

# Analysis of range expansion in two species undergoing character displacement: why might invaders generally 'win' during character displacement?

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## Keywords:

character displacement;  
coalescent-based analysis;  
competition;  
historical selection;  
phylogeography;  
population genetics;  
spadefoot toads.

## Abstract

Ecological character displacement occurs when interacting species diverge in resource use and associated traits in response to selection to minimize resource competition between them. Yet, when resource quality is asymmetric, the species that monopolizes the more profitable resource following character displacement may have higher fitness and therefore be deemed the 'winner'. Here, we ask: does the winner tend to be the resident species (i.e. the earlier inhabitant of the geographic region where character displacement occurred) or the invader (i.e. the subsequent inhabitant of the region)? We focus on two spadefoot toad species that have undergone character displacement. Previous studies revealed that *Spea bombifrons* gains the higher quality resource following character displacement; consequently, *Spea multiplicata* must use the lower quality resource, and as a result, experiences negative fitness consequences. Where the two species have undergone character displacement, three lines of evidence implicate *S. bombifrons* as the invader: *S. bombifrons* possess lower haplotype and nucleotide diversity; they do not exhibit isolation by distance (in contrast to *S. multiplicata*); and they display much higher population growth rates. We hypothesize that historical patterns of selection in its ancestral range pre-adapted *S. bombifrons* to evolve phenotypes capable of monopolizing the superior resource. Generally, because superior competitive abilities may facilitate successful invasions, invaders may be well positioned to win during character displacement.

## Introduction

Ecological character displacement occurs when competition between interacting species imposes divergent directional selection on each species' resource use and associated traits, causing them to diverge in these characters (Grant, 1972; Adams & Rohlf, 2000; Schluter, 2000; Day & Young, 2004; Rice & Pfennig, 2007). One consequence of character displacement is that interacting species will evolve to utilize different resources, which can sometimes create a 'winner' and a 'loser'. In particular, when asymmetries exist in resource quality, the species that monopolizes the higher quality resource

will potentially have higher fitness (and may therefore be deemed the winner) compared with the species that is displaced from this resource (the loser; Pfennig & Pfennig, 2005). These two species may enter into competition with each other through multiple scenarios: (1) sympatric speciation; (2) reciprocal expansions into a new geographical region; or (3) one species expanding into a geographic region already inhabited by the competitor. In cases of character displacement ignited by the last scenario, we ask: does the winner of character displacement tend to be the resident species (i.e. the earlier inhabitant of the geographic region where character displacement occurred) or the invader (i.e. the subsequent inhabitant of the region)?

There are theoretical reasons for predicting that either the resident or the invader may win during character displacement. Residents might generally win if they tend to have longer association with a more profitable

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resource, and, consequently, if they were pre-adapted to monopolize this resource in the face of competition. By contrast, if the success of an invasion depends on the invading species' superior competitive ability (Sakai *et al.*, 2001; Vila & Weiner, 2004; but see Bossdorf *et al.*, 2004), invaders might generally win in character displacement. For example, compared with noninvasive resident species, invasive species may forage more efficiently (Petren & Case, 1996; Holway, 1999; Rehage *et al.*, 2005), convert resources into tissue growth more effectively (Byers, 2000) or actively displace competitors from shared resources (Holway, 1999). Because so little is known about whether residents or invaders are more likely to win during character displacement, a critical first step in understanding why one species is able to monopolize the more profitable resource in the face of competition is to establish whether the winner in character displacement is the invader or the resident.

We used spadefoot toads as a model system to investigate whether the invader or resident species wins during character displacement. As we describe below, tadpoles of two species (*Spea multiplicata* and *S. bombifrons*) have undergone ecological character displacement in south-eastern Arizona (SE AZ) and south-western New Mexico (SW NM), USA (Pfennig & Murphy, 2000, 2003; Pfennig *et al.*, 2006). Additionally, because the resources the two species use following character displacement are asymmetric in quality, the species that uses the lower quality resource, *S. multiplicata*, is apparently experiencing negative fitness consequences of character displacement (Pfennig & Pfennig, 2005). Although displacement to the lower quality resource is better for *S. multiplicata* than competitive exclusion, the fitness costs of this displacement may increase the risk of eventual Darwinian extinction of this species in sympatry (Pfennig & Pfennig, 2005). Thus, *S. bombifrons* may be deemed the winner of this competitive interaction.

