

Distinctiveness in the face of gene flow: hybridization between specialist and generalist gartersnakes

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Abstract

Patterns of divergence and polymorphism across hybrid zones can provide important clues as to their origin and maintenance. Unimodal hybrid zones or hybrid swarms are composed predominantly of recombinant individuals whose genomes are patchworks of alleles derived from each parental lineage. In contrast, bimodal hybrid zones contain few identifiable hybrids; most individuals fall within distinct genetic clusters. Distinguishing between hybrid swarms and bimodal hybrid zones can be important for taxonomic and conservation decisions regarding the status and value of hybrid populations. In addition, the causes of bimodality are important in understanding the generation and maintenance of biological diversity. For example, are distinct clusters mostly reproductively isolated and co-adapted gene complexes, or can distinctiveness be maintained by a few 'genomic islands' despite rampant gene flow across much of the genome? Here we focus on three patterns of distinctiveness in the face of gene flow between gartersnake taxa in the Great Lakes region of North America. Bimodality, the persistence of distinct clusters of genotypes, requires strong barriers to gene flow and supports recognition of distinct specialist (*Thamnophis butleri*) and generalist (*Thamnophis radix*) taxa. Concordance of DNA-based clusters with morphometrics supports the hypothesis that trophic morphology is a key component of divergence. Finally, disparity in the level of differentiation across molecular markers (amplified fragment length polymorphisms) indicates that distinctiveness is maintained by strong selection on a few traits despite high gene flow currently or in the recent past.

Keywords: conservation genetics, hybrid zone, speciation, *Thamnophis butleri*, *Thamnophis radix*

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Introduction

A common assumption in evolutionary and conservation biology is that gene flow is a strong homogenizing force. This principle was emphasized by Mayr (1954) and Eldredge & Gould (1972) in their arguments for the importance of small isolated populations as sites of rapid evolution, and many others in arguments favouring allopatric speciation (Mayr 1963; Coyne & Orr 2004). Similarly, proponents of the biological species concept reason that the occurrence of distinct kinds of organisms is

possible only when successful interbreeding between them is rare (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004; Futuyma 2005). Following from these ideas, conservationists are often concerned that hybridization might cause the loss of distinctiveness of a protected taxon — a result that some authors have characterized as a kind of extinction (Rhymer & Simberloff 1996; Allendorf *et al.* 2001; Daniels & Corbett 2003; Allendorf & Luikart 2007; Fitzpatrick & Shaffer 2007a, b).

Much of this reasoning appears to be founded on Wright's (1931) rule of thumb that an average of one migrant per generation is adequate to counteract divergence via genetic drift. Specifically, populations are likely to fix alternative alleles by random drift only when the effective

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migration rate is lower than one individual every two generations (see Mills & Allendorf 1996; Vucetich & Waite 2000; Wang 2004). However, single-locus models involving divergent selection have shown that adaptive divergence in the face of gene flow requires only that the selection coefficient exceed the migration rate (Haldane 1930; Wright 1931; Bulmer 1972), or equivalently, the probability of random mating (Gavrilets 2003). Multilocus simulations also indicate that the necessary strength of selection per locus decreases asymptotically with increasing numbers of divergently selected loci (Fry 2003). Thus, the homogenizing effect of gene flow is not a foregone conclusion, but rather a hypothesis on the strength and prevalence of divergent selection.

In natural hybrid zones, the distribution of genetic variation provides considerable information on the effects of gene flow and selection. The shape of a cline can be used to estimate the overall strength of the barrier to gene exchange given certain assumptions about selection and dispersal (Barton & Gale 1993; Vines *et al.* 2003). Recent analyses using many molecular markers have focused on heterogeneity in patterns of differentiation across markers and what it might say about the genetic basis of reproductive isolation and ecological adaptation (Rieseberg *et al.* 1999; della Torre *et al.* 2002; Storz & Dubach 2004; Turner *et al.* 2005; Stinchcombe & Hoekstra 2007; Yatabe *et al.* 2007). These studies support the prediction that genomes can be 'porous', engaging in extensive gene flow while maintaining significant differentiation near loci responsible for divergently selected traits (Barton 1979a; Barton & Bengtsson 1986; Mallet 2005). This is in contrast to the conventional idea of 'the genome' as a delicately co-adapted gene complex that could be severely disrupted by gene exchange (Dobzhansky 1937; Mayr 1963).

The distribution of genotypes among individuals within localities is the most direct indication of whether distinct groups can co-exist at the same place and time. 'Genotypic clusters' has even been offered as a phenomenological definition of 'species' (Mallet 1995). The stable co-existence of distinct clusters requires barriers to gene exchange and is almost always associated with ecological and behavioural differentiation (Jiggins & Mallet 2000; Schluter 2000; Coyne & Orr 2004). An important question is whether ecological, morphological, and behavioural distinctiveness require barriers to gene exchange that have genome-wide effects or whether selection processes affecting only some genomic regions cause and maintain distinctiveness despite high levels of interbreeding and gene exchange across other genomic regions.

