviewed in Hudson 1990). However, Templeton *et al.* (1995) cautioned that certain conclusions drawn from the NCA inference key might sometimes be incorrect, as they had not yet been supported by either computer simulation or existing population genetic theory. The predictions used to detect range expansion were based upon only one empirical finding (Cann *et al.* 1987), that range expansion causes some older haplotypes to be found in the area they originated, whereas younger haplotypes that arose as the population was expanding can be widespread and dispersed from the ancestral area. Thus, Templeton *et al.* (1995) concluded that empirical data from organisms known to have expanded their ranges were needed to provide support for this aspect of the inference key.

In a subsequent study, Templeton (1998) addressed the validity of the inferences used to detect range expansion by examining empirical data from 13 organisms for which a priori evidence of range expansion existed. NCA categorizes range expansion into two types, contiguous expansion and long-distance colonization. Using NCA, Templeton found evidence for either contiguous expansion or long-distance colonization in 12 of the 13 organisms, and judged that NCA was a robust method for detecting range expansion. He concluded that NCA may not detect all range expansion events, especially those accompanied by severe bottlenecks (Templeton 1998), but that the method is conservative and does not generally yield false positives (Templeton 2002).

We argue that NCA may sometimes incorrectly infer long-distance movements, by mistaking the localized extinction of haplotypes in areas between two locations for the long-distance movement of a haplotype from one location to another. This is because NCA cannot detect localized extinction of haplotypes, which may result either from range contraction and expansion caused by biotic or climatic influences, or from genetic drift (lineage sorting) or selection. Repeated range contractions and loss of haplotypes between regions have likely occurred quite commonly,

particularly during eras of cyclic climatic change such as the late Pleistocene, with its repeated glaciation events. We therefore expect that a nonrandom geographical distribution of haplotypes may commonly be found in organisms whose ranges have gradually expanded and contracted, and hence that NCA may incorrectly interpret such patterns.

Here, we provide data from an organism with minimal dispersal abilities for which there is a priori evidence of range expansion, in order to assess the robustness of the NCA inference key for correctly assigning long-distance vs. contiguous range expansion. In particular, we focus on the possible effects that extinction of alleles can have on patterns of geographical association of haplotypes, and the problems this may create when interpreting the NCA inference key.

The Quaternary period has been characterized by repeated cycles of glacial advance and retreat in the Northern Hemisphere. The northern regions of North America were covered in ice sheets during much of the Pleistocene, leaving the northern Great Plains glacier-free as recently as only 18 000 years BP (Pielou 1991 and references therein). During the early glacial periods of the Pleistocene in the Great Plains, ice extended as far south as Nebraska and Kansas (Dreeszen 1970). In addition, a large region extending from Nebraska and eastern Colorado to eastern New Mexico and western Texas was characterized by periodically active blowing sand dunes during the late Pleistocene, with some dune activity extending into the mid-Holocene (Arbogast 1996; Holliday 1997; Muhs *et al.* 1997). Together, the glaciers and sand dune deserts covered large regions of the northern and western Great Plains during the late Pleistocene. At the same time, fossil pollen and vegetation data indicate that a short distance south of the glacial advance grew a savanna-like mix of trees with Rocky Mountain affinities and typical prairie grasses and shrubs (Kapp 1970; Wright 1970). Hence, this region likely served as a refugium for plains-adapted organisms.

The contraction of habitable areas during glacial maxima of the Quaternary and their subsequent expansion following the ice's retreat influenced the distribution and presumably the genetic structure of many organisms that currently occupy northern and central North America (reviewed in Hewitt 2000). The climatic history inferred from vegetation and soil analyses is complemented by a rich animal fossil record. Anuran fossils of Pleistocene age are absent from much of the northern Great Plains, including North Dakota and much of South Dakota, but are present in relative abundance further south from Kansas to Texas (reviewed in Holman 1995), suggesting this area served as a Pleistocene refugium. Fossils from these sites include species of bufonid toads that currently have distributions encompassing northern regions that were uninhabitable during much of the Pleistocene (Holman 1995).

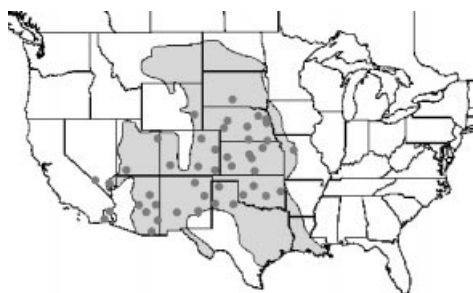


Fig. 1 Distribution of *Bufo woodhousii* in the USA, shaded grey (redrawn from Conant & Collins 1991). Collection sites for this study shown as dark grey dots.

Species that expanded into northern areas are expected to show lower genetic diversity there than in more southerly areas that served as refugia (reviewed in Hewitt 1996, 1999). This colonizing edge of low genetic diversity has been well-documented in Europe for multiple species, but the population structure of organisms inhabiting North America's Great Plains has not been examined as thoroughly. Birds of the Great Plains that have been surveyed for geographical variation generally show a lack of population structure (reviewed in Ball & Avise 1992; Zink 1996). Minimal genetic diversity and lack of geographical structuring have been ascribed by some authors to recent population expansion (Avise *et al.* 1988; Ball & Avise 1992; Zink & Dittmann 1993; Ellsworth *et al.* 1994).

Less is known about the population structure of other Great Plains organisms, especially those with lower vagility than birds. Tiger salamanders (*Ambystoma tigrinum*) show very low mitochondrial sequence divergence, suggesting relatively recent postglacial colonization (Routman 1993; Templeton *et al.* 1995; Shaffer & McKnight 1996). Although fish of the glaciated northern Great Plains also generally display low genetic diversity compared with fish from unglaciated regions (reviewed in Bernatchez & Wilson 1998), their post-Pleistocene distribution in unglaciated areas is probably due to a complex interplay of changing river and lake drainage patterns (Cross 1970), and may not reflect those of terrestrial taxa.

