MOLECULAR ECOLOGY

Molecular Ecology (2009) **18**, 4888–4903 doi: 10.1111/j.1365-294X.2009.04381.x

# The effect of marker choice on estimated levels of introgression across an avian (Pipridae: *Manacus*) hybrid zone

TAMAKI YURI,*† ROBERT W. JERNIGAN,‡ ROBB T. BRUMFIELD,*§¶ NIRMAL K. BHAGABATI*** and MICHAEL J. BRAUN*¶

**Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, 4210 Silver Hill Road, Suitland, MD 20746, USA, †Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK 73072, USA, ‡Department of Mathematics and Statistics, American University, Washington, DC 20016, USA, §Museum of Natural Science, 119 Foster Hall, Louisiana State University, Baton Rouge, LA 70803, USA, ¶Department of Biology and Behavior, Ecology, Evolution and Systematics Program, University of Maryland, College Park, MD 20742, USA, **Conservation Science Program, World Wildlife Fund, 1250 24th Street NW, Washington, DC 20037, USA*

## Abstract

**Hybrid zones are often characterized by narrow, coincident clines for diverse traits, suggesting that little introgression occurs across them. However, this pattern may result from a bias in focussing on traits that are diagnostic of parental populations. Such choice of highly differentiated traits may cause us to overlook differential introgression in nondiagnostic traits and to distort our perception of hybrid zones. We tested this hypothesis in an avian hybrid zone by comparing cline structure in two sets of molecular markers: isozyme and restriction fragment length polymorphism markers chosen for differentiation between parental forms, and microsatellite markers chosen for polymorphism. Two cline-fitting methods showed that cline centre positions of microsatellite alleles were more variable than those of isozyme and restriction fragment length polymorphism markers, and several were significantly shifted from those of the diagnostic markers. Cline widths of microsatellite alleles were also variable and two- to eightfold wider than those of the diagnostic markers. These patterns are consistent with the idea that markers chosen for differentiation are more likely to be under purifying selection, and studies focussed on these markers will underestimate overall introgression across hybrid zones. Our results suggest that neutral and positively selected alleles may introgress freely across many hybrid zones without altering perceived boundaries between hybridizing forms.**

*Keywords*: diagnostic markers, hybrid swarm, microsatellites, neutral markers, smoothing splines, stepped cline model

*Received 16 March 2009; revision received 24 July 2009; accepted 3 August 2009*

## Introduction

Hybrid zones have long been considered important arenas for the study of speciation (Endler 1977; Barton & Hewitt 1985; Harrison 1990; Arnold 1997). The perceived evolutionary significance of hybrid zones is, however, likely to be influenced by the methods chosen to characterize them. For example, our attention is often drawn to cases of hybridization between dramatically differentiated organisms because the hybrids are strikingly distinct from the parental forms. This tendency may have led to an overrepresentation, among well-studied hybrid zones, of those that are narrow, temporally stable and characterized by strong selection against hybrids. This bias could contribute to a common view of hybrid zones as 'black holes' where new alleles go in but do not come out (e.g. Gill *et al*. 1993).

Correspondence: Tamaki Yuri, Fax: 1-405-325-7699;
E-mail: tyuri@ou.edu

Furthermore, many stable, well-studied hybrid zones are characterized by steep, concordant and coincident clines for diverse traits, suggesting limited introgression across them (Moore 1977; Barton & Hewitt 1985; Arnold 1997). The uniformity of these clines, however, may be influenced by the tendency of investigators to focus on diagnostic genetic markers; those fixed for alternate alleles in the parental forms. Such diagnostic markers are useful for identifying hybrids but will typically represent a biased subset of genetic loci with high $F_{ST}$. This subset is more likely to be under purifying selection that opposes introgression than randomly selected loci; thus, they will tend to show steep and coincident clines across a hybrid zone (Sattler & Braun 2000; Brumfield *et al.* 2001). Because hybridizing organisms are generally closely related (e.g. Harrison & Arnold 1982; Robbins *et al.* 1986; Latta & Mitton 1999; Payseur *et al.* 2004; Mebert 2008), and their genetic incompatibilities are likely to be limited, diagnostic markers typically represent only a small fraction of the genome. To gain an unbiased view of genetic structure and introgression across a hybrid zone, one should sample representative markers from the entire genome (Rieseberg *et al.* 2002; Teeter *et al.* 2008).

One approach to assess the impact of this potential sampling bias is to use a control set of marker loci chosen without regard to their degree of differentiation between hybridizing forms. Such markers are more likely to be neutral themselves and to represent a random sample of the genome with respect to linkage to loci under selection. Introgression rates of neutral markers vary according to their physical linkage with selected genes (e.g. Rieseberg *et al.* 1999; Via & West 2008). In stable hybrid zones, therefore, a continuum in the degree of differentiation is expected among neutral loci, while selected loci would tend to represent the upper range of differentiation (Bierne *et al.* 2003).

To assess the impact of diagnostic markers on estimates of introgression, we investigated a hybrid zone between two closely related manakins (*Manacus vitellinus* and *Manacus candei*) in western Panama. These birds are strikingly different in their secondary sexual traits, but hybridize frequently at the range interface, producing highly variable individuals that are intermediate in colour, morphology and genetics (Parsons *et al.* 1993; Brumfield *et al.* 2001). The hybrid zone features clines for male plumage colour that are shifted about 50 km west of the steep, coincident clines for most molecular and morphometric markers. The differential introgression of male plumage colour traits in these sexually dimorphic, polygynous, lek-mating birds is likely a result of sexual selection (McDonald *et al.* 2001; Stein & Uy 2006).

Four isozyme and three restriction fragment length polymorphism (RFLP) markers used by Brumfield *et al.* (2001) were chosen based on their differentiation between parental populations. Four of these markers were diagnostic, whereas three were nondiagnostic (polymorphic in one or both parental forms). Six markers were characterized by steep, coincident clines, whereas the seventh, the only marker that was not fixed in either parental form, showed a much broader cline. The authors suggested that introgression across the hybrid zone is not uniform throughout the genome and that the diagnostic markers exhibiting narrow clines may be under selection that inhibits introgression.

Here, we test this hypothesis of Brumfield *et al.* (2001) by comparing clines of their loci with those of 13 microsatellite loci chosen for polymorphism alone. Although the exact processes of microsatellite evolution are uncertain, microsatellites are often regarded as neutral markers (Charlesworth *et al.* 1994; Schlötterer 2000). Therefore, the microsatellite loci are less likely to be under purifying selection and should compose a less biased sample than the genetic loci used by Brumfield *et al.* (2001). We predict that, on average, the microsatellite loci will reveal a wider range of differential introgression across the *Manacus* hybrid zone than diagnostic markers, with clines that are broader in width and more variable in position. The overarching goals of this study are to assess whether marker choice affects estimation of genome-wide allelic introgression across hybrid zones and, by extension, to examine how marker choice influences our perceptions on genetic connectedness of hybridizing forms and evolutionary roles of hybrid zones.

## Materials and methods

### *Samples*

The 213 samples used in this study are described in Brumfield *et al.* (2001, 2003) and in Supplementary Table S1. Blood or other tissues were collected from 187 individuals comprising 10 locations along a transect spanning the *Manacus vitellinus*– *Manacus candei* hybrid zone in western Bocas del Toro, Panama (Fig. 1). To serve as reference populations of the parental species, samples of *vitellinus* and *candei* were examined from 250 km east and 140 km west of the hybrid zone, respectively. Sample size per locality ranged from 4 to 26, with a mean of 17.8 (Table 1).

### *Laboratory methods*

We collected allele size data from 13 microsatellite loci, including loci AC2, AC5, AC7, AC8, AC10, AC12, AC13

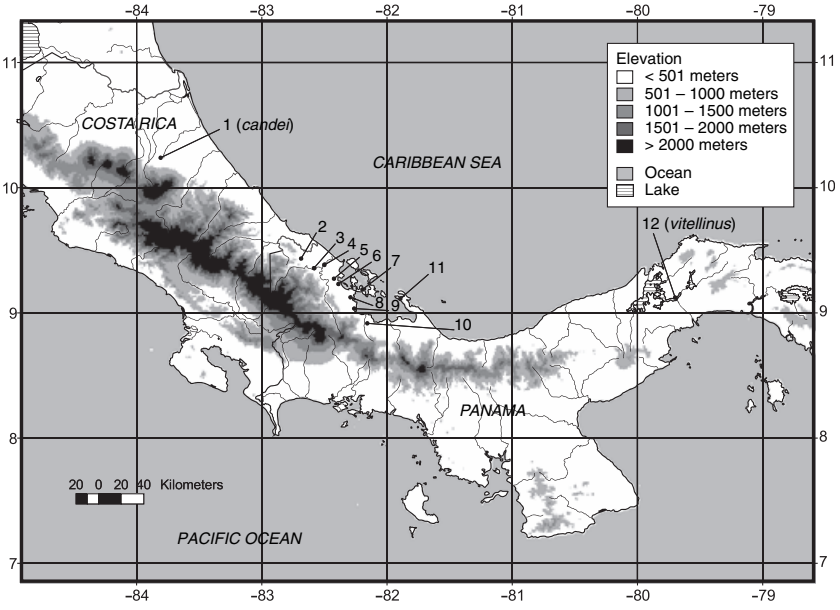


**Fig. 1** Map of 12 populations sampled across the *Manaus candei– Manaus vitellinus* hybrid zone.

and TCA1 from Brumfield (1999), Man3, Man6, Man7 and Man8 from Shorey *et al.* (2000) and LTMR8 from McDonald & Potts (1994). Primer sequences and polymerase chain reaction conditions are available from those sources. Data for nine loci from three sites (populations 2–4) were available from Brumfield (1999). DNA was isolated by the standard proteinase K/phenol–chloroform extraction and ethanol precipitation. Individual samples were amplified with fluorescently labelled primers (HEX, FAM, TET) and sized on ABI 373A or 3100 automated sequencers (Applied Biosystems, Foster City, CA). Chromatograms were analysed using GeneScan v.3.1 and Genotyper v.2.5 (Applied Biosystems). Genotypes for all loci were assigned to all individuals, except for one locus (Man8) from one individual in population 12.