Here we evaluate the ability of two closely related gartersnakes to remain distinct despite evident gene flow in a zone of geographical overlap in southern Wisconsin, USA. Butler's gartersnake, *Thamnophis butleri*, is a dietary specialist, consuming almost nothing but earthworms and

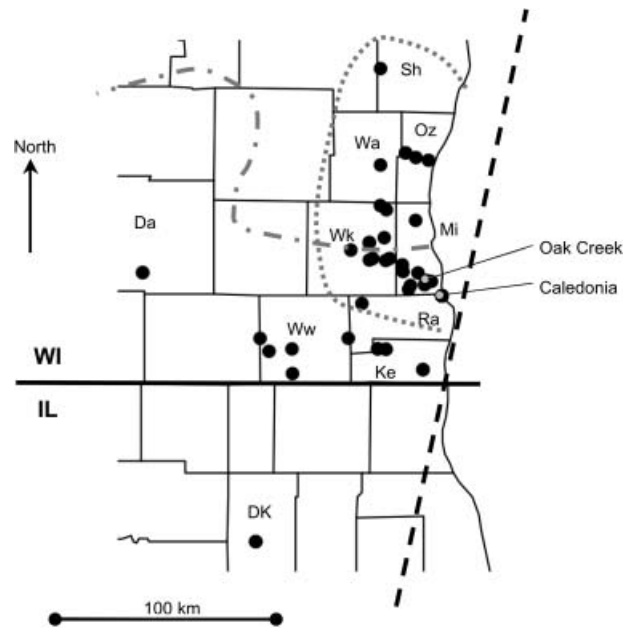


Fig. 1 Map of sample localities showing county boundaries (black lines), approximate species ranges (dotted line for *Thamnophis butleri* and dash-dot-dash for *Thamnophis radix*), and the best fit cline axis (dashed line). The heavy black line represents the border between Wisconsin (WI) and Illinois (IL). Lake Michigan is to the right. Counties of interest include: Sh, Sheboygan; Wa, Washington; Oz, Ozaukee; Wk, Waukesha; Mi, Milwaukee; Da, Dane; Ww, Walworth; Ra, Racine; Ke, Kenosha; DK, DeKalb.

occasional leeches in nature (Carpenter 1952; Burghardt 1967; Rossman *et al.* 1996). Morphological and molecular evidence identify the plains gartersnake, *Thamnophis radix*, as the sister group to *T. butleri* (Rossman *et al.* 1996; Alfaro & Arnold 2001; de Queiroz *et al.* 2002). Rossman *et al.* (1996) suggested that *T. butleri* is a dwarfed (neotenic) derivative of *T. radix*; the small body size (38–69 cm total length) and relatively small head of *T. butleri* may be adaptations to its annelid diet. *Thamnophis radix* is more generalized, consuming small vertebrates in addition to earthworms, with greater adult body length (51–102 cm) and a more robust head than *T. butleri* (Rossman *et al.* 1996). The geographical ranges of *T. butleri* and *T. radix* overlap in southern Wisconsin (Fig. 1) and individuals with intermediate or mosaic morphological and behavioural characteristics have been observed (Albright 2001; Casper 2003; Kirby 2005).

Butler's gartersnake is currently listed as Threatened in Wisconsin and Ontario, and as Endangered in Indiana (IDNR 2007; Lydersen 2006; WDNR 2006; Environment Canada 2008). Its decline is attributed mainly to habitat destruction; wet meadows and prairies are being developed rapidly in many areas (Vogt 1981; Joppa & Temple 2005). This development pressure is highest in certain areas within the contact zone between *T. butleri* and *T. radix* (e.g.

metropolitan Milwaukee). Therefore, the taxonomic status and conservation value of individuals and populations in the contact zone is of great interest among managers and developers. The treatment of hybrids under endangered species legislation is determined on a case-by-case basis (USFWS 1996; Haig & Allendorf 2006) and the research reported here is part of a larger multidisciplinary study examining the nature and scope of the issue. If extensive hybridization is found, legislative decisions regarding criteria for protection and designation of critical habitat will have to consider some interesting challenges. For example, is hybridization to be regarded as a threat to *T. butleri* or an important evolutionary process that is itself in danger of obliteration?

We used a combination of morphometrics and 285 amplified fragment length polymorphism (AFLP) markers to (i) test for evidence of gene flow across the contact zone between *T. butleri* and *T. radix*, (ii) evaluate support for bimodal vs. unimodal distributions of individuals within populations, and (iii) screen for heterogeneity among molecular markers, as might be expected if divergent selection and gene flow hold sway over different regions of the genome.

Methods

Sampling and morphometrics

Ninety-four individual gartersnakes were captured opportunistically from 34 sites in southern Wisconsin and northern Illinois (Fig. 1). Most sites are represented by 1–3 specimens; however, we obtained larger samples from two localities (Caledonia, 19 snakes; and Oak Creek, 32 snakes) to address questions about the distribution of genotypes within local populations. Tissues for DNA analysis were obtained by clipping slivers off several ventral scales or by removing up to 1 cm from the tip of an individual's tail. For the two larger population samples, we also included three mensural characters in analyses: snout-vent length (SVL, tip of the snout to the posterior terminus of the vent, measured by gently placing live snakes on a meter stick and waiting for relaxation), head length (HL, distance from snout to posterior margin of parietal scales), and head width (HW, widest point anterior to posterior margin of parietal scales) in millimetres. All measures were repeated three times and an average value used. HL and HW measurements were made with digital calipers.