The toad *Bufo woodhousii* dwells primarily in grasslands, from the north-central USA into Texas, the southwestern USA, and northern Mexico (Fig. 1). *B. woodhousii* from the southwestern USA has been described as a subspecies (*B. w. australis*), based on morphological distinctions from the Great Plains form, *B. w. woodhousii* (Shannon & Lowe 1955). *B. woodhousii* occupies varied habitats, but is typically found in grasslands and is absent both from very dry desert and from woodlands. It breeds in ponds, seasonal pools or streams with areas of slow-moving water. *Bufo* are not known to disperse long distances, and typically breed in their natal pond (Blair 1953; Breden 1987). During the Pleistocene, northern portions of the range currently inhabited

by *B. woodhousii* were either desert or beneath glacial ice. Clearly, *B. woodhousii* must have expanded its range into these areas once the climate became more moderate.

Based on this information, we expect that genetic analysis of this toad will reveal the genetic patterns of two population processes. First, we expect to detect a gradual range expansion into areas currently occupied by *B. woodhousii* that were previously glaciated or covered by xeric sand dunes in the late Pleistocene and into the Holocene. Second, we expect to see some genetic differentiation between the Great Plains populations and those of the southwest. We use NCA to help elucidate the genetic patterns left by population history, while also considering whether the inferences agree with the known dispersal abilities and habitat requirements of this toad. In particular, we focus on whether inferences regarding range expansion are compatible with the biology of this species.

Methods

Specimen collection and molecular techniques

We collected 347 individuals of *Bufo woodhousii* from 45 locations across most of its current contiguous range in the USA (Fig. 1, Table 1). Collecting locations were designed to be as evenly spaced as possible, ≈ 150 –250 km apart. A combination of tadpoles, juveniles and adults was used in this study. Because it has been shown that anuran tadpoles prefer to congregate with siblings (Rautio *et al.* 1991; Hokit & Blaustein 1997; Saidapur & Girish 2000), we sampled individuals from multiple locations within ponds, or from multiple ponds within an area, to minimize the chances of collecting siblings with identical mitochondrial haplotypes. Adults and juveniles we considered to be from a single location were typically collected within a radius of 8 km or less.

Total genomic DNA was extracted, and the mitochondrial region encoding ≈ 100 bp of the 3'-end of 16S, all of tRNA^{Leu(UUR)} and most of the NADH dehydrogenase subunit 1 (*ND1*) gene was polymerase chain reaction (PCR)-amplified and sequenced as described previously (Masta *et al.* 2002). Sequences were analysed on an ABI Prism 377 automated sequence analysis machine, and the resultant chromatograms were viewed and the sequences corrected with the aid of SEQUENCHER Version 3.0. Sequence accuracy was further assessed by aligning all sequences and rechecking the chromatograms for all sites that differed from the consensus sequence. The *ND1* region was translated into an amino acid sequence using MACCLADE Version 4.0 (Maddison & Maddison 2000).

Sequence summary statistics

To test whether this mitochondrial region was evolving neutrally and therefore suitable for making population

Table 1 Distribution and abundance of haplotypes. The number of individuals possessing each haplotype at a given geographical location is shown in parentheses (n) after the location name

Haplotype	GenBank Accession no.	<i>n</i>	Geographical occurrence*
A	AF462464	138	Greenlee Co., AZ (4), Montezuma (1), Morgan (4), Prowers (2), Pueblo (3), Yuma (1) Co., CO, Barton (3), Russell (1), Gray (3), Harper (1), Logan (6) Co., KS, Saline Co., MO (2), McPherson (4), Gage (1), Keith (20), Valley (4), Pierce (6), Cuming (18), Dodge (1) Co., NE, Chaves (5), Colfax (9), Socorro (1) Co., NM, Blaine (7), Greer (1), McClain (1), Muskogee (4) Co., OK, Hall (6), Hartley (9), Parmer (9) Co., TX, Platte Co WY (1)
B	AF462541	41	Apache (2), Cochise (5), Gila (2) Co., AZ, Imperial Co., CA (18), Clark Co., NV (6), San Miguel (4), Socorro (4) Co., NM
C	AF462537	19	Clark (13), Nye (6) Co., NV
D	AY226941	16	Webster Co., NE
E	AY226942	12	Harper Co., KS (8), Hall Co., TX (4)
F	AF462538	14	Apache (6), Cochise (3), Navajo (2), Yavapai (1) Co., AZ
G	AY226943	8	Colfax Co., NM (2), Hartley (1), Parmer (5) Co., TX
H	AY226944	6	Barton (2), Logan (4) Co., KS
I	AY226945	6	Gila Co., AZ (1), Riverside Co., CA (3), San Miguel Co., NM (2)
J	AF462546	5	Chaves Co., NM (5)
K	AF462547	6	Greenlee Co., AZ (1), Prowers (2), Pueblo (1) Co., CO, Colfax Co., NM (1), Hall Co., TX (1)
L	AY226946	5	Pierce (1), Valley (4) Co., NE
M	AY226947	5	Gray Co., KS (5)
N	AF462545	3	Barton Co., KS (1), Hall Co., TX (2)
O	AY226948	3	Prowers Co., CO (1), Keith Co., NE (2)
P	AY226949	3	Chaves Co., NM (1), Parmer Co., TX (2)
Q	AY226950	4	Greenlee Co., AZ (1), Gray (1), Washington (1) Co., NE, Stanley Co., SD (1)
R	AY226951	3	Keith Co., NE (3)
S	AF462549	2	Cochise Co., AZ (2)
T	AY226952	2	Hartley Co., TX (2)
U	AF462539	3	Apache (1), Navajo (2) Co., AZ
V	AY226953	2	McPherson Co., NE (2)
W	AY226954	1	Hall Co., TX
X	AY226955	1	Cochise Co., AZ
Y	AY226956	1	Uintah Co., UT
Z	AY226957	1	Baca Co., CO
AA	AY226958	1	Pierce Co., NE
BB	AY226959	1	McPherson Co., NE
CC	AY226960	1	Hall Co., TX
DD	AY226961	1	Valley Co., NE
EE	AY226962	1	Keith Co., NE
FF	AY226963	1	Parmer Co., TX
GG	AY226964	2	Yuma Co., CO (1), Gage Co., NE (1)
HH	AY226965	1	Baca Co., CO
II	AY226966	1	Keith Co., NE
JJ	AY226967	1	McPherson Co., NE
KK	AY226968	1	McPherson Co., NE
MM	AY226969	4	Yavapai Co., AZ (4)
NN	AY226970	3	Yavapai Co., AZ (3)
OO	AY226971	5	Washington Co., UT (5)
PP	AY226972	1	Washington Co., UT
QQ	AY226973	1	Russell Co., KS
RR	AY226974	2	Morgan Co., CO (2)
SS	AY226975	4	Platte Co., WY (4)
TT	AY226976	1	Yuma Co., CO
UU	AY226977	2	Yuma Co., CO (2)
VV	AF462540	2	Gila Co., AZ (2)