### *Analyses of genetic variation*

Tests of Hardy–Weinberg (HW) expectations were performed with Genepop (Raymond & Rousset 1995), using the method of Guo & Thompson (1992). Expected heterozygosity was calculated according to Nei (1978) using Genetic Data Analysis (GDA, Lewis & Zaykin 1999). Allelic richness and $F_{IS}$ were calculated using FSTAT v.2.9.3.2 (Goudet 1995). We tested significance of $F_{IS}$ values by randomizing alleles among individuals within populations, with sequential Bonferroni correction for multiple tests (Holm 1979). Pairwise estimates of the coancestry coefficient $\theta$ (Weir 1996) and the coancestry distance, $d = -\ln(1-\theta)$ (Reynolds *et al.* 1983), were calculated using GDA. The coancestry distance does not assume equal rates of divergence across populations or constant rates for loci, which is appropriate when drift is responsible for differentiation.

### *Cline analysis*

We analysed cline structure for the seven genetic marker loci of Brumfield *et al.* (2001) and for all microsatellite alleles that met the following criteria: (i) allele frequency showed a clinal change, which is necessary for demonstrating introgression; (ii) There was a minimum difference of 0.25 between the highest and lowest frequencies. Choosing such alleles would likely exclude most alleles that vary in frequency only by sampling error and/or random variation owing to environmental fluctuations; (iii) when two alleles at a locus appeared correlated (i.e. when they were clearly not independent from each other), only one was analysed. Initially, 17 alleles from 11 loci were chosen for analysis. Cline shape parameters were estimated by fitting curves to allele frequency data using two methods.

### *Stepped cline model*

We used a likelihood cline modelling approach that is described by Szymura & Barton (1986, 1991) and implemented in Analyse (Barton & Baird 1999), following Brumfield *et al.* (2001). The model consists of three separate sections of curves; a cline centre section fitted by a sigmoid curve and two tailing sections fitted by exponential curves. For each cline, goodness-of-fit tests were performed to identify a stepped cline model (SCM) that best described the data with the fewest number of parameters [Models I, II, III and the goodness-of-fit tests

**Table 1** Summary of expected heterozygosity ($H_E$), allelic richness (AR) and inbreeding coefficient ($F_{IS}$) for 13 microsatellite loci in 12 populations across the hybrid zone transect

| Locus (# alleles) | | | AC2 (18) | | | AC5 (15) | | | AC7 (3) | | | AC8 (7) | | | AC10 (9) | | | AC12 (18) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Population | $N$ | Distance (km) | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ |
| 1 | 6 | 0.00 | 0.74 | 4.15 | −0.14 | 0.94 | 6.56 | −0.07 | 0.41 | 1.98 | −0.25 | 0.68 | 2.96 | 0.02 | 0.83 | 4.47 | 0.00 | 0.79 | 4.22 | −0.06 |
| 2 | 22 | 138.25 | 0.61 | 2.77 | 0.33 | 0.89 | 5.69 | 0.03 | 0.36 | 1.90 | 0.25 | 0.59 | 2.77 | −0.08 | 0.45 | 2.56 | −0.12 | 0.65 | 3.23 | −0.12 |
| 3 | 20 | 151.75 | 0.45 | 2.46 | 0.22 | 0.79 | 4.55 | −0.15 | 0.43 | 1.96 | 0.07 | 0.61 | 2.72 | 0.18 | 0.70 | 3.49 | 0.14 | 0.69 | 3.41 | −0.32 |
| 4 | 20 | 159.50 | 0.68 | 3.07 | 0.13 | 0.85 | 5.05 | 0.00 | 0.43 | 1.96 | 0.07 | 0.68 | 3.34 | 0.12 | 0.53 | 3.10 | −0.04 | 0.61 | 2.76 | 0.09 |
| 5 | 21 | 182.25 | 0.69 | 3.40 | −0.03 | 0.81 | 4.47 | −0.05 | 0.43 | 2.12 | 0.12 | 0.66 | 3.22 | 0.13 | 0.37 | 2.28 | −0.17 | 0.60 | 2.75 | 0.05 |
| 6 | 4 | 188.25 | 0.75 | 4.00 | −0.41 | 0.75 | 4.00 | −0.41 | 0.25 | 2.00 | 0.00 | 0.61 | 3.00 | 0.20 | 0.82 | 4.00 | 0.10 | 0.86 | 5.00 | 0.14 |
| 7 | 12 | 198.50 | 0.79 | 4.50 | 0.05 | 0.72 | 3.60 | 0.20 | 0.39 | 1.94 | 0.15 | 0.77 | 3.69 | −0.09 | 0.67 | 3.52 | 0.00 | 0.72 | 3.60 | 0.08 |
| 8 | 22 | 201.25 | 0.74 | 3.86 | 0.02 | 0.65 | 3.36 | −0.26 | 0.27 | 1.78 | −0.17 | 0.70 | 3.39 | −0.25 | 0.58 | 3.31 | 0.14 | 0.53 | 3.18 | −0.04 |
| 9 | 26 | 210.00 | 0.90 | 5.75 | 0.06 | 0.84 | 4.81 | −0.01 | 0.50 | 2.57 | 0.01 | 0.79 | 4.30 | 0.03 | 0.74 | 3.93 | 0.22 | 0.90 | 5.76 | 0.02 |
| 10 | 20 | 230.75 | 0.72 | 4.21 | 0.03 | 0.71 | 3.82 | 0.16 | 0.54 | 2.55 | −0.22 | 0.73 | 3.45 | 0.11 | 0.70 | 3.37 | 0.00 | 0.77 | 4.09 | −0.04 |
| 11 | 20 | 319.50 | 0.81 | 4.55 | 0.14 | 0.81 | 4.35 | −0.05 | 0.47 | 2.47 | −0.06 | 0.77 | 3.81 | −0.04 | 0.64 | 3.07 | −0.01 | 0.85 | 4.96 | 0.06 |
| 12 | 20† | 569.50 | 0.75 | 4.43 | 0.07 | 0.85 | 4.88 | 0.12 | 0.61 | 2.68 | 0.02 | 0.62 | 3.26 | −0.04 | 0.67 | 3.17 | −0.20 | 0.86 | 5.20 | 0.13 |
| All | 213 | | 0.83 | | | 0.85 | | | 0.45 | | | 0.76 | | | 0.75 | | | 0.85 | | |