We used population genetic, phylogenetic and phylogeographic analyses to address two issues. First, we asked which species was the invader into SE AZ and SW NM: *S. bombifrons* (the winner) or *S. multiplicata* (the loser)? Second, after determining that *S. bombifrons* was the invader into this region, we sought to determine its ancestral range. Estimating the ancestral range provided insight into historical patterns of selection that may have predisposed this species to monopolize the superior resource following character displacement.

### Study system

Mexican spadefoot toads, *S. multiplicata*, and Plains spadefoot toads, *S. bombifrons*, co-occur in the south-western USA (Fig. 1). In a broad region of potential sympatry, both species may co-occur at intermediate elevations (hereafter termed 'syntopy'). However, at high elevations, only *S. multiplicata* is present (hereafter

termed 'allotopy') and at low elevations, only *S. bombifrons* is present (Pfennig *et al.*, 2006). Phylogenetic hypotheses suggest that among the four currently recognized species in the genus *Spea*, *S. multiplicata* is the basal species, with *S. bombifrons* as its most distantly related congener (Wiens & Titus, 1991; García-París *et al.*, 2003).

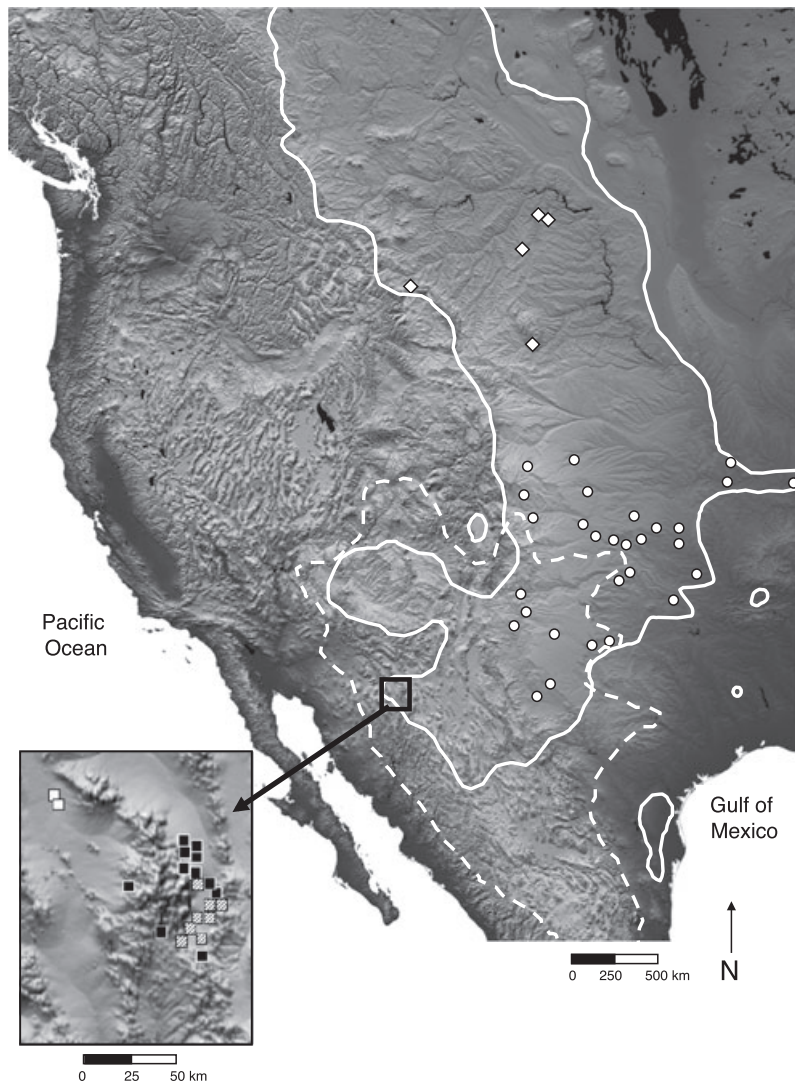
Larvae of both species exhibit trophic polyphenism: they develop either into an omnivore morph, which feeds mostly on organic detritus on the pond bottom, or a larger, morphologically distinct carnivore morph, which specializes on anostracan fairy shrimp (Pomeroy, 1981; Pfennig, 1990, 1992). The carnivore morph is induced by the ingestion of shrimp (Pomeroy, 1981; Pfennig, 1990). Moreover, both species grow better on shrimp (Pfennig & Murphy, 2000), suggesting that it is the more nutritious resource.