Molecular markers

DNA was extracted following the DNeasy protocol (QIAGEN). AFLP markers were obtained using the standard protocol described by Vos *et al.* (1995) using four different

selective primers (MseCTAG – GATGAGTCCTGAGTA-ACTAG, MseCTAC – GATGAGTCCTGAGTAACTAC, MseCTTC – GATGAGTCCTGAGTAACTTC, MseCTTG – GATGAGTCCTGAGTAACTTG). Fragment presence–absence was determined by running labelled selective polymerase chain reaction (PCR) product in an ABI 3100 automated sequencer. Fluorescent peaks were aligned and called using the DAX software (Van Mierlo Software Consultancy) and then verified by visually inspecting each individual chromatogram. To ensure repeatability, a complete technical replicate (from restriction–ligation through selective PCR) was performed for each individual. Repeatability was estimated for each marker as one minus the ratio of the number of differences between technical replicates to the total number of snakes genotyped (Bonin *et al.* 2004; Pompanon *et al.* 2005; Nosil *et al.* 2008). Putative markers with less than 90% repeatability were discarded from the analysis. While a restricted focus on diagnostic or ancestry-informative markers (AIMs) is desirable in some cases (Fitzpatrick & Shaffer 2007a; Mebert 2008), inclusion of all reliable markers is necessary to provide a fair evaluation of genomic heterogeneity (Stinchcombe & Hoekstra 2007; Nosil *et al.* 2008).

Data analysis

We evaluated evidence for hybridization per se using a Bayesian population genetic clustering algorithm in the software Structure 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2007). We used Structure to cluster individuals in the overlap zone according to two alternative models. First, the no-admixture model assumes that each individual belongs to one of the two populations. Here, the Markov chain Monte Carlo (MCMC) algorithm approximates the posterior probability of population membership for each individual in the sample. If each individual is assigned to a population with high confidence, then we may infer that the no-admixture model is a good fit. If many individuals cannot be assigned with high confidence, and particularly if those individuals are from the putative hybrid zone, then we may prefer the admixture model. Under the admixture model, the MCMC estimates the fraction of each individual's genome that is derived from each population. After fitting each model to the data, we evaluated the weight of evidence in favour of admixture using an approximate Bayes factor (ratio of the estimated marginal likelihood of the admixture model to that of the no-admixture model). Bayes factors of 20:1 may be considered strong evidence in favour of one hypothesis over another, and over 100:1 may be considered very strong or definitive (Kass & Raftery 1995; Goodman 1999).

Analyses were run for 100 000 burn-in Markov chain generations and the posterior distributions estimated from 100 000 samples of 1 million generations after the

burn-in. These analyses were repeated with and without the prior specification of certain sites as reference *Thamnophis butleri* and *Thamnophis radix* samples (*T. butleri* sites were the most northerly sites, including Sheboygan, Ozaukee, northern Waukesha counties, Wisconsin. *Thamnophis radix* sites were the most southerly, including DeKalb Co., Illinois, and Dane and Walworth counties, Wisconsin). In practice, the resulting conclusions and individual assignment/admixture scores did not depend on the prior population information, but the overall mean posterior likelihoods were higher for the analyses run without the prior specification of *T. butleri* and *T. radix* reference samples. Therefore, only these results are reported.

We plotted individual ancestry estimates (proportion of alleles derived from *T. butleri*) against a single spatial axis (the cline axis) following Yanchukov *et al.* (2006). This involves estimation of a trend surface relating ancestry to latitude and longitude followed by projection of the two-dimensional spatial coordinates onto a single axis that is perpendicular to the dividing line between expected ancestry $> 1/2$ and $< 1/2$ on the trend surface.

To address the question of whether individuals within populations fall predominantly into two genotypic clusters corresponding to *T. butleri* and *T. radix*, we compared Bayesian ancestry proportions based on the analysis of AFLPs in Structure with morphometric data for the two sites where we had substantial sample sizes (Caledonia and Oak Creek). We entered SVL, HL, and HW into a *k*-means cluster analysis with $k = 2$. The *k*-means algorithm partitions individuals into two groups along the first principal component axis in such a way as to minimize the within-group variance (Ding & He 2004). Numerical simulations show that *k*-means (with $k = 2$) will tend to split a bimodal distribution such that each group contains one of the modes, whereas a unimodal distribution will simply be split down the middle. Thus, additional information must be applied to evaluate the usefulness of putative clusters (e.g. Loganantharaj *et al.* 2006).