| AC13 (12) | | | TCA1 (10) | | | LTMR8 (6) | | | Man3 (13) | | | Man6 (11) | | | Man7 (21) | | | Man8 (13) | | | All |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $H_E$ | AR | $F_{IS}$ | $F_{IS}$ |
| 0.62 | 2.89 | 0.21 | 0.00 | 1.00 | NA | 0.56 | 3.24 | −0.21 | 0.71 | 3.56 | −0.19 | 0.70 | 3.33 | 0.05 | 0.76 | 3.63 | −0.36 | 0.77 | 3.80 | −0.33 | −0.11 |
| 0.68 | 3.21 | −0.08 | 0.09 | 1.36 | −0.01 | 0.68 | 3.31 | 0.14 | 0.78 | 4.26 | 0.25 | 0.69 | 3.46 | −0.05 | 0.85 | 5.00 | −0.07 | 0.75 | 3.78 | −0.16 | 0.02 |
| 0.66 | 3.04 | 0.01 | 0.40 | 2.63 | −0.14 | 0.64 | 3.18 | −0.10 | 0.77 | 4.01 | 0.09 | 0.63 | 3.08 | −0.12 | 0.82 | 4.70 | 0.09 | 0.77 | 4.17 | −0.10 | −0.01 |
| 0.73 | 3.47 | 0.11 | 0.10 | 1.40 | −0.01 | 0.64 | 2.98 | −0.26 | 0.81 | 4.43 | 0.20 | 0.66 | 3.33 | 0.02 | 0.85 | 4.90 | 0.06 | 0.82 | 4.56 | −0.11 | 0.03 |
| 0.72 | 3.62 | 0.14 | 0.30 | 2.05 | 0.20 | 0.74 | 3.58 | −0.03 | 0.82 | 4.50 | −0.11 | 0.72 | 3.53 | −0.06 | 0.87 | 5.19 | 0.01 | 0.82 | 4.58 | −0.04 | 0.00 |
| 0.82 | 4.00 | −0.26 | 0.71 | 3.00 | 0.33 | 0.61 | 3.00 | 0.20 | 0.86 | 5.00 | −0.20 | 0.68 | 3.00 | 0.29 | 0.93 | 6.00 | −0.09 | 0.75 | 4.00 | 0.37 | 0.02 |
| 0.73 | 3.78 | −0.03 | 0.65 | 3.23 | 0.24 | 0.77 | 3.89 | 0.25 | 0.83 | 4.79 | 0.10 | 0.72 | 3.69 | −0.16 | 0.89 | 5.58 | 0.17 | 0.88 | 5.27 | 0.06 | 0.08 |
| 0.75 | 4.03 | −0.09 | 0.52 | 2.51 | 0.14 | 0.70 | 3.45 | −0.10 | 0.83 | 4.80 | 0.02 | 0.77 | 3.91 | 0.06 | 0.87 | 5.17 | −0.05 | 0.84 | 4.87 | 0.14 | −0.03 |
| 0.87 | 5.26 | −0.06 | 0.74 | 3.85 | −0.04 | 0.59 | 2.67 | −0.11 | 0.84 | 4.81 | −0.05 | 0.86 | 5.10 | −0.03 | 0.92 | 6.21 | 0.00 | 0.87 | 5.32 | 0.12 | 0.01 |
| 0.83 | 4.65 | 0.10 | 0.79 | 4.26 | 0.12 | 0.64 | 2.83 | −0.17 | 0.86 | 5.16 | 0.13 | 0.86 | 5.22 | −0.04 | 0.92 | 6.11 | −0.09 | 0.89 | 5.62 | 0.16 | 0.03 |
| 0.89 | 5.43 | 0.10 | 0.65 | 3.42 | −0.24 | 0.57 | 2.67 | −0.05 | 0.86 | 5.12 | 0.07 | 0.85 | 5.07 | 0.06 | 0.92 | 6.07 | 0.07 | 0.91 | 5.89 | 0.07 | 0.02 |
| 0.81 | 4.66 | 0.08 | 0.61 | 2.93 | 0.19 | 0.50 | 2.56 | −0.10 | 0.89 | 5.53 | −0.01 | 0.84 | 5.03 | −0.14 | 0.91 | 5.81 | −0.05 | 0.87 | 5.35 | −0.03 | 0.01 |
| 0.81 | | | 0.54 | | | 0.68 | | | 0.86 | | | 0.81 | | | 0.90 | | | 0.88 | | | |

†For locus Man8, $N$ for population 12 was 19.

are described in Brumfield *et al*. (2001)]. Model parameters were estimated by searching the parameter space for the maximum likelihood (ML) model, using the Metropolis-Hastings algorithm implemented in Analyse. Three thousand iterations were run from 10 different starting points for each model. The test statistic was calculated as twice the absolute difference in log-likelihood between the models under comparison. Significance ($\alpha = 0.05$) was determined by comparison to the chi-squared distribution, with degrees of freedom equivalent to the difference in number of parameters between models.

Effective sample sizes that take into account ML estimates of $F_{IS}$ and $F_{ST}$ (the standardized variance of allele frequency fluctuations between sites around the fitted cline, Szymura & Barton 1986, 1991) were calculated for each sample at each locus (Phillips *et al*. 2004). $F_{IS}$ is used to correct effective sample sizes in cases where there are deficiencies of heterozygotes. Reduced variance as a result of relatedness among individuals in a sample or random drift ($F_{ST}$) also reduces effective sample sizes. If these factors are not taken into account, the cline-fitting algorithms overweigh large samples and inflate confidence in the inference. Because numbers of alleles per locus vary among populations, suggesting heterogeneity in effective population size (or relatedness), likelihood-based $F_{ST}$ was calculated with two models, using either one global $F_{ST}$ value for all populations or two separate $F_{ST}$ values for two groups of populations. We selected an $F_{ST}$ model for each allele using the goodness-of-fit test mentioned before. Because Brumfield *et al*. (2001) used observed sample sizes, we repeated their cline analyses using effective sample sizes.

Cline parameters were estimated with the best-fit model for each locus by running 30 000 iterations of the

Metropolis-Hastings algorithm with five different starting points. We checked the validity of the fitted model by comparing repeat runs, which should always converge to the same global optimum (Barton & Baird 1999). For two alleles (loci AC2 and LTMR8), five Metropolis-Hastings searches failed to converge on a single ML value. This suggests that there is insufficient data to make sensible parameter estimates, and these alleles were not considered further.

Support limits (SL) for each parameter estimate were determined by projecting the volume of the explored parameter space that lies within two log-likelihood units of the optimum onto the plane of a given parameter, which are approximately equivalent to 95% confidence intervals (CI). In all cases, these estimates of SL were made by the examination of model parameters generated by making 20 000 random parameter changes with the best-fit model as a starting point for all parameters. For three of the seven loci from Brumfield *et al.* (2001) and six of the fifteen microsatellite loci, Metropolis-Hastings searches did not stay around the optimum, and 2–20 repeats of 20 000 iterations of Metropolis-Hastings resampling routines were necessary to produce enough samples that lie within two log-likelihood units of the optimum. In addition, Metropolis-Hastings search parameters (initial temperature and step size) were lowered to prevent the sampling system from wandering to states far from the optimum. Significance of differences between parameter estimates from different markers was determined from the SL; when SL of two parameter estimates were not overlapped, the difference was regarded as statistically significant.

### *Monotonic smoothing splines and parametric bootstrap*

Cline shape parameters were also estimated by fitting smoothing splines (SS; Schluter 1988) to allele frequency. Statistical comparisons of cline parameters among markers were made with a parametric bootstrap method (Efron & Tibshirani 1993). This approach can exploit the binomial model of sampling gene frequencies (Schluter 1988), but has the advantage of flexibility in not being constrained by a fixed underlying functional form.

Owing to the strong monotonic pattern of many of our markers and a prior expectation that a smooth monotonic function best describes many clines (Endler 1977; Barton & Hewitt 1985), we chose the smoothing parameter to minimize smoothing that guaranteed monotonicity of the logit-transformed frequency values weighted by the inverse of the estimated logit variances. The fitted SS were then transformed back to a cline of estimated allele frequencies. Using the estimated SS curve, the cline centre was calculated as the location where the estimated allele frequency $p = (p_{max} + p_{min})/2$, and the cline width as 1/maximum slope.

Two hundred parametric bootstrap samples of each marker were generated using a binomial model with probability of success being the spline-estimated gene frequency at each location. Then, the resulting bootstrap samples were logit transformed, and fitted by SS weighted by the inverse of the estimated logit variances. On each of the 200 resulting curves, we estimated sample cline centres and widths. The distribution of these 200 cline centres and widths model the variability in shape among the bootstrap samples. Pairwise statistical significance (*P*-values) of the difference in the cline parameter estimates between markers were calculated as the percentage of the difference values that occur beyond zero using 200 paired bootstrap cline parameter estimates from the two markers under comparison. In other words, if most of the difference values are positive, the *P*-value is the percentage of the difference values that are less than zero. If most of the difference values are negative, the *P*-value is the percentage of the difference values that are greater than zero. A significance level ($\alpha$) of 0.05 was applied for all comparisons.

### *Approximate marginal probability density of cline parameters*

Using Metropolis-Hastings and bootstrap resampling routines respectively, we collected 20 000 values for each parameter per allele in SCM analysis and 200 values in SS analysis. To illustrate probability distributions of cline shape parameter values, we calculated approximate marginal densities from the resampled parameter values using the program Locfit (Loader 1996) implemented in R (http://www.r-project.org/). Locfit estimates density using local likelihood fitting (Tibshirani 1984; Tibshirani & Hastie 1987), where the log-link function $\log[f(x)]$ is modelled by local polynomials. The procedure is applicable when an additive Gaussian model is inappropriate as an error structure, as is the case with posterior probability distributions.