*Further information on specimens and collection locations are available upon request from the first author.

genetic inferences, we performed the McDonald-Kreitman (McDonald & Kreitman 1991) test of neutrality on the ND1 portion of the sequence. Unlike most tests of neutrality, this does not assume that all individuals are from a panmictic population, and therefore is appropriate to use even if populations are subdivided. The ratio of polymorphic nonsynonymous to synonymous substitutions within a species is compared with this same ratio of substitutions that are fixed in relation to an outgroup species. Comparisons should be made between closely related species, or the test may falsely reject the null hypothesis of neutral evolution (Ford & Aquadro 1996; Wayne & Simonsen 1998; Gerber *et al.* 2001). Therefore, we used previously published ND1 sequences from 22 *B. americanus*, the sister group to *B. woodhousii* (Masta *et al.* 2002), to determine the number of fixed differences.

Population genetic and phylogenetic interpretation of sequence data

To examine whether *B. woodhousii* populations are at equilibrium, we performed Tajima's D-test (Tajima 1989) and Fu's *F_s* test (Fu 1997). Because these tests assume that populations are in both mutation-drift and migration-drift equilibrium, significant values for either may indicate that the populations experienced past population growth, were previously subdivided or that sequences are not evolving in a neutral manner. Tajima's test can detect deviations from equilibrium due to these forces, using critical values established by Simonsen *et al.* (1995). Fu (1997) has shown *F_s* is very good at detecting population growth. All tests of neutrality/nonequilibrium were implemented in DNASP Version 3.53 (Rozas & Rozas 1999).

We dated the divergence of major clades using Nei and Li's formula for *d_A* (equation 10.21, Nei 1987). Divergence and standard error calculations were carried out with DNASP 3.53, using a Jukes-Cantor (Jukes & Cantor 1969) correction. We used a molecular clock rate of 1.18% divergence/million years as calibrated for *Bufo* ND1-ND2 (Macey *et al.* 1998).

To estimate sequence relationships, we constructed gene trees with maximum likelihood (ML). Because many haplotypes were identical, we first merged all redundant taxa using MACCLADE 4.0 (Maddison & Maddison 2000). The model of evolution for use in ML analysis was determined by comparing the likelihood scores of trees generated under increasingly parameter-rich models. The model that yielded a tree with the statistically lowest likelihood score was identified with the use of the program MODELTEST 3.06 (Posada & Crandall 1998). The HKY85 (Hasegawa *et al.* 1985) with gamma shape parameter model best fit the data, and thus was used in the ML analyses. We implemented an iterative series of ML searches by obtaining an initial tree

by neighbour-joining, using it to estimate the transition/transversion ratio and the shape parameter, then using these values in a ML search. A series of searches was conducted, iterating between obtaining parameter values for a tree, fixing those values, and searching for a new tree. Searches were continued until multiple identical trees with the same lowest log likelihood scores were found. To test whether these sequences have been evolving in a clock-like manner, we performed a ML search, using the above parameters, but enforcing a molecular clock. A likelihood ratio test was then used to determine if the likelihood scores of the trees found from these two searches differed significantly from each other, as outlined by Felsenstein (1981). One hundred ML bootstrap replicates were performed by setting the values for the transition/transversion ratio and shape parameter to those found for the most likely trees in the previous searches. All phylogenetic analyses were implemented in PAUP* 4.0 (Swofford 2000). Trees were rooted with two *B. americanus* sequences known to be outside the *B. woodhousii* clade (Masta *et al.* 2002). Pairwise haplotype mismatch distributions (Rogers & Harpending 1992; Rogers 1995) using a model of population growth/decline were performed in DNASP 3.5 to detect whether population expansion or contraction had occurred in clades recovered by the phylogenetic analyses.

Nested cladistic analysis

Sequences from all 347 individuals were used to construct a haplotype network with the program rcs Version 1.06 (Clement *et al.* 2000). This yielded a network that formed ambiguous connections or closed loops. Ambiguous connections were resolved using information from the maximum likelihood tree. While the choice of the single maximum likelihood topology does not consider all possible connections, this method of resolving the loops did not affect our interpretation of the results, as the closed loops were at low nesting levels and not found among haplotypes that influenced the NCA inferences. Nesting of the haplotype network was performed following rules in Templeton *et al.* (1987), Templeton & Sing (1993) and Crandall (1996).

Geographic coordinates were obtained for all populations in the field by GPS. Owing to their close geographical proximity, we pooled populations from Cuming Co., NE, with Dodge Co., NE, and those from Russell Co., KS, with Barton Co., KS, using the geographical centre between each of the pairs of populations for the geographical coordinates, yielding a total of 43 locations.

Nested cladistic analysis was performed with the program GEODIS Version 2.0 (Posada *et al.* 2000) available at http://InBio.byu.edu/faculty/kac/crandall_lab/geodis.htm. This program determines the clade distance (*D_c*) of a given group of haplotypes, which is a measure of the geographical

range of a haplotype group (in NCA terminology, a 'clade' represents a haplotype group). It also determines the nested clade distance (Dn) of a given group of haplotypes. Dn measures how a haplotype group is distributed relative to all the haplotypes present in the nested haplotype group. The statistical significance of Dc and Dn are then evaluated by random permutation analysis. We used the 2001 revised version of the inference key available on the GeoDis website to interpret the results for statistically significant patterns of geographical distribution of haplotypes.

Results

Sequence summary statistics

We obtained ≈ 1108 bp of 16S–ND1 sequence for each of the 347 *Bufo woodhousii*, and found 47 unique mitochondrial haplotypes; these sequences have been deposited in GenBank (new Accession nos AY226941–AY226977; see Table 1). We found 57 variable sites with a maximum sequence divergence among haplotypes of 1.26%. The tRNA^{Leu(UUR)} sequence is completely conserved, with all variable sites within either the 16S or ND1 genes. Within ND1, no stop codons are present, and most changes (41) are at third positions of codons, with six changes at first positions and two at second positions. This level of sequence conservation is expected from functional mitochondrial genes, suggesting these are not mitochondrial sequences that have been transposed to the nucleus and become pseudogenes. McDonald–Kreitman test results are consistent with neutral evolution of ND1 ($P = 0.8$ with G -test with Yates' correction; $P = 1.00$ with Fisher's exact test), with a ratio of 1:4 fixed nonsynonymous/synonymous substitutions and 8:54 polymorphic nonsynonymous/synonymous substitutions. A 3 bp deletion was found in three *B. woodhousii* haplotypes, resulting in the loss of an amino acid at position 259. This deletion is present in animals from two locations (southern Utah and central Kansas), with haplotypes that differ by up to nine sites (0.8% sequence divergence), suggesting the deletion occurred independently in these populations. This implies that the evolution of these sequences violates the infinite sites model (although no sites had more than two alternate nucleotides present, consistent with an infinite sites model).