In the San Simon Valley of SE AZ, the two species exhibit ecological character displacement in tadpole morph production (Pfennig & Murphy, 2000, 2003; Pfennig *et al.*, 2006). In ponds where each species occurs alone, both species produce similar, intermediate frequencies of each morph. However, in ponds where they co-occur, *S. multiplicata* produce almost entirely omnivores, whereas *S. bombifrons* produce almost entirely carnivores (Pfennig & Murphy, 2000, 2003; Pfennig *et al.*, 2006). Experiments reveal that this sympatric divergence in morph production has evolved because of interspecific resource competition (Pfennig & Murphy, 2002; Pfennig *et al.*, 2007).

Because *S. bombifrons* outcompetes *S. multiplicata* for the more nutritious resource (fairy shrimp), *S. bombifrons* can be deemed the winner of this competitive interaction. Indeed, character displacement appears to be costly for *S. multiplicata*: *S. multiplicata* are significantly smaller in adult body size in syntopy than in nearby allotopy (Pfennig & Pfennig, 2005). This shift in body size probably reflects, at least in part, character displacement in tadpole morph production (Pfennig & Pfennig, 2005). As noted above, *S. multiplicata* produce mostly omnivores in sympatric populations; omnivores are smaller at metamorphosis than carnivores and probably also mature at smaller size. Smaller body size, in turn, is associated with lower survival and fecundity (Pfennig & Pfennig, 2005).

### Materials and methods

This study had two goals. First, we sought to determine which species more recently invaded the San Simon Valley of SE AZ (where character displacement has been documented). Second, we sought to identify the approximate ancestral range of the invader, *S. bombifrons*. This information was used to infer possible historical patterns of selection on *S. bombifrons* that may have predisposed this species to monopolize the superior resource.



**Fig. 1** Potential geographic ranges and sampling locations of *Spea bombifrons* and *S. multiplicata* (see Tables S1 and S2 for more detailed location information). The solid white line surrounds the range of *S. bombifrons*; the dashed white line indicates the range of *S. multiplicata* (ranges based on Stebbins, 2003). The inset shows the San Simon Valley and the immediately surrounding valleys in south-eastern Arizona and south-western New Mexico. Symbols represent sampling locations: solid white symbols are *S. bombifrons* sampling locations, solid black symbols are *S. multiplicata* sampling locations, and white squares hatched with black lines are sampling locations where both species were present (syntopy). For closely clumped sampling locations, one symbol may be used to represent multiple locations. More than one individual may have been collected at each sampling location (see Tables S1 and S2). Symbol shapes designate the geographic group to which each *S. bombifrons* sampling location was assigned (see Materials and methods): white diamonds represent the northern group; white circles the central group; white squares (solid and hatched) the south-western group.

## Sampling

We collected adults and tadpoles of both species during summers 1999–2006 in SE AZ and SW NM (Fig. 1). Adults were collected at or near breeding aggregations; tadpoles were sampled from random sites throughout natural ponds using a hand-held dip net 7–15 days post-hatching. We sampled three types of ponds, which differed in their species composition: (1) ponds in which *S. multiplicata* was the only species of *Spea* present (pure *S. multiplicata* ponds;  $n = 17$ ); (2) ponds in which *S. bombifrons* was the only species of *Spea* present (pure *S. bombifrons* ponds;  $n = 6$ ); and (3) ponds in which both species of *Spea* were present (syntopic ponds;  $n = 10$ ; see Fig. 1 and Tables S1 and S2 in Supplementary material for additional collection information). Within a few hours of collection, tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methanesulpho-

nate (MS 222), and preserved in 95% ethanol. We also obtained from museums and individual collectors additional *S. bombifrons* tissue samples from throughout their geographic range (number of locations = 38; Fig. 1, Table S1).

## DNA extraction, amplification and sequencing

We extracted genomic DNA from adult and tadpole tissues (Appendix S1). We then amplified and sequenced a 663-basepair portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome (mtDNA; Appendix S1). We sequenced an average of 15.5 *S. multiplicata* individuals from each of 27 locations (five to 36 individuals per location; Table S2) and an average of 6.4 *S. bombifrons* individuals from each of 54 locations (one to 33 individuals per location; Table S1).

## Determining order of invasion

To determine which species invaded SE AZ more recently, we used three approaches. First, we calculated and compared haplotype and nucleotide diversities for the two species across the same region. Second, we examined patterns of isolation by distance (IBD) in the two species. Finally, using a coalescent-based analysis, we estimated population growth in SE AZ populations of *S. bombifrons* and *S. multiplicata*.