## Results

### *Analyses of genetic variation and population structure*

All 13 microsatellite loci were polymorphic, with 3–21 alleles per locus, and values of expected heterozygosity ($H_E$) ranging from 0.45 to 0.90 (Table 1). $H_E$ was generally higher in the eastern (*Manacus vitellinus*) end of the transect (populations 10–12) than the western (*Manacus candei*) end (populations 1–3). Populations 10–12 also

had more alleles at 10 of 13 loci than populations 1–3, and their allelic richness was on average 1.3 times higher. After sequential Bonferroni corrections, no microsatellite locus showed significant deviation from HW expectations in any populations based on the exact HW tests or the tests using alternative hypotheses of heterozygote deficiency or excess (Rousset & Raymond 1995). Also, pairwise allelic combinations were in linkage equilibrium at all microsatellite loci. The isozyme and RFLP loci also met HW expectations, and individuals in population 9 showed a wide range of genetic and morphological intermediacy (Brumfield *et al.* 2001). Together, these observations demonstrate that hybridization and backcrossing are common in this system.

To explore patterns of differentiation in microsatellites vs. traditional loci used in Brumfield *et al.* (2001), we compared the pairwise coancestry coefficient $\theta$ estimated from the two data sets (Supplementary Table S2). Within either side of the hybrid zone (among populations 1–8 or populations 10–12), $\theta$ for the isozyme and RFLP markers was essentially zero (mean: –0.01, range: –0.03–0.05), whereas microsatellite $\theta$ was consistently larger (mean: 0.04, range: –0.01–0.08). In contrast, the reverse was true between populations across the hybrid zone; $\theta$ for isozyme and RFLP markers (mean: 0.60, range: 0.52–0.72) was much larger than that for microsatellites (mean: 0.14, range: 0.10–0.18). Trees constructed by Unweighted Pair Group Method with Arithmetic (UPGMA) with coancestry distances illustrate the difference between the two marker sets (Fig. 2). These observations are consistent with the hypothesis that the isozyme and RFLP markers chosen for differentiation compose a biased sample of the genome and overestimate differentiation between hybridizing taxa.

## *Cline analyses*

Our reanalyses of the isozyme and RFLP clines confirmed the findings of Brumfield *et al.* (2001) that SCM was best-fit with Model II for most loci, $\lambda 5$ required the more parameterized Model III and the less parameterized Model I was sufficient for *Ada* (Tables 2 and 3). In general, clines estimated with the modified SCM were very similar to those found by Brumfield *et al.* (2001) but produced broader SL on parameter estimates (Fig. 3 and Tables 2, 3) because estimated effective sample sizes were smaller than the observed sample sizes used in the original analyses. In contrast, clines estimated with the SS method consistently had greater cline widths, more widely shifted cline centres and wider CI.

Two microsatellite loci (AC7 and Man8) had no alleles that exhibited pronounced clinal variation across the transect. Each of the remaining 11 loci showed clinal variation in one to four alleles. The 15 microsatellite alleles analysed in detail (see 'Materials and methods' for the criteria of marker choice) consist of one or two alleles from each of 11 loci (Fig. 4). None of the microsatellite alleles required the most parameterized SCM (Model III). Model I produced the best fit for 12 alleles, and Model II for 3 alleles (Tables 2 and 3). SS parameter estimates were similar to SCM estimates in some cases (e.g. Man6-196 and AC8-137; Fig. 4), but again the SS method tended to estimate greater cline widths, more variable cline centres and wider CI (Fig. 4 and Tables 2, 3).

## *Cline centres*

Taking all markers and both methods of analysis into account, cline centre estimates spanned a relatively wide range, from 92 to 256 km (Table 2). With few exceptions, these estimates fall within the densely sampled portion of the transect (138–231 km; Table 1), suggesting that the population samples adequately covered the main range of genetic transitions.

The four diagnostic isozyme and RFLP markers had remarkably coincident cline centres that fell within

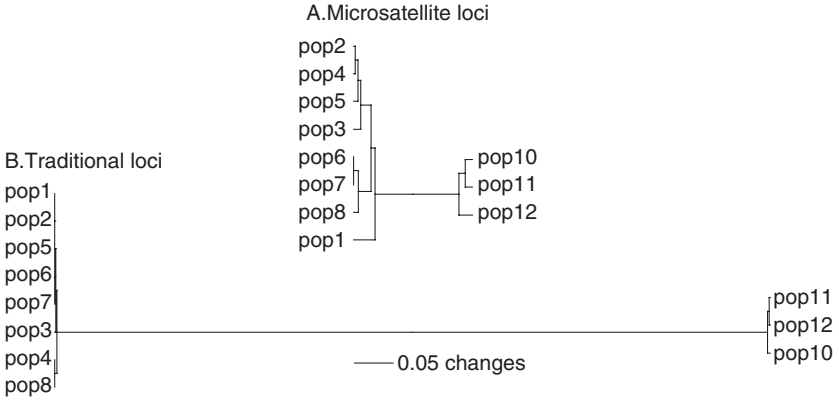


**Fig. 2** UPGMA trees estimated using coancestry distances based on two data sets; (a) 13 microsatellite loci from this study and (b) 4 isozyme and 3 restriction fragment length polymorphism loci from Brumfield *et al.* (2001). The negative distances were set to zero before constructing trees. Population 9 is located near the centre of the hybrid zone and was excluded from the analyses to elucidate comparisons within and between the two sides of the hybrid zone.

**Table 2** Estimates of cline centres using stepped cline model (SCM) and smoothing spline (SS) cline-fit for 15 microsatellite markers and 7 genetic markers from Brumfield *et al.* (2001). Support limits for SCM estimates and 95% confidence intervals (CI) using parametric bootstrap for SS estimates are shown

| | SCM | | | | | SS | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Support limits | | | 95% CI | |
| | Model | ln *L* | Cline centre (km)† | Low | High | Cline centre (km)† | Low | High |
| Microsatellite markers | | | | | | | | |
| AC2-216 | 2 | −2.4 | 205.2 | 198.4 | 211.3 | 195.3 | 159.3 | 216.4 |
| AC5-213 | 1 | −3.8 | 213.1 | 189.0 | 255.7 | 220.4 | 157.1 | 429.0 |
| AC8-129 | 1 | −4.9 | 218.4 | 202.7 | 254.4 | 238.4 | 186.3 | 378.7 |
| AC8-137 | 1 | −2.7 | 181.8 | 165.3 | 194.6 | 182.3 | 142.2 | 203.4 |
| AC10-282 | 2 | −1.6 | 202.4 | 201.3 | 209.9 | 216.4 | 197.4 | 274.5 |
| AC12-100 | 2 | −2.1 | 209.6 | 204.8 | 230.3 | 227.4 | 170.2 | 279.5 |
| AC13-161 | 1 | −2.0 | 207.5 | 198.1 | 284.3 | 254.5 | 132.2 | 358.8 |
| AC13-162 | 1 | −3.5 | 114.5 | 18.9 | 153.9 | 92.2 | 77.1 | 205.4 |
| TCA1-202 | 1 | −1.9 | 207.2 | 193.9 | 229.1 | 213.4 | 192.3 | 342.7 |
| TCA1-203 | 1 | −5.3 | 190.6 | 176.2 | 207.3 | 181.3 | 134.2 | 263.6 |
| LTMR8-143 | 1 | −3.1 | 214.3 | 155.6 | 273.1 | 232.4 | 163.3 | 278.5 |
| Man3-187 | 1 | −2.7 | 197.8 | 177.5 | 202.2 | 178.3 | 104.2 | 206.4 |
| Man6-196 | 1 | −2.2 | 202.1 | 189.4 | 212.4 | 199.4 | 165.3 | 214.4 |
| Man6-216 | 1 | −3.8 | 220.8 | 210.6 | 240.0 | 222.4 | 210.4 | 292.7 |
| Man7-174 | 1 | −1.9 | 185.1 | 153.0 | 207.2 | 176.3 | 90.1 | 356.7 |
| Diagnostic markers from Brumfield *et al.* (2001) | | | | | | | | |
| *Ak-2* | 2 | −2.5 | 208.8 | 205.8 | 212.7 | 221.4 | 214.4 | 229.4 |
| λ5 | 3 | −1.0 | 208.5 | 202.3 | 210.4 | 223.4 | 214.4 | 231.4 |
| mtDNA | 2 | −1.6 | 208.3 | 205.9 | 211.0 | 222.4 | 211.4 | 233.4 |
| pSCN-3 | 2 | −2.0 | 209.3 | 206.1 | 210.3 | 221.4 | 211.4 | 230.4 |
| Nondiagnostic markers from Brumfield *et al.* (2001) | | | | | | | | |
| *Ada* | 1 | −3.4 | 169.2 | 24.9 | 232.2 | 170.3 | 120.2 | 320.7 |
| *Gsr* | 2 | −1.9 | 210.0 | 200.9 | 257.6 | 256.5 | 176.2 | 391.7 |
| *Pgm-2* | 2 | −2.3 | 206.5 | 201.4 | 210.0 | 224.4 | 196.3 | 261.5 |

†Distance from population 1.

1–2 km of each other and were estimated with narrow SL or CI by either curve-fitting method (Table 2 and Fig. 3). SS estimates of diagnostic cline centres were all shifted about 13 km eastward from the SCM estimates. This shift may have more to do with how the methods fit the data rather than any biological significance (see 'Discussion'). Because the two methods resulted in substantially different cline parameter estimates for some alleles, our discussion hereafter will focus only on those comparisons for which both methods agree on the statistical significance of the differences in estimates.