We plotted morphological principal component scores against DNA-based ancestry estimates and fit linear models to assess (i) whether ancestry and morphology are correlated, and (ii) the extent to which any such correlation can be accounted for by cluster membership as opposed to a continuum of intermediates. The first model is a simple regression of ancestry on the principal component (PC) scores, the second is a regression of ancestry on inferred morphological cluster (a two-state factor), and the third is a multiple regression including both PC scores and cluster assignments. We compared these three models using Akaike's information criterion (AIC).

Within the Caledonia and Oak Creek samples, we also tested the empirical distribution of individual ancestry estimates against the expected distribution after one and two generations of random mating. We simulated random

mating by randomly forming pairs from the observed data and calculating their offspring ancestry as the average (midparent) ancestry of the pair (McKeigue *et al.* 2000). We performed 10 000 replicates to estimate the expected distribution of ancestry after one and two generations for each site. We then used Kolmogorov–Smirnov tests to evaluate the difference between observed and expected distributions. R code for performing these tests is available at <http://web.utk.edu/~bfitzpa1/under 'R scripts'>.

As a check on the ancestry estimates from Structure, we also used Buerkle's (2005) maximum-likelihood method to estimate ancestry, again using the northernmost and southernmost sites as putative reference samples. This ancestry index was highly correlated with the Bayesian estimates ($r = 0.96$) and did not lead to different patterns in other analyses.

To address the question of whether distinctiveness persists in the face of extensive gene flow, we follow the logic of Lewontin & Krakauer (1973; see also Beaumont 2005; Stinchcombe & Hoekstra 2007), that the demographic process of gene flow should be reflected in an overall or background level of genetic differentiation while divergent selection may cause a greater level of differentiation at a subset of loci. We evaluated heterogeneity among markers using two strategies based on the approach of Beaumont & Nichols (1996) and Beaumont & Balding (2004). They identified markers with exceptional β (Cockerham & Weir's 1993 estimator of F_{ST}) by comparing the distribution of β estimated from the observed data to a simulated null distribution. Their method takes advantage of the fact that β is highly dependent on f_1 , the probability that alleles drawn from different populations are the same (they refer to the quantity $1 - f_1$ as 'heterozygosity', but we find this misleading because heterozygosity usually refers to the gene diversity within populations or the actual frequency of heterozygous genotypes). Outliers are identified with respect to the joint distribution of β and $1 - f_1$ simulated in the program `FDIST2`. The null hypothesis being simulated is migration-mutation-drift balance with no selection. We used this approach to evaluate heterogeneity among markers in their contributions to divergence between the clusters identified by Structure. The simulation implemented in `FDIST2` uses an infinite alleles or stepwise-mutation model and may give nonsensical results for biallelic markers. Therefore, we repeated the analysis using Simcoal2 to generate biallelic [RFLP (restriction fragment length polymorphism)] data. Following the recommendations of Beaumont & Balding (2004), we tuned the migration and mutation rates to obtain median β and $1 - f_1$ similar to those calculated from the observed data and used their program `Cplot` to estimate quantiles of β conditioned on $1 - f_1$.

Finally, we used the false discovery rate estimation procedure of Pounds & Morris (2003) to estimate the fraction of markers that deviate from the null hypothesis of no

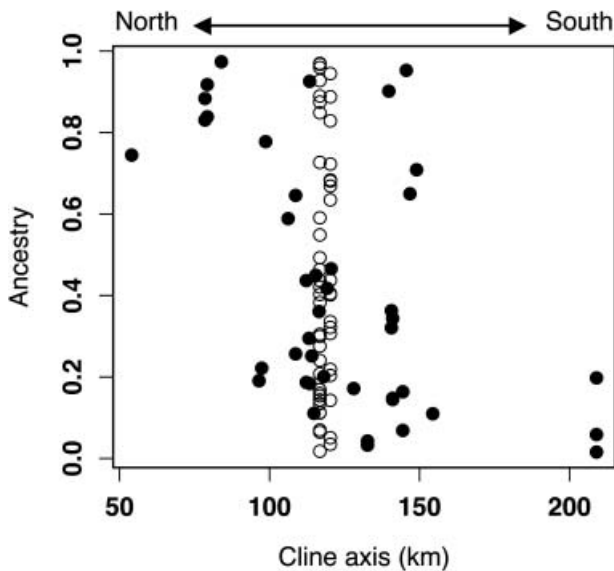


Fig. 2 Distribution of ancestry estimates (proportion of individual genomes derived from *Thamnophis butleri*) along the cline axis (Fig. 1 and results) between Sheboygan County, Wisconsin (at the far north) and DeKalb County, Illinois (at the far south). Oak Creek and Caledonia samples are shown as open circles.

difference in allele frequency between *T. butleri* and *T. radix*, and the fraction of markers for which this null hypothesis is true. In this procedure, we performed χ^2 tests for the difference in estimated allele counts between species for each marker (Weir 1996). Under the null hypothesis of no difference between *T. butleri* and *T. radix*, the *P* values from a large number of markers are expected to follow a uniform distribution (Pounds & Morris 2003). The beta-uniform mixture (BUM) analysis estimates the best partition of a set of *P* values into a uniform (flat) distribution, corresponding to the null hypothesis, and a beta distribution with high density at low *P* values, corresponding to the alternative hypothesis (Pounds & Morris 2003).