Population genetic and phylogenetic interpretation of sequence data

The maximum likelihood tree in Fig. 2 shows two major clades, one composed primarily of sequences from individuals in the western USA (designated clade IV-1) and the other (clade IV-2) consisting largely of sequences from individuals in the Great Plains. Roughly half the individuals in the western clade belong to a subclade nested within it.

The divergence between the two major mitochondrial clades seen in the ML tree suggests that *B. woodhousii* populations may have been fragmented in the past. However, because the two clades currently overlap geographically, subsequent range expansion may have occurred. To explore this further, we analysed these two clades with several methods for detecting population growth, which may be expected to accompany range expansion.

Clade IV-1 of the ML tree displays several measures consistent with a population that has maintained a constant population size. First, both Tajima's D and Fu's F_s statistic (Table 2) are marginally not significant, consistent with neutral evolution and a lack of either population bottlenecks or rapid growth. Also, the pairwise differences mismatch curve (not shown), has a somewhat bimodal distribution as would be expected of a stable population, with mean absolute error of 0.452. Clade IV-2 of the ML tree displays properties consistent with a population that has undergone exponential growth. Both Tajima's D and Fu's F_s statistic (Table 2) are significantly negative, and the pairwise mismatch distribution curve (not shown) is consistent with expectations under population growth, with mean absolute error of 0.262. Rogers (1995) found that the mean squared error between observed and fitted mismatch distributions tends to be smallest in populations that have grown. However, both clades IV-1 and IV-2 display star-like phylogenies (Fig. 2), a property consistent with past population growth (Slatkin & Hudson 1991).

The sequences do not deviate significantly ($P = 0.72$, not significant at the 0.01 level) from a clock-like model of evolution ($-\ln L = 2134$ with molecular clock enforced, $-\ln L = 2114$ without enforcing a clock). The number of substitutions per site between clades IV-1 and IV-2 is 0.00544, which yields a divergence time of $\approx 461\,000$ years BP (± 2 SE of 27 000 years), using a molecular clock rate of 1.18% division/Myr. Divergence of the two major clades within clade IV-1 was estimated at 177 000 years BP (± 2 SE of 22 000 years).

Table 2 Measures of nucleotide polymorphism among sequences from the clades illustrated in Fig. 2

	Pi	theta/site	Tajima's D	sig.	Fu's F_s	sig.
clade IV-1	0.00178	0.00306	−1.1598	no ($P > 0.10$)	−3.031	no
clade IV-2	0.00128	0.00510	−2.0748	yes ($P < 0.05$)	−21.794	yes

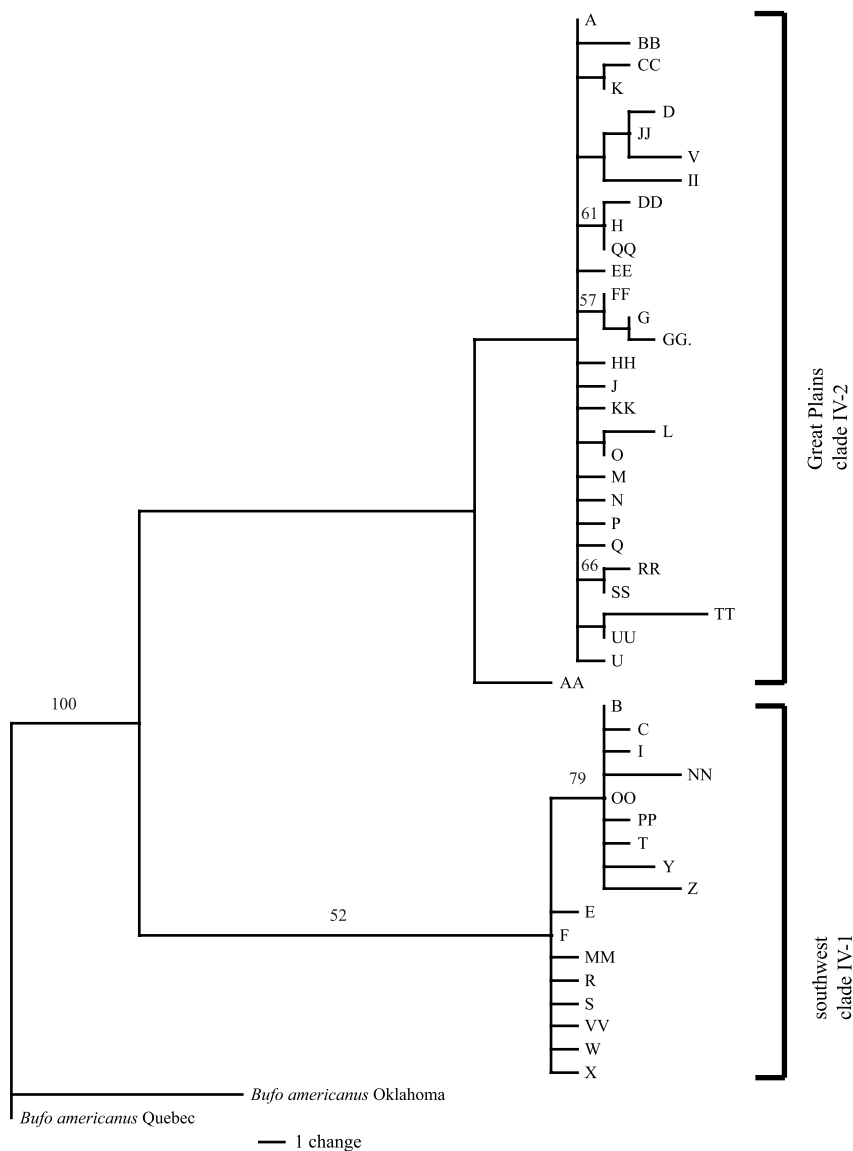


Fig. 2 Maximum likelihood tree of *B. woodhousii* haplotypes, with HKY85 (Hasegawa *et al.* 1985) with gamma model of evolution. ($-\ln L = 2113.83$; $Ti/Tv = 8.91$; shape parameter = 0.078). Bootstrap values from 100 maximum likelihood replicates shown over the branches. See text for explanation of clade designations.