Because it probably experienced a population bottleneck more recently as a result of colonization, we predicted that the more recent invader to this geographic region should exhibit lower genetic variation. Although numerous factors (e.g. selection, mutation rate, gene flow and demography) may affect levels of genetic variation in different species differentially, the fact that these two species experience similar ecological selection pressures and are similar in their phylogenetic position, generation times and dispersal capabilities suggests that a cross-species comparison should provide useful information about differences in recent demographic history. We used ARLEQUIN 2.0 (Schneider *et al.*, 2000) to calculate haplotype diversity and nucleotide diversity for each species. We then compared haplotype and nucleotide diversities for the two species over the entire region. We also calculated haplotype and nucleotide diversities for each species in each sampled pond separately (Tables S1 and S2) and compared the two species' mean diversity values by using nonparametric Wilcoxon rank sum tests. We employed nonparametric tests because the data did not meet parametric assumptions.

We tested both species for IBD (Slatkin, 1993) – a positive correlation between geographic distance and genetic distance. A signature of IBD should be evident for populations in migration-drift equilibrium. A nonequilibrium population, such as a recent invader, would not be expected to exhibit IBD, however (Slatkin, 1993). To control for any differences between species in geographic spread of the samples, we tested for IBD only across 10 syntopic ponds in the San Simon Valley (Fig. 1, Tables S1 and S2). We used Mantel tests in ARLEQUIN 2.0 to assess any correlation between population pairwise log-transformed geographic distance and population pairwise genetic distance ( $F_{ST}$ ).

We predicted that a more recent invader should exhibit a higher rate of population growth. We used a coalescent-based Bayesian analysis, as implemented in LAMARC 2.1.2b (Kuhner, 2006), to estimate  $\Theta$  ( $= 2N_f\mu$ , where  $N_f$  is the effective number of females in the population and  $\mu$  is the mutation rate per site per generation) and exponential growth rates ( $g$ , in units of  $\mu^{-1}$ ) for each of the two species in SE AZ. These parameters can be used to estimate the relative population sizes of *S. multiplicata* and *S. bombifrons* at a given time in the past (Wares & Cunningham, 2001; Marko, 2004; see Appendix S1). For each species, we sampled 100 000 genealogies with a

sampling interval of 100 after discarding 10 000 genealogies as burn-in. We replicated these analyses three times.

## Estimating *Spea bombifrons*' ancestral range

To determine the ancestral range of *S. bombifrons*, we compared levels of genetic variation from populations across the range of the species. We predicted that the ancestral range should exhibit higher molecular diversity values than more newly colonized regions (Begun & Aquadro, 1993; Hewitt, 2000). Before we did this, we identified discrete populations to compare by examining hierarchical population structure using an AMOVA (Analysis of Molecular Variance) in ARLEQUIN 2.0. We grouped samples from each collection location into subpopulations, and then grouped the subpopulations together until the maximum amount of variation was explained by the groupings (Fig. 1, Table S1). We then compared both haplotype and nucleotide diversities qualitatively among these regions.

Because range expansions often produce distinctive tree topologies (i.e. a star-burst pattern; Ball *et al.*, 1988; Avise, 2004), we estimated a phylogenetic tree to determine both whether *S. bombifrons* showed signatures of range expansion and how widespread the expansion may have been. For comparison, we also included *S. multiplicata* samples from SE AZ. We estimated the phylogenetic relationships among the sampled cyt *b* haplotypes using a Bayesian analysis as implemented by MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). To root the tree, we included in the analysis three partial cyt *b* sequences from *Spea*'s sister genus *Scaphiopus* (GenBank accession numbers: AY236791–AY236793; García-París *et al.*, 2003). We implemented the Hasegawa *et al.* (1985) model of DNA substitution with equal rates among sites (HKY), which MODELTEST 3.6 (Posada & Crandall, 1998) identified as the most likely model for our data. We performed two runs of the Bayesian analysis with four chains each, lasting  $4.0 \times 10^6$  generations. From these runs, 80 002 trees were produced (40 001 for each run), of which 8000 were discarded as burn-in.