Results

Hybridization and spatial structure

The approximate marginal log likelihoods of the admixture and no-admixture models fitted using Structure (with no prior population information) were -14965.9 and -15116.8 , respectively; the approximate Bayes factor favouring admixture was 3.7×10^{65} . As Bayes factors greater than 100 may be considered 'decisive' (Kass & Raftery 1995; Goodman 1999), we conclude that the data strongly support the hypothesis that *Thamnophis butleri* and *Thamnophis radix* have been hybridizing in southern Wisconsin. This result is illustrated in Fig. 2. Including prior designation of *T. butleri* and *T. radix* reference sites did not alter this

conclusion, but lowered each likelihood estimate, perhaps owing to the occurrence of individuals with some degree of mixed or ambiguous ancestry in some designated reference sites.

Gartersnakes with inferred mixed ancestry between 25% and 75% *T. butleri* were found throughout southern Waukesha and Milwaukee counties and in northern Racine County (Fig. 2). Individuals with high (> 90%) and low (< 10%) *T. butleri* ancestry were also found throughout the contact zone. There was no evidence supporting a change in mean ancestry across the contact zone; linear and quadratic regressions of ancestry on geographical coordinates (including only the 19 sites within Waukesha, Milwaukee and Racine counties) fit the data poorly (multiple regression of *T. butleri* ancestry on UTM coordinates within the contact zone gave $r^2 = 0.006$, $F_{2,78} = 0.25$, $P = 0.79$). While a trend may be detectable with larger sample sizes, the key observation remains that individuals spanning the full range of ancestry proportions can be found throughout the contact zone (Fig. 2).

Bimodality

The frequency distributions of individual ancestry estimates (fraction of the genome derived from *T. butleri*) appear bimodal within the Oak Creek and Caledonia sites (Fig. 3). Kolmogorov–Smirnov tests did not distinguish the observed distribution of ancestry from the expected distribution after one generation of random mating ($D = 0.222$ and $P = 0.307$ for Caledonia, $D = 0.198$ and $P = 0.193$ for Oak Creek). But the observed distributions were significantly more bimodal than the expected distributions after two generations of random mating ($D = 0.314$ and $P = 0.048$ for Caledonia, $D = 0.283$ and $P = 0.0169$ for Oak Creek). In addition, the relationship between the genetic ancestry estimates and morphological variation strongly suggests clustering (Fig. 3).

The *k*-means clustering algorithm split the combined sample into groups of 17 and 32 along the first principal component (Fig. 3a). The first principal component (PC1) accounted for 84% of the variance and had roughly equal positive loadings for all three morphometrics (0.577 SVL, 0.569 HW, and 0.586 HL). Results were similar for analyses run with raw data and log-transformed data and whether the covariance or correlation matrix was used; we report results from the raw data analysed via the correlation matrix. While this principal component is likely a good indicator of overall 'size', it also clearly includes aspects of shape (Bookstein 1989; Somers 1989). The loadings are multivariate coefficients indicating contributions of head width and length given their covariances with SVL.

There is a significant regression of individual ancestry estimates on principal component scores ($F_{1,47} = 32.01$, $P < 0.0001$), however, the AIC of this linear regression was

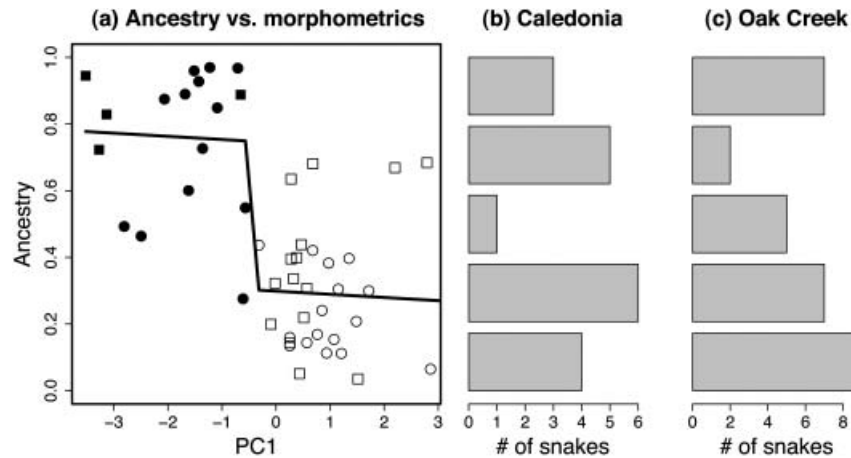


Fig. 3 (a) Comparison of molecular and morphological clustering in two population samples. High ancestry means a large fraction of an individual's genome is inferred to be *Thamnophis butleri*. The morphometric coordinate (PC1) distinguishes shorter snakes with relatively small heads (negative values) and longer snakes with relatively large heads (positive values). Black symbols represent individuals placed in the smaller group by *k*-means cluster analysis. Circles are samples from Oak Creek and squares are from Caledonia. The fitted regression line is: (expected ancestry) = $0.2987 - 0.0095(\text{PC1}) + 0.4456(X)$, where $X = 1$ for individuals falling into the smaller morphometric cluster and $X = 0$ otherwise. The overall model fit is: $r^2 = 0.573$, $F_{2,46} = 30.86$, $P < 0.0001$. Only the coefficient on X was significantly different from zero ($t = 4.252$, $P = 0.0001$ cf. the coefficient on PC1: $t = 0.296$, $P = 0.7682$). (b) and (c) histograms illustrate bimodal frequency distributions of individual genetic ancestry estimates at Caledonia and Oak Creek, respectively.