Both methods produced more variable cline centre estimates for the three nondiagnostic isozyme and RFLP markers (Table 2 and Fig. 3). For *Ada*, cline centre estimates were shifted by about 40 (SCM) to 50 (SS) km from the diagnostic cline centres. The SL and CI were wider for the nondiagnostic markers (Table 2 and Fig. 5c,d), and none of their cline centres was significantly shifted from those of the diagnostic markers (Supplementary Table S3).

The 15 microsatellite alleles had even more variable cline centre estimates with both methods, and several were shifted by as much as 94–130 km from the diagnostic cline centres (Table 2). SL and CI for microsatellites were again generally wider than those for the diagnostic markers (Table 2 and Fig. 5a,b). Nevertheless, cline centres of three microsatellite alleles (AC8-137, AC13–162 and Man3–187) were significantly displaced from all diagnostic cline centres as well as centres of several other microsatellite and/or nondiagnostic clines by both methods (Supplementary Table S3). All significant shifts of microsatellite cline centres were westward into the range of *candei* (Fig. 5).

### *Cline widths*

Best estimates of cline widths ranged from 1.2 to 281.3 km (Table 3). Width estimates based on SS were consistently larger than those based on SCM (21 of 22 cases), whereas SCM estimates exhibited a wider range

**Table 3** Estimates of cline widths using stepped cline model (SCM) and smoothing spline (SS) cline-fit for 15 microsatellite markers and 7 genetic markers from Brumfield *et al.* (2001). Support limits for SCM estimates and 95% confidence intervals (CI) using parametric bootstrap for SS estimates are shown

| | SCM | | | | | SS | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Support limits | | | 95% CI | |
| | Model | ln *L* | Cline width (km) | Low | High | Cline width (km) | Low | High |
| Microsatellite markers | | | | | | | | |
| AC2-216 | 2 | −2.4 | 11.5 | 0.1 | 53.5 | 112.9 | 64.1 | 176.9 |
| AC5-213 | 1 | −3.8 | 82.8 | 36.3 | 407.1 | 192.3 | 75.4 | 376.7 |
| AC8-129 | 1 | −4.9 | 59.3 | 28.1 | 166.6 | 238.6 | 87.8 | 307.4 |
| AC8-137 | 1 | −2.7 | 65.4 | 29.5 | 128.2 | 107.2 | 53.0 | 162.1 |
| AC10-282 | 2 | −1.6 | 1.9 | 0.0 | 16.8 | 143.5 | 66.0 | 250.1 |
| AC12-100 | 2 | −2.1 | 3.3 | 0.0 | 39.0 | 144.6 | 71.0 | 241.8 |
| AC13-161 | 1 | −2.0 | 9.5 | 0.1 | 408.5 | 208.5 | 115.0 | 329.8 |
| AC13-162 | 1 | −3.5 | 228.5 | 118.4 | 527.9 | 139.3 | 79.7 | 173.9 |
| TCA1-202 | 1 | −1.9 | 40.1 | 12.9 | 177.6 | 98.5 | 63.7 | 262.4 |
| TCA1-203 | 1 | −5.3 | 83.9 | 51.3 | 173.6 | 242.1 | 97.8 | 290.7 |
| LTMR8-143 | 1 | −3.1 | 281.3 | 119.1 | 715.0 | 167.3 | 117.3 | 300.5 |
| Man3-187 | 1 | −2.7 | 21.7 | 10.0 | 81.1 | 88.2 | 50.5 | 165.6 |
| Man6-196 | 1 | −2.2 | 39.9 | 7.0 | 118.6 | 62.5 | 59.1 | 203.0 |
| Man6-216 | 1 | −3.8 | 44.9 | 22.4 | 100.9 | 60.8 | 38.6 | 276.1 |
| Man7-174 | 1 | −1.9 | 81.4 | 9.7 | 301.4 | 241.5 | 110.8 | 331.1 |
| Diagnostic markers from Brumfield *et al.* (2001) | | | | | | | | |
| *Ak-2* | 2 | −2.5 | 9.0 | 0.2 | 22.8 | 49.1 | 41.2 | 98.3 |
| λ5 | 3 | −1.0 | 10.8 | 1.1 | 19.3 | 77.3 | 46.8 | 110.7 |
| mtDNA | 2 | −1.6 | 11.0 | 6.5 | 20.3 | 69.6 | 35.7 | 110.1 |
| pSCN-3 | 2 | −2.0 | 3.9 | 0.2 | 18.2 | 60.4 | 50.9 | 118.0 |
| Nondiagnostic markers from Brumfield *et al.* (2001) | | | | | | | | |
| *Ada* | 1 | −3.4 | 247.2 | 68.6 | 1566.1 | 213.6 | 101.6 | 326.0 |
| *Gsr* | 2 | −1.9 | 1.2 | 0.0 | 82.8 | 267.0 | 86.7 | 305.6 |
| *Pgm-2* | 2 | −2.3 | 7.6 | 0.1 | 17.9 | 201.4 | 81.8 | 218.7 |

of cline widths from extremely narrow to extremely wide (SCM range 1.2–281.3; SS range 49.1–267 km).

For the diagnostic markers, cline width estimates made by either method were consistently among the narrowest of the clines examined (Table 3 and Fig. 3). However, there were marked differences between the two methods in width estimates. SCM cline width estimates for these four loci ranged from 3.9 to 11.0 km, whereas SS estimates for the same four loci ranged from 49.1 to 77.3 km. The difference between the methods may have to do with the model used to fit the data and the inherent difficulty in estimating the maximum slope of a curve when the transition is not strictly monotonic, as well as our sampling methods (see 'Discussion'). The biological significance of these results lies in the fact that both methods consistently estimated narrow cline widths and tight SL and CI for the diagnostic loci compared with the other classes of markers (Table 3). The distributions of resampled cline widths were very similar for all of the diagnostic loci, whether derived from SCM or SS (Fig. 6c,d).

The three nondiagnostic markers were more variable in cline width (Table 3 and Fig. 3). Both analyses showed that the width of *Ada* cline was significantly broader than any diagnostic cline, with point estimates of 247.2 (SCM) and 213.6 (SS) km (Supplementary Table S4 and Fig. 6c). However, the two methods fit quite different curves to the *Gsr* and *Pgm-2* data; although the cline width estimate for neither marker differed significantly from those of the diagnostic markers by SCM, SS estimated their widths significantly wider than any of the diagnostic loci (Supplementary Table S4 and Fig. 6d). Based on the visual inspection of the cline fit to the data points, SS appeared to have overestimated the cline widths with these markers.

Cline widths for microsatellite alleles were also variable, ranging from 1.9 to 281.3 km for SCM estimates, and from 60.8 to 242.1 km for SS analysis (Table 3 and Fig. 4). The SCM estimates of microsatellite cline widths averaged eightfold wider than those of the four diagnostic markers (70.4 vs. 8.7 km). For SS estimates, the difference in average width was more than twofold