## Results

### Determining order of invasion

In comparing *S. multiplicata* and *S. bombifrons* from the region where they are undergoing character displacement, *S. bombifrons* showed lower overall haplotype and nucleotide diversities than *S. multiplicata* [mean (SD) haplotype diversity: 0.239 (0.040) vs. 0.543 (0.028); mean (SD) nucleotide diversity: 0.00038 (0.00047) vs. 0.00197 (0.00136) respectively]. Indeed, *S. bombifrons* exhibited significantly lower haplotype

**Table 1** Estimates of  $\Theta$  and  $g$  for *Spea multiplicata* and *S. bombifrons* in south-east Arizona.

Species	$\Theta$ (95% HPD)	$g$ (95% HPD)	Relative $N_t$ 100 000 years ago
<i>S. multiplicata</i>	0.007 (0.0042–0.0123)	12.58 (–413.2–602.4)	0.99 (0.89–1.09)
<i>S. bombifrons</i>	0.004 (0.0011–0.0159)	10 510 (1803–15 100)	0.12 (0.05–0.7)

The confidence intervals presented are 95% highest posterior density credible regions (HPD). Relative  $N_t$  was calculated using the point estimate for  $\Theta$  and the endpoints of the 95% HPD for  $g$ , a generation time of 2 years and a mutation rate of  $4.0 \times 10^{-9}$  substitutions per site per generation (Tan & Wake, 1995). Any discrepancy between this rate and the actual mutation rate for *cyt b* in *Spea* will only affect the time estimate; it will not affect the comparison between the two species. Likewise, while estimates of  $g$  tend to be biased upward when based on one gene (Kuhner *et al.*, 1998), the relative estimates for the two species should not be affected. Details of the calculations performed can be found in Appendix S1.

( $W_{17,27} = 754$ ,  $P = 0.0004$ ) and nucleotide diversities ( $W_{17,27} = 786$ ,  $P < 0.0001$ ) than *S. multiplicata*. These values are consistent with more recent colonization by *S. bombifrons*.

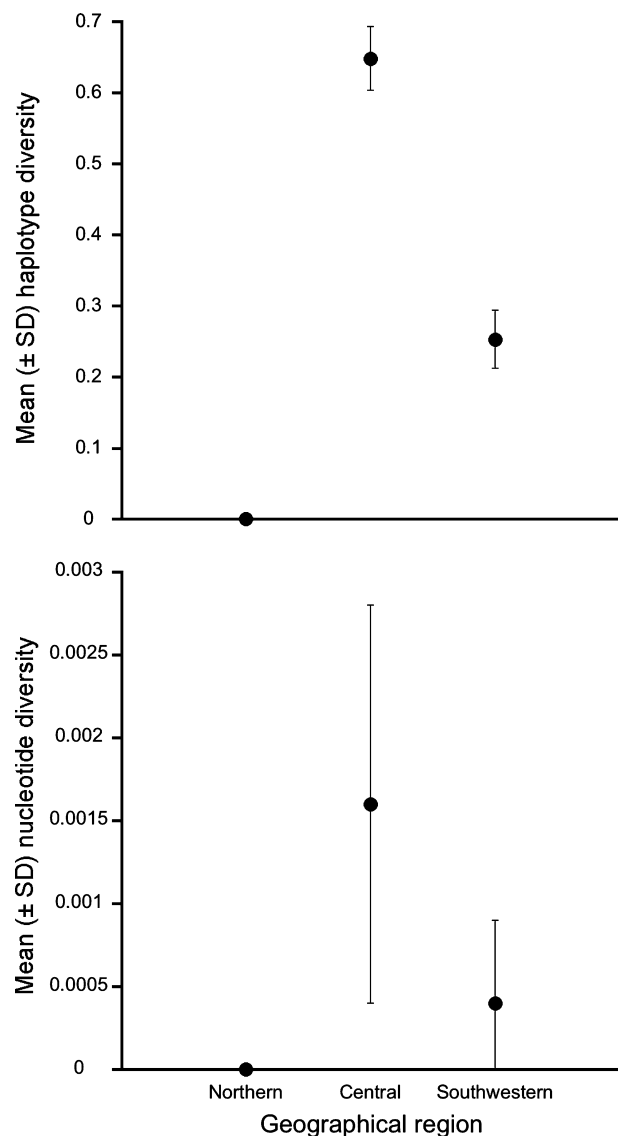
The two species exhibited different patterns of IBD across the 10 syntopic ponds in SE AZ. Whereas *S. multiplicata* exhibited a significant signature of IBD ( $r = 0.48$ ,  $P = 0.009$ , based on 100 000 permutations), *S. bombifrons* showed no significant pattern of IBD ( $r = 0.25$ ,  $P = 0.15$ , based on 100 000 permutations), again, suggesting that this species may have more recently invaded.

*Spea bombifrons* exhibited a significantly higher population growth rate than *S. multiplicata* (Table 1). This suggests that the *S. bombifrons* population in SE AZ is growing very quickly, as might be expected by a species that recently invaded. Conversely, the population growth rate for *S. multiplicata* is not significantly different from zero, a value that indicates a stable population size. Moreover, the relative female effective population size 100 000 years ago for *S. bombifrons* is significantly smaller than for *S. multiplicata* (Table 1). This suggests that *S. bombifrons* in SE AZ have experienced a more recent population bottleneck, as would be expected from the more recent invader.