0.4344 as compared to -13.8096 for a model including both the PC1 score and inferred morphological *k*-means cluster membership and -15.7161 for a model including only cluster membership. Figure 3(a) illustrates the full model, showing only a subtle (and statistically unsupported) relationship between ancestry and PC1 within clusters. The 17 smaller snakes with relatively smaller heads tended to have higher *T. butleri* ancestry and the 32 larger snakes with relatively larger heads tended to have higher *T. radix* ancestry (Fig. 3a). The clustering is by no means perfect, with many intermediate individuals on both morphological and genetic axes. However, the relationship between the inferred morphometric and genetic clusters is strong and there is little or no relationship between morphology and ancestry within clusters (Fig. 3a).

Genomic heterogeneity

Most markers showed only low levels of differentiation (Fig. 4a). The median of F_{ST} was 0.071 and 90% of markers had $F_{ST} < 0.2$. The distribution of β vs. $1 - f_1$ appeared \angle shaped, with many markers near $\beta = 0$, and many markers near their maximum possible value given $1 - f_1$ (the 1:1 diagonal of Fig. 4). The two-allele mutation model was a better fit for the data because the relationship between β and $1 - f_1$ is far more constrained when there are only two alleles (Fig. 4b,c). There was no evidence of outliers relative to the 95% envelopes of the simulated distributions (Fig. 4b, c), and the observed and simulated (two-allele) medians were not significantly different (randomization tests: $P = 0.796$ for the median of $1 - f_1$ and $P = 0.547$ for

the median of β). However, the observed and simulated distributions are different (Kolmogorov–Smirnov $D = 0.2377$, $P < 0.00001$ for $1 - f_1$ and $D = 0.2167$, $P < 0.00001$ for β). The \angle shape of the observed bivariate distribution is a substantial deviation from the simulated null distributions. The distribution of β becomes bimodal as $1 - f_1$ increases.

The BUM analysis of population differentiation P values estimated a mixture of 85.4% markers where the null hypothesis of no difference is true and 14.6% with significant differences in allele frequencies between *T. butleri* and *T. radix* (Fig. 5). The empirical distribution of P values was significantly different from the expected uniform distribution (Kolmogorov–Smirnov $D = 0.1446$, $P = 0.00001$). This and the distribution of β vs. $1 - f_1$ (Fig. 4) lead us to conclude that the data are inconsistent with a neutral model of migration–drift balance. The empirical distribution appears to be a mixture of a set of markers with very little differentiation (low β) at any level of f_1 and a set of markers that are close to their maximum possible β given $1 - f_1$.

Discussion

Our analyses of 285 AFLP markers support the hypothesis that *Thamnophis butleri* and *Thamnophis radix* form a hybrid zone in southeastern Wisconsin. The Bayes factor of the admixture model over the no-admixture model was decisive. Individuals with inferred mixed ancestry were found in a roughly 70-km wide zone corresponding closely to Casper's (2003) hypothesized hybrid zone (Figs 1 and 2). However, there is no evidence of a spatial trend in ancestry within