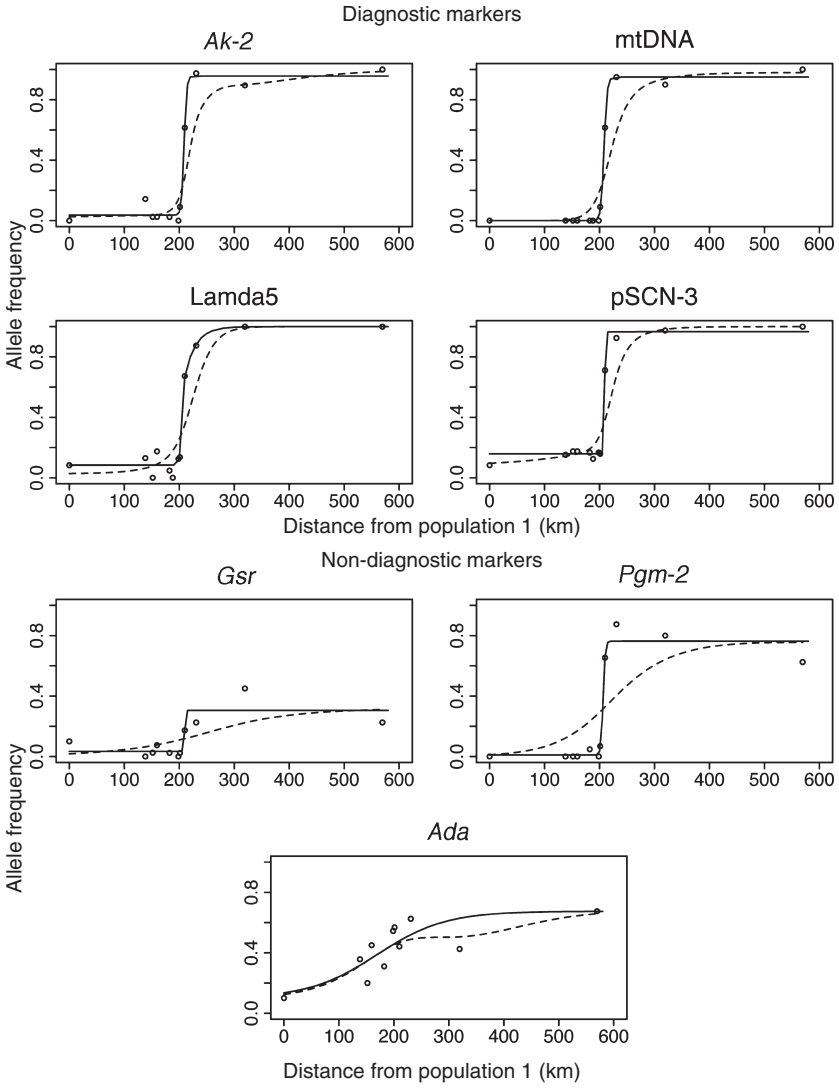


**Fig. 3** Fitted clines estimated from frequencies of seven isozyme and restriction fragment length polymorphism alleles from Brumfield *et al.* (2001) using stepped cline model (solid line) and smoothing spline (dotted line) methods. We defined two categories of markers: diagnostic markers are fixed or nearly fixed in both parental populations, and nondiagnostic markers are polymorphic in either one or both parental populations.

(149.9 vs. 64.1 km). Five microsatellite clines (AC5-213, AC8-129, AC13-162, TCA1-203 and LTMR8-143) were significantly broader than any diagnostic cline by both analyses (Supplementary Table S4 and Fig. 6a,b).

## Discussion

### *Cline structure variation*

In contrast to the markers chosen for differentiation by Brumfield *et al.* (2001), polymorphic microsatellite markers displayed clines that were broader in width and more variable in position in the *Manacus* hybrid zone. All the diagnostic loci were marked by narrow, coincident clines, suggesting a strong barrier to introgression for those loci. Yet, the wide range of microsatellite cline widths indicates that introgression can occur relatively freely for alleles at other loci. These results are consistent with the idea that microsatellites that evolve neutrally can diffuse across this hybrid zone, whereas diagnostic markers are likely to be under strong purifying selection opposing their introgression. Other microsatellites did have narrow clines similar to the diagnostic loci; introgression for those microsatellites may be impeded by linkage to traits under purifying selection. Furthermore, introgression was not restricted to microsatellite loci; at least one nondiagnostic isozyme cline (*Ada*) was found to be significantly broader than the diagnostic clines by both cline-fitting methods. This may also be caused by a lack of purifying selection, as *Ada* was the only nondiagnostic locus that was polymorphic in both parental populations.

In addition, at least three microsatellite clines centres were significantly displaced from the diagnostic clines. These markers may themselves be neutral but could be linked to loci under positive selection. Under this

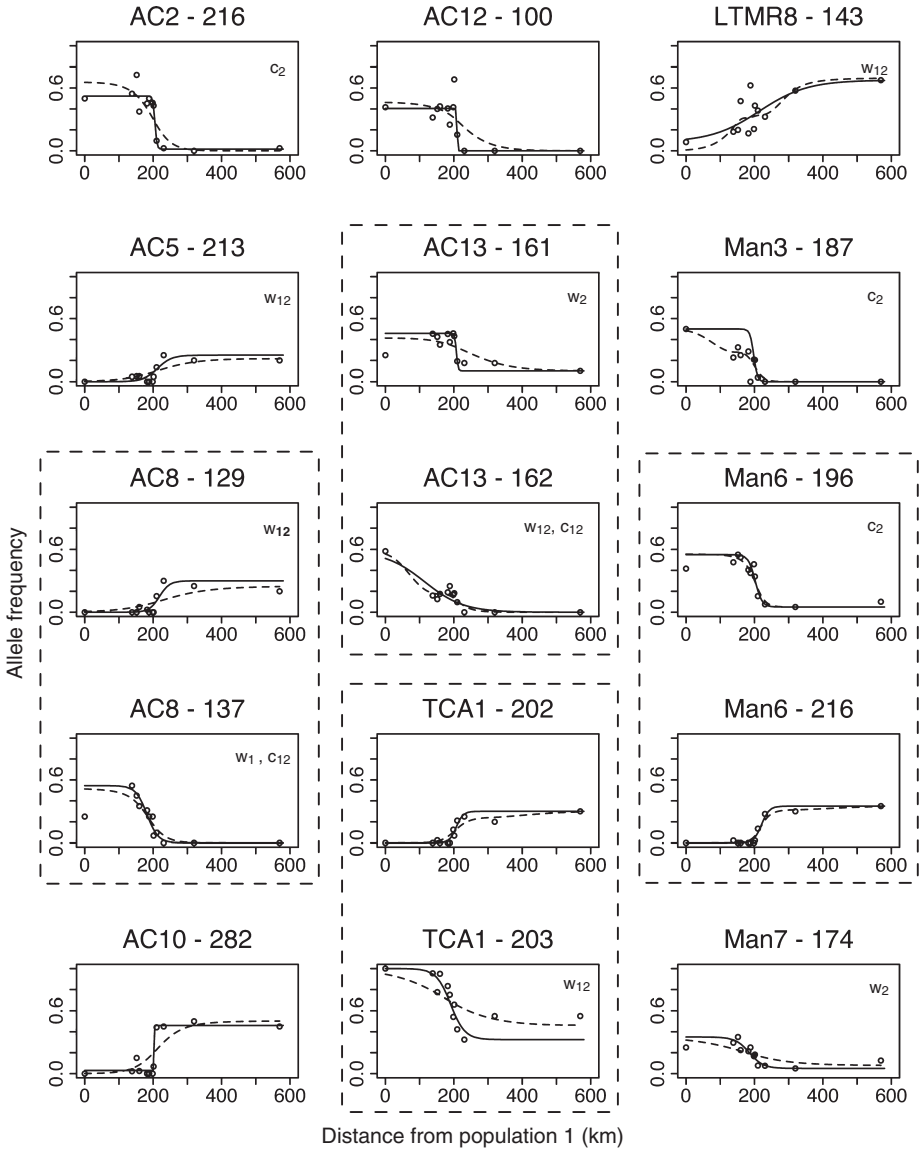


**Fig. 4** Fitted clines estimated from frequencies of 15 microsatellite alleles using stepped cline model (SCM; solid line) and smoothing spline (SS; dotted line) methods. The letters *c* and *w* appear in the upper right corner of those panels where the cline centre (*c*) or cline width (*w*) is significantly different from the four diagnostic markers of Brumfield *et al*. (2001). The subscripts 1 and 2 indicate significant differences by SCM analysis (1) or SS analysis (2). Dotted squares enclose alleles within the same locus.

scenario, the microsatellite cline centres would move with the selected traits as they introgress across the hybrid zone, but eventually stop when linkage between the neutral marker and the selected loci breaks down through recombination. Interestingly, all of the displaced microsatellite clines have moved westward into the range of *Manacus candei*. Male plumage colour clines have also introgressed westward in this system, apparently owing to positive sexual selection (McDonald *et al*. 2001; Stein & Uy 2006), providing one possible explanation for microsatellite cline movement.

The different patterns of introgression between our marker sets might be attributed to the fact that four of the seven markers of Brumfield *et al*. (2001) were isozyme loci; the selective status of enzymatic markers has long been debated (Eanes 1999). For example, Riginos *et al*. (2002) compared allele frequencies of allozyme and nonallozyme loci among mussel populations, and found that the allozyme loci were much more differentiated. They presented the results as evidence of purifying selection on allozyme loci. However, Bierne *et al*. (2003) found no statistical difference in the range of differentia-