Nested cladistic analysis

Figure 3 shows the haplotype network. The size of each oval is scaled to the number of individuals possessing that haplotype, with the most common haplotype A ($n = 138$) represented by a square. The nesting design is given in Fig. 4. The inference that clade IV-2 is the interior clade was justified by several criteria. First, the likelihood tree (Fig. 2) indicated that the haplotypes AA plus A diverged earliest, indicating these are more similar to the ancestor than clade IV-1 haplotypes. Trees that were slightly less likely placed clade IV-1 nested within clade IV-2. Second, the most common and widespread haplotype (A) is within clade IV-2. Templeton and others (Crandall & Templeton 1993) have shown empirically and theoretically (Castelloe & Templeton 1994) that haplotypes of high frequency that are

geographically widespread tend to be near the root of a genealogy.

Twelve nested haplotype groups yielded significant associations of haplotype and geography. NCA inferences for these groups are given in Table 3. Overall, there is evidence for two instances of contiguous range expansion and several long-distance colonization events. Contiguous range expansion is inferred to have occurred among two lower level nesting groups; these are from southeast Wyoming into northeastern Colorado and from south and central Nebraska into the Sandhills area of west-central Nebraska. Long-distance colonization is inferred at multiple clade levels, in each case indicating movement from the southwestern USA into northeastern Texas, south-central Kansas and west-central Nebraska (see dark grey circles in Fig. 5). There were three inferences of long-distance dispersal

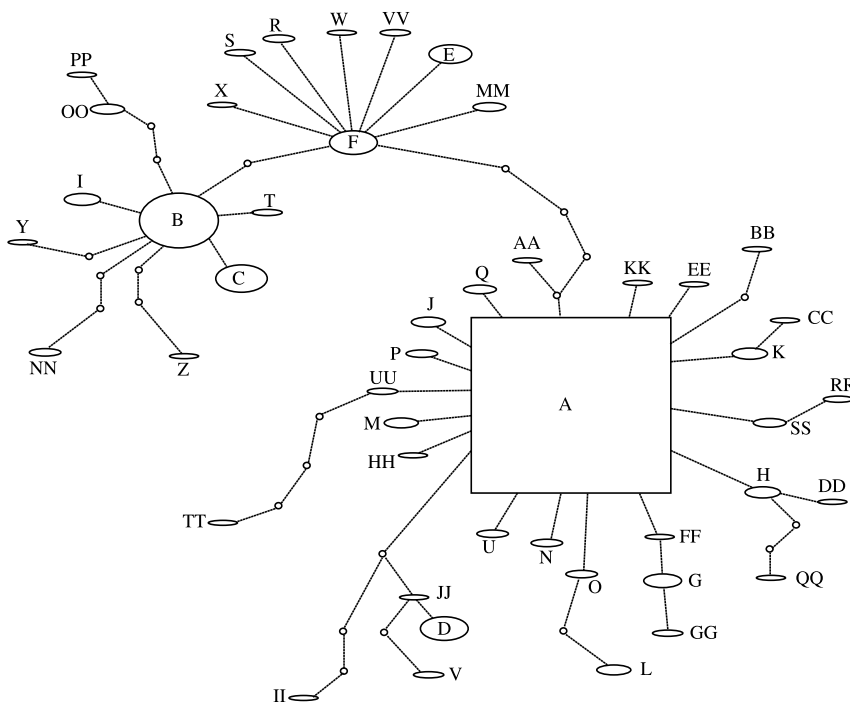


Fig. 3 Network of haplotypes of *Bufo woodhousii*, with gaps coded as a fifth state. Each haplotype is designated with a unique one- or two-letter combination.

Table 3 Nested cladistic analysis results for *Bufo woodhousii*

Clade	χ^2 statistic	Probability*	Inference chain	Inferred pattern†
I-1	685.48	0.001	1-2-3-5-6-7-YES	RGF with long distance dispersal into northeast AZ
I-5	8.00	0.036	1-2-3-4-9-10-NO	Fragmentation or IBD between central NE and western NE + eastern CO
I-6	17.00	0.058	1-2-3-4-9-10-NO	Fragmentation or IBD between southern NE and western NE
I-17	156.52	0.000	1-2-3-5-6-7-8-NO	Long distance dispersal or IBD between southwest and TX
I-18	149.30	0.000	1-2-11-12-13-YES	Long distance colonization from the southwest into southwest NE, south-central KS and north TX
I-19	6.00	0.063	1-2-11-12-NO	Contiguous range expansion from southeast WY to northeast CO
II-1	316.00	0.000	1-2-3-5-6-7-YES	RGF with long distance dispersal from southwest into southeast CO
II-3	380.35	0.046	1-2-3-4-NO	RGF with IBD for area encompassing corners of WY, CO and NE
II-5	11.92	0.021	1-2-11-12-NO	Contiguous range expansion from south-central NE to west-central NE
III-2	69.44	0.013	1-2-3-4-NO	RGF with IBD for central NE and KS
IV-2	193.59	0.000	1-2-3-4-NO	RGF with IBD for south and western NE
Cladogram				
Total	274.25	0.000	1-2-11-12-13-YES	Long distance colonization from southwest into the Great Plains

*The probability refers to the frequency with which the 1000 randomly generated chi-square statistics were equal to or greater than the observed chi-square.
†RGF = restricted gene flow; IBD = isolation by distance. Haplotype groups without genetic and/or geographical variation are not listed.

(recurrent processes) at three different locations (Table 3). NCA also found evidence for restricted gene flow with isolation-by-distance among groups of populations found primarily in central and western Kansas and Nebraska.