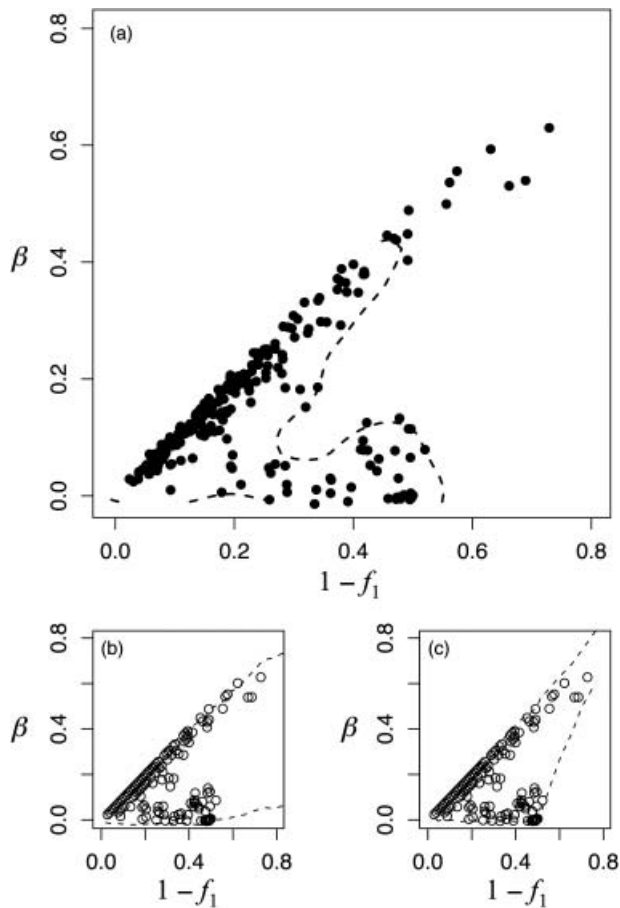


Fig. 4 (a) Empirical distribution of β vs. $1-f_1$ with a small amount of noise ('jitter') added to each point to avoid overlap and better illustrate the density of points. The dashed line is the 95% density contour estimated with the bivariate kernel density estimation function `kde2d` in R (Venables & Ripley 2002). In (b) and (c) the same points are shown in comparison to the 95% envelopes of the null distributions simulated with `Fdist2` (b) and with the two-allele mutation model in `Simcoal2` (c).

the zone and individuals estimated to be > 90% *T. butleri* and > 90% *T. radix* were both found throughout the contact zone (Fig. 2). This unusual configuration suggests that hybridization has failed to merge local populations of these two groups of gartersnakes into homogeneous admixtures. The distribution of genotypes across the zone and within populations indicates that this is a contact zone with limited gene flow between overlapping populations rather than a spatial gradient of intermediates between *T. butleri* and *T. radix*.

Interbreeding is expected to result, not in elimination of variation, but in randomization of variation (Mendel 1866; Hardy 1908; Slatkin 1987). The outcome of secondary contact without strong assortative mating or divergent selection should be formation of populations with unimodal distributions of individual ancestry clustered around

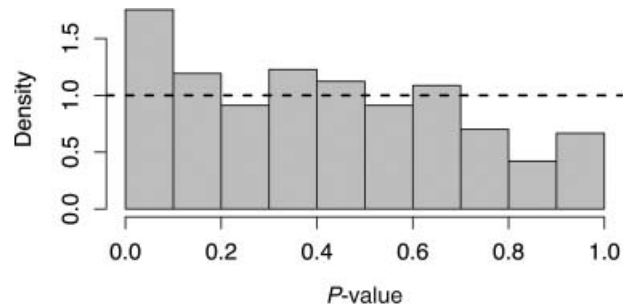


Fig. 5 Frequency distribution of *P* values for the null hypothesis of no difference in allele frequency between *Thamnophis butleri* and *Thamnophis radix*. Dashed line illustrates the expected density (uniform distribution) if the null hypothesis were true for all markers.

a population mean admixture proportion (Long 1991; Harrison & Bogdanowicz 1997; Jiggins & Mallet 2000). The mean admixture proportion is expected to vary geographically along a smooth cline that may be maintained by a balance between dispersal, genetic drift, and selection (Barton 1979b; Barton & Gale 1993). The occurrence of distinctly *T. butleri* and *T. radix* individuals throughout the contact zone in southern Wisconsin (Fig. 2) represents a nonrandom partitioning of the total genetic variation. Such nonrandomness occurs under two circumstances: (i) in the early generations of secondary contact, ancestry will be overdispersed (but not bimodal) owing to the gradual decay of linkage disequilibria (Lewontin 1974; Long 1991), and (ii) nonrandom distributions of genotypes can be maintained indefinitely in a balance between gene flow and divergent selection, including mate choice (Barton 1979b; Barton & Gale 1993; Jiggins & Mallet 2000). These two scenarios can be distinguished based on their effects on the distribution of variation among individuals and on heterogeneity among molecular markers.

Distinctiveness: bimodality within populations

Figure 3 suggests that individual ancestry estimates within populations tend to be clustered on either side of 50%. Two generations of random mating would eliminate such bimodality therefore these populations are probably affected by divergent selection or assortative mating. These bimodal distributions of ancestry indicate that *T. butleri* and *T. radix* occur as distinct clusters of genotypes even where they locally co-occur.

Clusters of individuals with predominantly *T. butleri* and *T. radix* ancestry based on AFLP data show remarkable concordance with morphological clusters corresponding to a group of smaller snakes with relatively smaller heads (*T. butleri*) and larger snakes with relatively larger heads (*T. radix*). This association of clusters is not a consequence

of arbitrarily subdividing a data set with an underlying covariance between continuous variables. The linear model treating morphological cluster membership as a predictor of AFLP-based ancestry fit the data better than a direct regression of ancestry on the morphometric PC1, and including both cluster membership and PC1 in the same model did not substantially improve the fit. These results are more consistent with a discontinuity between two groups than a continuous hybrid swarm.