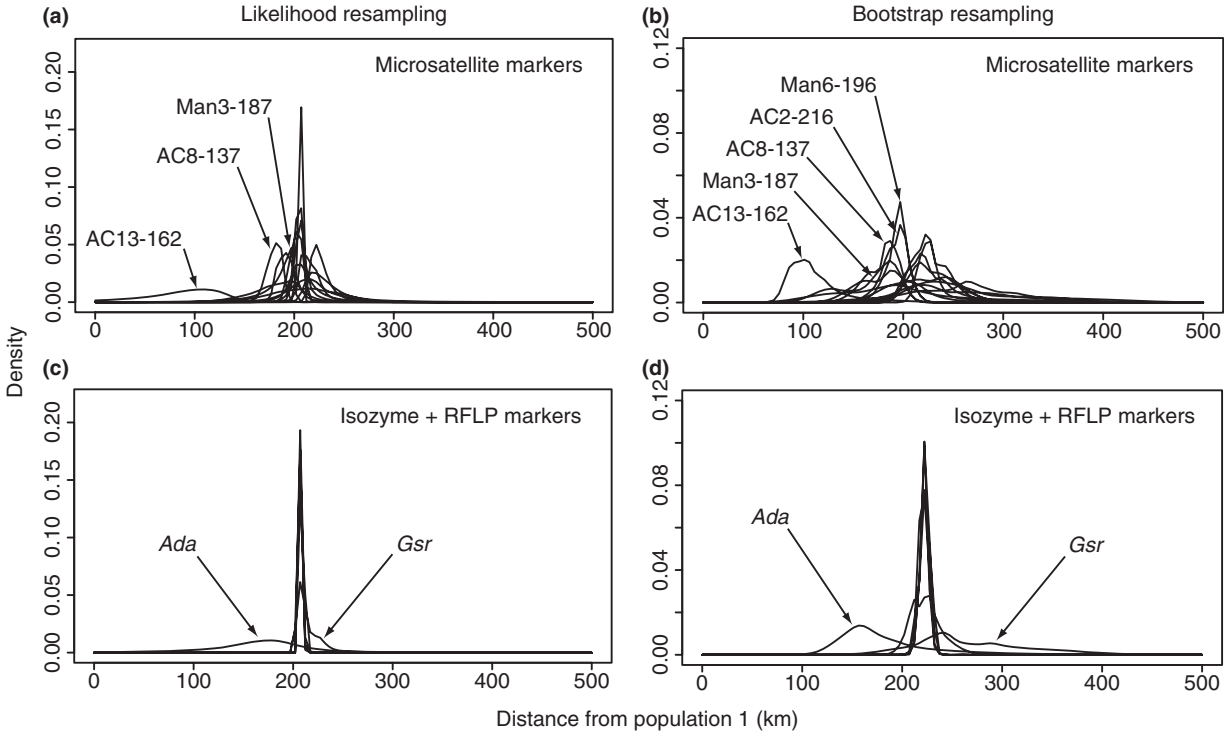


**Fig. 5** Density of cline centre estimates using likelihood (stepped cline model) and bootstrap (smoothing splines) resampling for 15 microsatellite loci from this study and seven genetic markers from Brumfield *et al.* (2001). Microsatellite markers that differ significantly from all diagnostic markers are labelled.

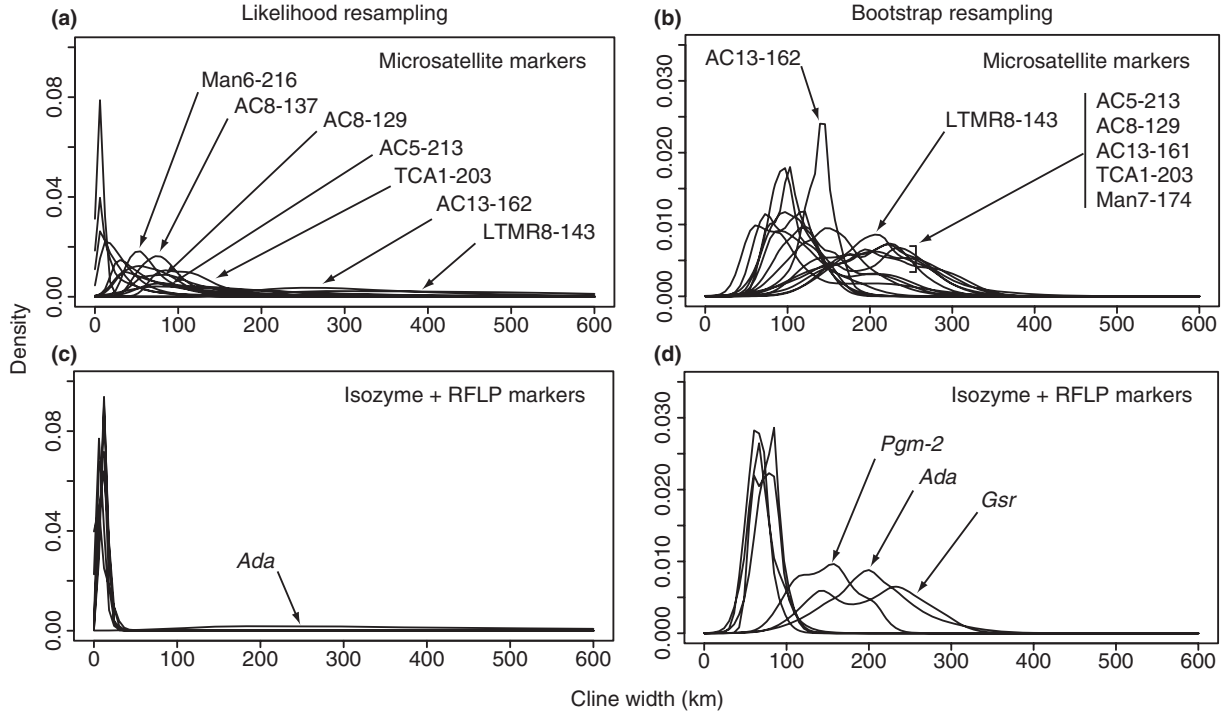


**Fig. 6** Density of cline width estimates measured by 1/maximum slope using likelihood (stepped cline model) and bootstrap (smoothing splines) resampling for 15 microsatellite loci from this study and 7 genetic markers from Brumfield *et al.* (2001). Microsatellite markers that differ significantly from all diagnostic markers are labelled.

tion between allozyme and nonallozyme markers, when the data set was expanded to include more loci. Consequently, Bierne *et al.* (2003) concluded that the original markers consisted of a biased sample of allozyme loci under strong selective pressure. The differences in introgression between the Brumfield *et al.* (2001) markers and the microsatellites are also likely to reflect the original sampling bias for differentiated loci over and above any systematic difference between marker types.

Alternatively, it might be supposed that broader clinal structures of nondiagnostic alleles could be formed from shared ancestral polymorphism either by random variations or sampling errors or by disruptive selection owing to environmental change. However, such scenarios are unlikely to explain our observations because there is no reason for many unlinked alleles that are shared ancestrally to form generally coincident clines of various widths in either of the scenarios.

### *Differential introgression across hybrid zones*

Multi-locus studies have increasingly revealed patterns of introgression that vary dramatically from locus to locus (e.g. Rieseberg *et al.* 1999; Wilding *et al.* 2001; Emelianov *et al.* 2004; Harr 2006; Carling & Brumfield 2008; Teeter *et al.* 2008). These studies suggest that hybridizing taxa vary significantly in the proportion of the genome that is subject to introgression. That proportion may be near zero in the case of hybrid sterility, intermediate in other cases (Rieseberg *et al.* 1999; Martinsen *et al.* 2001) or may include most of the genome (e.g. Flanagan *et al.* 2004; Vallender *et al.* 2007).

The genomic scope of introgression may be affected by degree of divergence, strength of selection and linkage relationships. When two hybridizing forms are genetically divergent, many incompatibilities may exist, and selection against hybrids is likely to be strong. Together with physical and epistatic linkage, selection at many dispersed loci can result in a generalized barrier to gene flow, effectively opposing introgression throughout most or all of the genome (Barton & Hewitt 1985). In such cases, diagnostic markers may be sufficient to gain a sense of the prevalence of introgression throughout the genome. However, many natural hybrid zones involve closely related taxa with fewer genetic incompatibilities. Hybridizing bird taxa, in particular, typically show low levels of genetic divergence, and low levels of hybrid dysfunction (Avise 1994; Grant & Grant 1994; Price & Bouvier 2002). In these cases, the strength of purifying selection is likely to vary among chromosomal regions depending on the number and distribution of loci under selection and the degree of linkage among them (Barton & Bengtsson 1986). Therefore, introgression may be unimpeded in some chromosomal regions, and assessments by diagnostic markers alone are likely to seriously underestimate average introgression genome-wide in many hybrid zones.

The mode or mechanism of divergence may also be important. Many avian hybrid zones are thought to have originated through secondary contact (Price 2008). In such cases, divergence has occurred in allopatry, and much of it may be because of neutral genetic drift. Once in contact, the gene pools of hybridizing forms will tend to merge for all loci that are not under purifying selection. This scenario contrasts with that of ecological speciation, where much of the accumulated divergence is because of direct selection for adaptive traits (e.g. Via & West 2008).

The geographic extent of introgression may be affected by dispersal potential, strength of selection, age of hybrid contact and linkage. Birds are vagile organisms, and thus, the potential for rapid and long distance gene flow is ever-present. Alleles with even a slight selective advantage will be able to quickly penetrate most hybrid zone barriers (Barton 1979; Piálek & Barton 1997). For a stable hybrid zone that may be hundreds or thousands of generations old, globally advantageous alleles initially confined to one form would have crossed the hybrid zone long ago and may have swept to fixation in the second form. This process would quickly eliminate variation and make ongoing gene flow more difficult to detect. However, it is possible for neutral markers linked to the selected loci to hitchhike until linkage breaks down and to leave observable shifts of their cline centres.