Discussion

Some of our NCA inferences are in accord with what may be inferred from Pleistocene and post-Pleistocene climatic

history. The Nebraska Sandhills were huge, extremely arid, unvegetated sand dunes as recently as the late Holocene, with activity probably extending back in time over more than one glacial-interglacial cycle (Muhs *et al.* 1997). NCA indicates that *Bufo woodhousii* shows a recent historical contiguous range expansion into this region from areas further south. Also, the area of northern Colorado (Morgan County) into which NCA inferred recent expansion from Wyoming was covered by the Fort

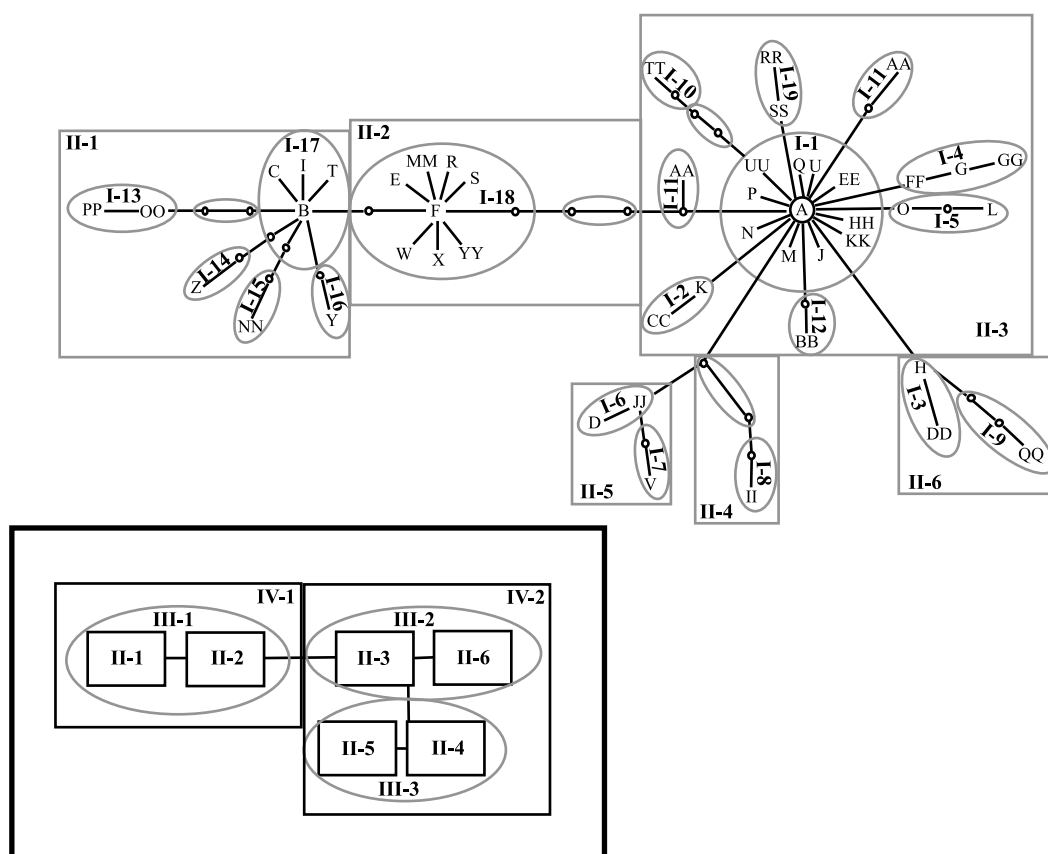


Fig. 4 Nested haplotype design. Each line represents a single mutation, and a small open circle indicates a haplotype not present in the samples. The haplotype letter designations are the same as in Fig. 3, with grey ovals enclosing one-step nested haplotypes, and grey boxes enclosing two-step nested haplotype groups. The inset depicts the nesting design of the three- and four-step nested haplotype groups, with grey ovals enclosing three-step nested haplotype groups and boxes enclosing four-step nested groups. All nested groups are designated first with a Roman numeral corresponding to the nesting level, and second by an Arabic numeral to distinguish between groups at the same nesting level.

Morgan sand dunes during the Holocene (Muhs *et al.* 1997). It is not possible to date these range expansion events precisely, as only a single shared mutation in a few individuals delimits the movement into these areas. If we assume a divergence rate of 1.18%/Myr, this expansion could have occurred 75 000 years BP, or as recently as a few years ago. However, an inference of recent range expansion into these areas is compatible with climate and landscape history.

We find no NCA evidence for range expansion into areas that were most recently glaciated, in particular north-east Nebraska and South Dakota. However, this may be due both to the short duration of time since these areas became ice-free ($\approx 18\,000$ years ago), and to the limited sampling of areas further north. Because NCA can only infer a range expansion event when new mutations have occurred in the populations that have expanded, many relatively recent post-Pleistocene range expansions may go undetected, unless extremely rapidly evolving genes are

examined. This limitation to make inferences about late Pleistocene and Holocene range changes has been noted for other organisms thought to have undergone range expansions (Seddon *et al.* 2001; Alexandrino *et al.* 2002). Even though mitochondrial DNA evolves relatively rapidly, it seems plausible that at most only one or two mutations may have occurred in the ≈ 1000 bp fragment sequenced for this study, since the most recent glacial retreat.

Although lack of mutations may explain why NCA did not detect more range expansion events, it is less clear why it did not detect allopatric fragmentation between the two major clades. Five mutational differences separate clades IV-1 and IV-2, and these two clades are centred in well-separated geographical areas, although there is considerable geographic overlap (Fig. 5). These observations, together with the apparent morphological differentiation of at least some southwestern populations (Shannon & Lowe 1955), are consistent with what would be expected had two

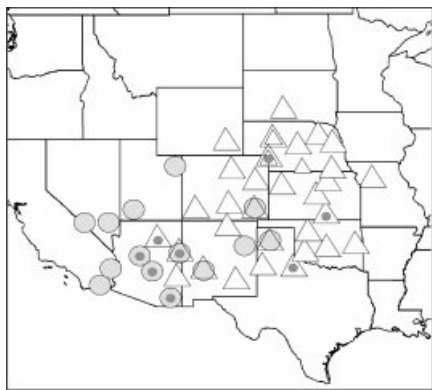


Fig. 5 Geographic distribution of the haplotypes from the four highest-level nesting clades from NCA. Circles designate clade IV-1 haplotypes, with the largest light-grey circles representing clade II-1, and the smallest dark-grey circles representing clade II-2 (which is equivalent to clade I-18). Triangles represent clade IV-2, with the largest triangles representing clade III-2, and the smallest triangles in Nebraska representing clade III-3.

populations fragmented and diverged during the Pleistocene ($\approx 460\,000$ years BP) and subsequently come into secondary contact. However, instead of detecting former allopatric fragmentation, the only significant NCA inferences for the southwestern clade are of restricted gene flow, and for long-distance movement to the Great Plains. No inference is made for secondary range expansion into the southwest by the Great Plains clade, despite the fact that many individuals with Great Plains haplotypes are found in the southwest. An alternative interpretation might be that *B. woodhousii*, rather than fragmenting into two populations, diverged morphologically *in situ* due to restricted gene flow that enabled some differentiation along the southwestern edge of its range. However, this alternative seems unlikely, given climatic, fossil and genetic evidence.