The small size and relative head size of *T. butleri* may be functionally related to its specialized diet (predominantly earthworms). The concordance of molecular and morphological clusters in the contact zone is consistent with a causal relationship between ecological differences and resistance to the homogenizing effects of gene flow in this system. Mathematical and verbal theories of divergence in the face of gene flow hold frequency-dependent selection caused by ecological trade-offs as the most reliable way to generate and maintain distinct groups that can co-exist in sympatry (reviewed by Coyne & Orr 2004; Gavrillets 2004; Bolnick & Fitzpatrick 2007). However, ecological differences may be simply 'protected' from the homogenizing effect of gene flow if co-existing groups are strongly reproductively isolated by intrinsic barriers such as assortative mating, hybrid infertility, or developmental incompatibilities (Futuyma 1989; Coyne & Orr 2004). Thus, it is important to distinguish between distinctive clusters occurring as a consequence of strong genome-wide barriers to gene flow vs. distinctive clusters maintained by strong selection despite substantial gene flow.

Male gartersnakes use pheromone trails for trailing and locating females; most gartersnakes strongly prefer to follow conspecific female trails (Ford 1986). For example, *T. butleri* males from Lower Michigan preferred conspecific female trails over those of sympatric *T. sirtalis* females. On the other hand, these same *T. butleri* males showed no preference for conspecific female trails over female trails from *T. radix* captured in Central Illinois. Ford (1986) hypothesized that *T. butleri* speciated from *T. radix* relatively recently and allopatrically, without selection pressure to evolve pheromone specificity. In passing, he also mentioned preliminary data indicating that in Milwaukee County, Wisconsin, *T. butleri* did not discriminate conspecific from sympatric *T. radix*. Without voucher specimens or genetic data, it is unclear exactly what animals Ford tested, although he stated to G.M.B. that morphological identification was confusing (personal communication, March, 2008). Such studies should be repeated with animals of known genetic composition from the hybrid zone.

Gene flow: heterogeneity among markers

Demographic processes such as fluctuations in population size and levels of dispersal among populations affect all

parts of the genome in the same way; in the absence of selection, differences in population genetic patterns among loci result from the simple randomness inherent in genealogical and mutational processes (Lewontin & Krakauer 1973; Hey & Machado 2003). Divergent selection (viability, fecundity, or mate choice) has the potential to single out certain loci, resulting in genomically localized barriers to gene exchange with little effect on unlinked loci (Barton & Bengtsson 1986; Chan & Levin 2005; Turner *et al.* 2005). Thus, a signature of divergent selection in the face of gene flow is the occurrence of relatively few loci with exceptional levels of divergence relative to the genomic 'background' (Beaumont 2005; Savolainen *et al.* 2006; Nosil *et al.* 2008).

Figure 4 illustrates just such genomic heterogeneity in the comparison of allele frequencies between *T. butleri* and *T. radix*. Most markers showed only low levels of differentiation, but several showed large differences in allele frequency. The BUM estimation results were consistent with a mixture of distributions where about 85% of markers have no differentiation between taxa and about 15% are differentiated. We infer that recent or ongoing gene flow between *T. butleri* and *T. radix* in Wisconsin has been sufficient to distribute genetic variation fairly evenly across taxa for most of the genome. However, approximately 15% of markers were associated with divergently selected traits maintaining two distinct genetic and morphological clusters corresponding to *T. butleri* and *T. radix*.

Conclusions

Instead of meeting in a clinal hybrid zone, *T. butleri* and *T. radix* appear to overlap in southern Wisconsin, where they engage in limited gene exchange. Hybridization between 'good species' is more common than once thought and its consequences for evolution, ecology, and conservation are subjects of considerable debate and research (Allendorf *et al.* 2001; Arnold 2006; Gompert *et al.* 2006; Fitzpatrick & Shaffer 2007a; Mallet 2005; Mallet 2007; Mavarez *et al.* 2006; Mebert 2008). The weight of evidence in this case indicates that natural hybridization between *T. butleri* and *T. radix* occurs but does not result in uniformly admixed populations. The distinctiveness between these two gartersnakes is evident throughout the contact zone. Most important for conservation management, individuals genetically identified as the protected taxon, *T. butleri*, are likely to occur in some proportion at any suitable locality in the hybrid zone. Answering the question of whether or not these individuals can be distinguished reliably in the field awaits more detailed morphometric analyses.

Hybridization between *T. butleri* and *T. radix* may be a result of secondary contact or a stage in nonallopatric divergence-with-gene-flow (Clarke 1966; Endler 1977; Futuyma 2005). A broader phylogeographical analysis of

both taxa will be necessary to evaluate those alternatives. The present study documents distinctiveness in the face of gene flow, joining a number of other studies (for reviews, see Jiggins & Mallet 2000; Berlocher & Feder 2002; Bolnick & Fitzpatrick 2007) that appear to be at odds with the notion of gene flow as a strong conservative force preventing the origin and maintenance of biological diversity. This and other examples of bimodal hybrid zones (Jiggins & Mallet 2000) and divergence-with-gene-flow (Rice & Hostert 1993; Jiggins & Mallet 2000; Bolnick & Fitzpatrick 2007; Niemiller *et al.* 2008) support the contention that natural selection can be more important than genetic drift in the origin of species (Schluter 2000; Fitzpatrick 2002; Coyne & Orr 2004).

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