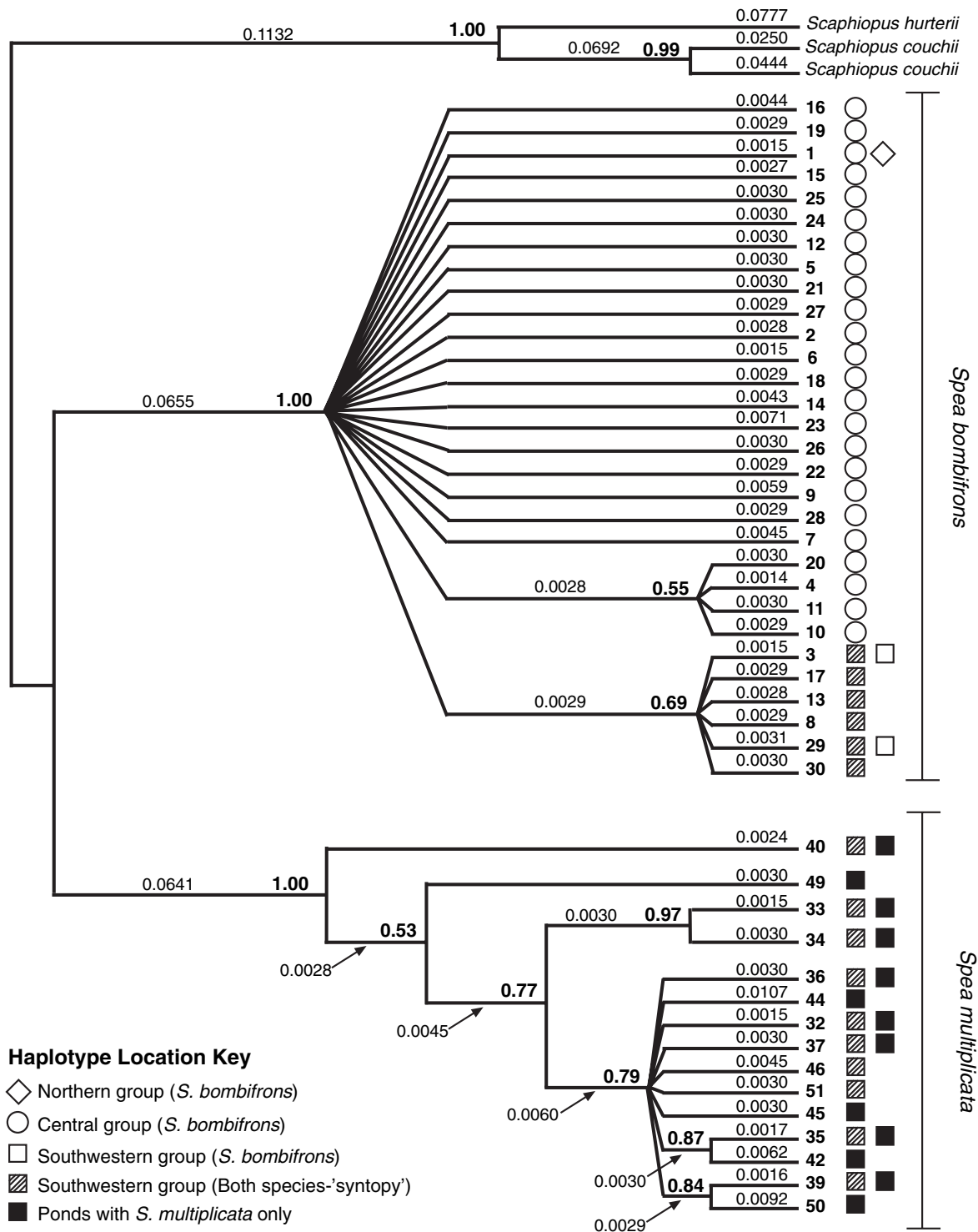
### Estimating *Spea bombifrons*' ancestral range

Our hierarchical population structure analysis revealed three discrete population groups across the range of *S. bombifrons* (Fig. 1; Appendix S1): a northern group, a central group and a south-western group. Of these three groups, the central group, located in the southern Great Plains (Fig. 1), exhibited the highest haplotype and nucleotide diversities (Fig. 2). This suggests that the ancestral range of *S. bombifrons* was in the southern Great Plains.