Such lines of evidence, although not completely ruling out the alternative scenario of *in situ* divergence, support best the idea that the Great Plains harbour the oldest populations of *B. woodhousii*, whereas populations arrived in the southwest more recently. *B. woodhousii* diverged from its closest extant sister taxon, the eastern-dwelling *B. americanus*, an estimated 1.3 Myr BP (Masta *et al.* 2002). Fossil bufonids identified as *B. woodhousii* have been found in the northern Great Plains and dated at 1.9 Myr BP to 900 000 years BP, whereas the oldest *B. woodhousii* fossils from the southwest (from Nevada) date only to $\approx 150\,000$ years BP (Holman 1995). Numerous fossils from the southwest have more recent late Pleistocene and Holocene dates, suggesting *B. woodhousii* has continuously occupied this region during glacial maxima, even though it was cooler and moister, with more extensive juniper or pinyon-juniper woodland habitat than found today

(Van Devender & Mead 1978). These data suggest that *B. woodhousii* originated in the Great Plains and spread to the southwest by the mid- to late Pleistocene.

It is not clear what, if any, climatic event might have caused fragmentation between Great Plains and southwest populations. The Rocky Mountains are currently a barrier to dispersal for these toads, which are not found in rocky high-altitude habitats. Therefore, the geographical connectivity between the Great Plains and western populations is primarily through New Mexico (see Fig. 1). It is possible that the xeric conditions leading to sand dune formation in the southern plains during the late Pleistocene (Holliday 1997) may have produced a barrier between *B. woodhousii* populations in the southwest and eastern Great Plains. We should also note that, despite our finding that *B. woodhousii* is differentiated into southwestern and Great Plains clades, our data does not definitively support the *B. w. australis* subspecies designation as proposed by its authors. The range described for the subspecies does not closely correspond with that of our southwestern clade or either of its subclades, which have much larger geographical distributions. Of course, this could merely reflect the fact that mitochondrial loci may move further and faster than nuclear loci coding for morphological features. But in addition, there has been recent gene flow between the major clades, as recognized by NCA and by the fact that haplotypes closely related to those on Great Plains are found in Arizona.

Although failure to detect likely range fragmentation may be judged acceptable on the basis that NCA seeks to be conservative in its conclusions, our NCA results also include apparent false positives – inferences of multiple recurrent long-distance dispersal events, and historical long-distance colonization from the southwest into the Great Plains. Long-distance movement probably never occurs naturally in these toads. They do not disperse far from their natal ponds, which limits their dispersal distance over short time intervals. Because toads are reliant on water in which to breed, their distribution is also greatly influenced by climatic changes that create conditions too arid or cold for their reproduction.

Long-distance movement is inferred by NCA when a large nesting distance (D_n) is found for a clade with a small clade distance (D_c). This can occur when a peripheral population possesses a unique haplotype but a limited geographical distribution with respect to that of the haplotype from which it was derived. In contrast, concordance between D_n and D_c suggests short-distance movement such as occurs during contiguous range expansion or isolation-by-distance. As pointed out by Templeton *et al.* (1995), these predictions assume adequate geographical sampling; inadequate sampling with sizeable geographical gaps can lead to large nested clade distances, even if range expansion was actually contiguous. In this study we

performed extensive sampling so as to eliminate large geographical gaps. Therefore, the results that led us to infer long-distance movements are not due to lack of power. Indeed, we arrived at the same inference of long-distance colonization at two different nesting levels, both with strong significance values, involving the same three populations in Texas, Kansas and Nebraska that possess haplotypes belonging to clades I-18 and IV-1 (see Fig. 5).

A more likely explanation for finding large nested clade distances (Dn) but small clade distances (Dc) in organisms with low vagility is that populations in the intervening areas have been subject to extinction of haplotypes. This could result from either stochastic genetic drift (lineage sorting) or biotic or climatic influences, followed by recolonization of the areas by individuals with haplotypes from another clade. Such haplotype loss in intervening areas could be interpreted by NCA as long-distance colonization: small Dc accompanied by large Dn . The current NCA inference key does not include as one of its possible outcomes that significant associations found may be due to, or influenced by, haplotype extinction.

We expect that extinction and replacement of alleles can influence the geographical and genetic patterns left by lineages of organisms. For example, a species expanding its range may contain a lower density of individuals along the leading edge of expansion, possibly due to suboptimal habitat or climate. Population contraction would leave a similar low-density population at the trailing edge. Such marginal populations would thus be most prone to extirpation due to biotic factors. Also, populations composed of few individuals on the edge of a range are more likely to lose haplotypes due to population bottlenecks and genetic drift (Nei *et al.* 1975). If extinction of populations was common on the edge of an organism's range, it could result in a checkerboard pattern of different alleles becoming fixed in different populations. Hewitt and others (Nichols & Hewitt 1994; Hewitt 1996; Ibrahim *et al.* 1996) have explicitly modelled expansion of populations with different types of dispersal. Although long-distance colonization did leave a patchwork of alleles in the invaded area (Nichols & Hewitt 1994) that would probably be correctly interpreted with the NCA inference key, both uniform and stepping-stone dispersal models also yielded a geographical patchwork of alleles, although they persisted for fewer generations. We expect that geographical patchworks of haplotypes may commonly be found in organisms that have gradually expanded or re-invaded parts of their range, and that such patterns may generally lead to incorrect inference of long-distance movements by NCA.

In general, given a random pattern of haplotype extinction, NCA should yield no geographical association, and no inferences will be made. In such cases, the method is conservative. However, haplotype and geography may be

significantly associated, even when the current structure does not allow accurate inference of past geographical associations. We suggest that the processes of lineage sorting and range contraction/expansion need to be acknowledged in interpreting geographical and genetic patterns.