The phylogenetic analysis suggests that *S. bombifrons* has probably undergone expansion throughout its entire geographical range. The clade as a whole forms a starburst pattern, exhibiting very little genetic differentiation or geographic structure (Fig. 3). This phylogeny also illustrates the greater degree of genetic differentiation in *S. multiplicata* from SE AZ compared with *S. bombifrons* (Fig. 3).



**Fig. 2** Mean genetic diversity measures for three populations across the geographic range of *Spea bombifrons*. From left to right on the x-axis, latitude decreases (see Fig. 1). Top, mean haplotype diversity  $\pm$  standard deviation. Bottom, mean nucleotide diversity  $\pm$  standard deviation.



**Fig. 3** Phylogenetic relationships among 47 unique *Spea bombifrons* and *S. multiplicata* cytochrome *b* haplotypes (663 bp). The tree shown is the majority-rule consensus cladogram based on a Bayesian analysis. Clade support values (boldface proportions) at each node are Bayesian posterior probabilities. Each branch is labelled with the branch length, in units of substitutions per site. Haplotype numbers at the tips of the cladogram follow the numbering scheme from Tables S3 and S4. The symbols at the end of each branch indicate the location(s) where each haplotype was found, corresponding with the geographic groupings in Fig. 1.

## Discussion

Three independent lines of evidence implicate *S. bombifrons* (the winner) as the more recent invader into SE AZ, where character displacement is taking place. First, *S. bombifrons* has lower haplotype and nucleotide diversity values than *S. multiplicata*. Reduced genetic diversity may indicate historically small population sizes or bottlenecks, characteristic of a colonization event. Second, patterns of IBD suggest that *S. multiplicata* is at equilibrium, whereas *S. bombifrons* is not, possibly because it has undergone a recent range expansion. Third, whereas *S. multiplicata* from SE AZ have a stable, or at most a slowly growing, population, *S. bombifrons* from the same area have a rapidly growing population. Moreover, *S. bombifrons* exhibits signs of a more recent population bottleneck, perhaps because of a founding event. Fast population growth following a bottleneck may characterize recent invaders. These growth rates are also consistent with a previous study that found a recent increase in the relative frequency of *S. bombifrons* at breeding aggregations in SE AZ (Pfennig, 2003).

Although multiple lines of evidence implicate *S. bombifrons* as the more recent invader into SE AZ, our data do not allow us to entirely rule out an alternative hypothesis: that *S. bombifrons* was resident in SE AZ and underwent a demographic expansion after *S. multiplicata* invaded. In this alternative scenario, however, the impact of *S. bombifrons* on the competitor, *S. multiplicata*, is nearly equivalent to what it would be were *S. bombifrons* the invader. For character displacement to occur, population sizes of competing species must be large enough to deplete shared resources, generating interspecific competition (Grant & Grant, 2006). Either scenario would therefore have produced new selective pressures favouring interspecific divergence in resource use and associated traits. Moreover, both scenarios are consistent with the idea that species able to very quickly increase population size in the face of competition, as invasive species do, might tend to win in character displacement.

Two lines of evidence indicate that *S. bombifrons* underwent a widespread range expansion out of its ancestral range in the southern Great Plains. First, high levels of genetic variation in the southern Great Plains (i.e. the central group, Figs 1 and 2) suggest that this region is probably the ancestral range for *S. bombifrons*. Second, the haplotype tree (Fig. 3) shows a starburst-shaped *S. bombifrons* clade with a widespread haplotype (haplotype 1) and an excess of rare haplotypes (Table S3). This topology is consistent with recent expansion throughout the entire range, and suggests that populations have been relatively recently connected genetically (Ball *et al.*, 1988; Avise, 2004).

Much of *S. bombifrons*' expansion from their ancestral range northward is probably the result of post-Pleistocene expansion after the glaciers receded. Recent south-

ward expansion may have been driven, in part, by anthropogenic changes to the environment. Because *S. bombifrons* tadpoles develop more slowly than do *S. multiplicata* tadpoles (Pfennig & Simovich, 2002), *S. bombifrons* was probably unable to breed in the highly ephemeral ponds that historically characterized much of SE AZ. Beginning in the 1880s, however, ranchers began to excavate longer lasting 'cattle tanks' (Gehlbach, 1981; Bock & Bock, 2000), which now serve as *Spea*'s primary breeding sites (A. Rice and D. Pfennig, personal observation). Consequently, slower developing species (such as *S. bombifrons*) that normally do not live in arid regions occur in SE AZ. Other possible causes for the southward expansion remain unclear.