One way to do this is to consider various other methods that complement NCA. Currently, no single method is entirely capable of discriminating incomplete lineage sorting from other population genetic processes. Thus, it is necessary to consider multiple approaches and to determine whether concordant results are given by various methods that can be used in conjunction with NCA to help determine whether long-distance colonization likely occurred. If two genetically distinct lineages partially overlap geographically, it could be due to previous range fragmentation followed by range expansion of one or both lineages. Maximum likelihood coalescent approaches, such as those developed by Kuhner *et al.* (1998) and available in the program FLUCTUATE, can be used to estimate the population growth rate (Kuhner *et al.* 1998). Coalescent simulations of data under different population history models could also be performed, as illustrated by Knowles & Maddison (2002). The program GENIE utilizes the topologies of gene trees to visualize 'skyline plots' of past demographic changes in population size (Strimmer & Pybus 2001). The pairwise distributions of sequence differences can also be used to make inferences about past population growth (Slatkin & Hudson 1991; Rogers & Harpending 1992), and can be implemented using ARLEQUIN (Schneider *et al.* 2000) and DNASP (Rozas & Rozas 1999). Methods that test for neutral evolution, such as Tajima's D or Fu's F_s can also be useful for assessing deviations from the null hypothesis of a population in equilibrium. Ideally, one would want to find concordance among the inferences from different methods such as these, before feeling confident that a population has experienced a past history of population growth.

We used several approaches to determine whether the inferences made by NCA for populations of *B. woodhousii* are consistent with inferences from other methods. Had clade IV-1 expanded its range into the Great Plains, as suggested by the NCA inference of long-distance colonization, then we may expect that that population had grown, and we therefore should see indications of population growth using appropriate methods. However, we do not find strong evidence of population growth in clade IV-1. Both Tajima's D and Fu's F_s statistic are compatible with a population in equilibrium, and do not deviate from neutral expectations. (The star-like phylogenetic structure of our data accompanied by low sequence divergences prevented us from making inferences with either FLUCTUATE or GENIE.) Thus, alternative population genetic methods do not support the NCA inference of long-distance

colonization that other lines of evidence had already led us to question.

With clade IV-2, NCA made no inferences suggesting significant range expansion, so we should not expect to find evidence of population growth in this clade with other methods if this inference is correct. However, once again results from other tests differ from NCA inferences. In contrast to clade IV-1, clade IV-2 shows significantly negative values for both D and F_s , consistent with a population that has undergone significant growth. Although it is technically possible that the significant values could be due to non-neutral evolution of these mitochondrial genes, our *B. woodhousii* ND1 data were clearly consistent with neutrality by the McDonald–Kreitman test. In addition, it seems improbable that selection is acting on this region of mitochondrial DNA very strongly for populations in only certain parts of their range, especially given that these clades now inhabit the same areas.

A more parsimonious interpretation of these results is that the Great Plains and southwestern populations of *B. woodhousii* became fragmented in the past and diverged, and that the two populations underwent subsequent climate-induced range expansions and contractions. Some remnant populations of clade IV-1 remain in Nebraska, Kansas and Texas, but the predominant history of *B. woodhousii* in the recent past has been growth and range expansion of clade IV-2 into the southwest.

The multiple inferences of long-distance dispersal may not be as problematic as inferences of long-distance colonization. Looking for congruence among multiple unlinked loci, a process referred to by Templeton (2002) as 'cross-validation', could reduce or eliminate the possibility of incorrectly inferring long-distance dispersal, because stochastic events should not affect multiple loci in the same way. Indeed, Templeton (2002) argues that 'Any event that is not cross-validated is regarded as tentative and questionable.' However, if separate isolates undergo range fragmentation and subsequent expansion such that they overlap secondarily, alleles at different loci may still give a concordant (but incorrect) NCA conclusion of long-distance colonization, because the loci will be correlated due to previous divergences in allopatry. Therefore, it may be possible to overcome false positives due to lineage sorting by sampling more loci for NCA, but cross-validation may not be as helpful in overcoming false positives that are due to historical events.

Based upon the predicted geographical and genetic patterns that lineage sorting and range contraction followed by expansion can leave, we suggest changes to the NCA inference key. Because these processes can leave the pattern of a large D_n coupled with a small D_c , we suggest that when these are found (leading to a YES conclusion in step 12 of the NCA inference key), that step 13 should be modified as follows:

13 Are the clades with significantly large D_n 's (or tip clades in general when D_n for I–T is significantly small) separated from the geographical centre of the other clades by intermediate geographical areas that were sampled?

NO – Go to step 14.

YES – Long-distance colonization may have occurred. Go to step 13.5.

13.5 Are all of the following true?

- (a) Is it biologically realistic that the organism could have undergone long-distance movement?
- (b) Are the nested haplotypes inferred to have undergone long-distance colonization within a clade that shows evidence of population growth by other methods?
- (c) At the level of the entire cladogram, does the clade *not* inferred to have produced long-distance colonization *not* show evidence of past population growth with other methods?

YES – Long-distance colonization.

NO – Insufficient evidence to discriminate between long-distance colonization and past fragmentation followed by range expansion.

The addition of this step to the NCA inference key should help alleviate potential for false-positive inferences of long-distance colonization due to localized extinctions of individuals in an area, followed by recolonization by individuals possessing haplotypes from another clade.

Conclusions

The histories of organisms influenced by Pleistocene climate gyrations are probably very complicated, and it may thus never be possible to fully and accurately describe all processes involved. History can be erased via the extinction of haplotypes, or it can go unrecorded due to a lack of mutations over short periods to mark recent or rapid historical change. NCA attempts to examine which of multiple processes are compatible with one's data, which should make the method useful for making inferences about complex histories. However, inferring processes from patterns can still be an ambiguous exercise, particularly if haplotype extinctions are common. When haplotype loss seems likely, it will be necessary to incorporate as much information as possible about one's organism, including, where possible, ecological and physiological knowledge, life-history data and genetic information from multiple unlinked loci. Combining such data with historical information on climate, landscape and geography is ideal. In addition, we suggest incorporating alternative complementary tests, such as those that examine population growth or decline, to help assess the confidence one should place in NCA inferences of past long-distance colonization.

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This work is part of a broader study designed to empirically evaluate the robustness of the nested cladistic analysis method. Susan E. Masta studies processes contributing to population divergence and speciation in amphibians and arachnids, and Nicole M. Laurent is interested in the use of DNA profiling in forensics. Eric J. Routman's research has focused on population structure and phylogeography of amphibians in the Great Plains.