Given that *S. bombifrons* appears to be the invader into SE AZ, we also sought to understand why this species, as opposed to *S. multiplicata*, won during character displacement. For at least two reasons, historical patterns of selection in the ancestral range may have pre-adapted *S. bombifrons* to monopolize the superior shrimp resource. First, because *Spea* follows Bergmann's rule (adult body size increases with increasing latitude; R. Martin and D. Pfennig, unpublished data), *S. bombifrons* invading from the north were probably larger than the resident *S. multiplicata* (as shown in Fig. 1, *S. multiplicata* has a more southerly distribution; thus, allopatric *S. multiplicata* are smaller than allopatric *S. bombifrons*). Because larger females produce larger tadpoles (R. Martin and D. Pfennig, unpublished data), which, in turn, are better predators of shrimp (Frankino & Pfennig, 2001), *S. bombifrons* may have been predisposed to monopolize the superior shrimp resource. Second, the lineages of *S. bombifrons* that invaded SE AZ probably had an historical association with *S. multiplicata*; they would have encountered the north-eastern edge of *S. multiplicata*'s range in an earlier stage of the expansion from their ancestral range (Fig. 1). By contrast, *S. multiplicata* in SE AZ would not have previously encountered *S. bombifrons*. Consequently, *S. bombifrons* had probably experienced prolonged selection to outcompete *S. multiplicata* for the superior shrimp resource. Generally, why one species wins during character displacement may depend on a variety of factors, including, but not limited to, historical patterns of selection on behaviour or morphology that pre-adapt individuals for competitive interactions with naïve interspecifics.

Do invaders generally win during character displacement? We cannot answer this question definitely because the fitness consequences of character displacement are not known in most other systems that have undergone character displacement. In at least one other case, however, the invader appears to have won. The medium ground finch, *Geospiza fortis*, was already present on the Galápagos island of Daphne Major when the large ground finch, *G. magnirostris*, invaded. Following this invasion, the two species underwent character displacement in resource use and beak morphology that enabled

the invader to monopolize the more nutritious seed resource (Grant & Grant, 2006). Thus, as in spadefoots, the invader has apparently won during character displacement in *Geospiza* finches. Further research into additional cases of character displacement is necessary to determine if invaders generally win, however. Because successful invaders may often be superior competitors (Sakai *et al.*, 2001; Vila & Weiner, 2004; Rehage *et al.*, 2005), invasive species may generally be more likely to win during character displacement. Moreover, we may only detect character displacement when the invader monopolizes the more profitable resource; because population sizes should be smaller for recent invaders in general, any invaders that fail to monopolize the more profitable resource are more likely to go extinct.

In sum, population genetic, phylogenetic and phylogeographic analyses, when combined with information about fitness trade-offs, can shed light on the outcome of character displacement. Ultimately, this historical perspective may help us to understand whether invaders generally win during character displacement, and, if so, why.

## Acknowledgments

We thank P. Marko, K. Pfennig, R. Martin, C. Ledon-Rettig, A. Chunco, S. Dhole and E. Wojtowicz for helpful discussion and comments; E. Myers and one anonymous reviewer for helpful suggestions; R. Martin and K. Pfennig for help with collections; R. Newman, J. Jones and S. Lowe for supplying *S. bombifrons* tissue samples; the American Museum of Natural History's (AMNH) Southwestern Research Station (SWRS) for housing and logistical support; and the Game and Fish Departments of Arizona, Colorado, New Mexico and Texas for providing scientific collecting permits. We thank the following museums for *S. bombifrons* tissue samples: the Sternberg Museum of Natural History, the Museum of Vertebrate Zoology (UC-Berkeley), the Texas Natural History Collection of the Texas Memorial Museum and the Sam Noble Oklahoma Museum of Natural History. This work was funded by the National Science Foundation (DEB-0234714 and DEB-0640026), Sigma Xi, SWRS and the AMNH.

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Received 15 November 2007; revised 5 February 2008; accepted 8 February 2008

### Supplementary material

The following supplementary material is available for this article:

**Appendix S1** Supplementary methods and results.

**Table S1** *Spea bombifrons* collection localities and sample sizes.

**Table S2** *Spea multiplicata* collection locations and sample sizes.

**Table S3** Distribution and occurrence of cytochrome *b* haplotypes from *Spea bombifrons* collection locations.

**Table S4** Distribution and occurrence of cytochrome *b* haplotypes from *Spea multiplicata* collection locations.

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