All of the factors mentioned before suggest that both the geographic extent of introgression and the proportion of the genome that is free to introgress across avian hybrid zones may be larger than is currently appreciated. For example, Gay *et al.* (2008) recently described another avian hybrid zone with remarkably wide and heterogeneous clines. We believe that with thorough consideration of taxon-specific characteristics in genetics and life history, studies of introgression that specifically target neutral markers will result in a deeper understanding of patterns of divergence, cryptic gene flow and differential introgression.

### *SCM vs. SS methods*

Both cline analysis methods detected significant differences in cline position and width for microsatellite markers vs. diagnostic markers. Although both methods assume monotonic clines, most alleles did not show strictly monotonic frequency changes, and this led to differences in how the methods fit clines to the data. On average, the SS method estimated wider clines that

were shifted further away from the centre of the hybrid zone than those estimated by the SCM method. The SS method generally 'smoothes out' sudden changes in frequency, and the monotonic constraint further flattens the curve, especially when the allele frequency changes are sharp at the cline centre (e.g. diagnostic markers), and/or the end points do not show extreme frequencies (e.g. *Gsr* and *Pgm*-2). Unlike the SS cline fitting, the SCM method uses a model with three separate sections: a cline centre section fitted by a sigmoid curve and two tailing sections fitted by exponential curves. Therefore, the SCM method is less likely to be influenced by the end data points. Varying the amount of smoothing and relaxing the assumption of monotonicity will result in closer fit of SS curves to the raw data and may have advantages in fitting curves to more complex clines influenced by spatially varying environmental or demographic conditions (e.g. Man3-187). However, the question of whether the relaxation of the assumption reflects the true underlying structure of hybrid zones needs further exploration.

Additionally, both methods are limited by the sample size including the number of birds at each capture site and the number of sites (populations), although the SCM accounts for sampling errors and random variations caused by environmental fluctuations (Szymura & Barton 1986). For a given capture site, accuracy of allele frequencies are based on the number of birds sampled. These sample sizes varied from $2n = 8$ to 52 (as they are diploid), and random sampling variability for such small sample sizes can make it difficult to accurately measure the true allele frequency at a given site. Looking across all 12 sites, effectively $2N = 426$ observations of the presence or absence of an allele were used in estimating the cline. Both likelihood and bootstrap sampling approaches evaluate the sampling variability, which is then quantified with SL and CI on the cline parameters. To be conservative, we inferred a significant difference in two clines only when indicated by both bootstrap results and nonoverlapping SL. Greater sample sizes would reduce SL and CI of cline parameter estimates and yield greater power to declare a larger number of clinal differences as significant (hence, they would lend stronger support to our conclusions). Larger sample sizes would also smooth the random variation in allele frequencies and improve the estimates of the clines.

The centre of the *Manacus* hybrid zone appeared to be a good example of hybrid swarm, where a large variety of genotypes, with a wide range of fitness, are produced. If hybrids have lower viability (i.e. higher genetic incompatibility), purifying selection is typically strongest at the centre of the hybrid zone, where many incompatibility loci are heterozygous, and will become progressively weaker at populations further away from the centre, as hybrids backcross and become genetically closer to the pure parental forms. These conditions can cause allele frequencies to change rapidly and may create nonmonotonic patterns in which allele frequencies at the populations proximate to the hybrid zone centre become more extreme than those in the parental populations (e.g. *Gsr*, *Pgm*-2, AC12-100 and TCA1-203).

Therefore, in hybrid zones between forms with moderate to high compatibility, genetic markers may tend to show cline structures that are not strictly monotonic, except for those that are diagnostic (i.e. fixed in parental forms) and likely to be under strong selection across the transect. Despite the relatively complex cline structures revealed in this study, the SCM method appeared to have fitted the curves well at the central parts of the clines and thus performed well on estimating the cline parameters for most cases. In future studies, however, it will be desirable to develop nonmonotonic models and other cline-fitting methods that account for the conditions such as hybrid swarm and varied levels of selection across hybrid zones.

## Conclusions

Hybrid zone studies have typically focussed on diagnostic or highly differentiated markers (e.g. Brumfield *et al.* 2001; Payseur *et al.* 2004; Yanchukov *et al.* 2006; Macholan *et al.* 2007). The uniformity of cline structures in such markers has led to the conclusions that (i) strong barriers to introgression exist in these hybrid zones, and (ii) there is reproductive isolation between the taxa (e.g. Johnson *et al.* 1999; Helbig *et al.* 2002; de Queiroz 2005). Our study showed that such conclusions might have been artefacts caused by biased choices of markers. Although we focussed on the markers whose allele frequencies change in a clinal fashion, assessments of differential introgression in markers that are randomly sampled across the genome will be likely to provide greater insight into the genetic and evolutionary complexity found in hybrid zones.

Selection against reproductive incompatibility in hybrid swarms, even with moderately strong endogenous selection on hybrids, may result in genome-wide shifts in allele frequencies (Schilthuizen *et al.* 2001, 2004). In conjunction with recombination, this system will generate a varied and continuously changing pool of recombinants with a wide range of fitness. Thus, hybridization may increase the potential for adaptive trait combinations that can spread beyond the hybrid zone (Lewontin & Birch 1966; Piálek & Barton 1997; Barton 2001). Selective sweep of these adaptive traits, together with neutral gene flow, provide a mechanism to hold most species together (Rieseberg & Burke 2001a,b). In other cases, new trait combinations may

lead to speciation or even rapid radiation (Arnold 1997; Seehausen 2004; Grant *et al*. 2005; Baack & Rieseberg 2007; Mallet 2007). Therefore, not only are hybrid zones actively involved in evolutionary processes (i.e. hybrid zones are not dead ends), but hybridization may also stimulate adaptive evolution and promote species cohesion on the whole, while still allowing for geographic differentiation between populations in response to spatially varying ecological conditions.

## Acknowledgements

Storrs L. Olson and Thomas J. Parsons led the early fieldwork on the *Manacus* hybrid zone, and collected the majority of the samples used here. J. P. Angle, J. Blake, R. Clay, R.I. Crombie, J. P. Dean, T. Glenn, F. M. Greenwell, C. Handley, L. Horth, D. McDonald, E. S. Morton, A. Pineda, Sr, A. Pineda, Jr, M. Varn and D. A. Wiedenfield also provided valuable field assistance. Louisiana State University kindly provided the tissue loan. N. H. Barton, S. J. E. Baird and A. Yanchukov provided expert advice on the SCM analyses using Analyse. R. T. Kimball, E. L. Braun, their lab group members, Staffan Bensch and three anonymous reviewers provided helpful comments.

## References

Arnold ML (1997) *Natural Hybridization and Evolution*. Oxford University Press, New York.

Avise JC (1994) *Molecular Markers, Natural History, and Evolution*. Chapman and Hall, New York.

Baack EJ, Rieseberg LH (2007) A genomic view of introgression and hybrid speciation. *Current Opinion in Genetics and Development*, **17**, 513–518.

Barton NH (1979) Gene flow past a cline. *Heredity*, **43**, 333–339.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology*, **10**, 551–568.

Barton NH, Baird SJE (1999) *Analyse: Software for Analysis of Geographic Variation and Hybrid Zones* (version 1.0.3). Available at http://www.biology.ed.ac.uk/research/institutes/evolution/software/Mac/Analyse/index.html.

Barton NH, Bengtsson BO (1986) The barrier to genetic exchange between hybridising populations. *Heredity*, **56**, 357–376.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113–148.

Bierne N, Daguin C, Bonhomme F, David P, Borsa P (2003) Direct selection on allozymes is not required to explain heterogeneity among marker loci across a *Mytilus* hybrid zone. *Molecular Ecology*, **12**, 2505–2510.

Brumfield RT (1999) *Evolution of brilliant male plumage traits in Manacus*. PhD Thesis, University of Maryland, Maryland, USA.

Brumfield RT, Jernigan RW, McDonald DB, Braun MJ (2001) Evolutionary implications of divergent clines in an avian (*Manacus*: Aves) hybrid zone. *Evolution*, **55**, 2070–2087.

Brumfield RT, Jernigan RW, McDonald DB, Braun MJ (2003) Errata. *Evolution*, **57**, 2919.

Carling MD, Brumfield RT (2008) Haldane's rule in an avian system: using cline theory and divergence population genetics to test for differential introgression of mitochondrial, autosomal, and sex-linked loci across the *Passerina* bunting hybrid zone. *Evolution*, **62**, 2600–2615.

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. *Nature*, **371**, 215–220.

Eanes WF (1999) Analysis of selection on enzyme polymorphisms. *Annual Review of Ecology and Systematics*, **30**, 301–326.

Efron B, Tibshirani RJ (1993) *An Introduction to the Bootstrap*. Chapman and Hall, New York.

Emelianov I, Marec F, Mallet J (2004) Genomic evidence for divergence with gene flow in host races of the larch budmoth. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **271**, 97–105.

Endler JA (1977) *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, NJ.

Flanagan NS, Tobler A, Davison A *et al*. (2004) Historical demography of Mullerian mimicry in the neotropical *Heliconius* butterflies. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 9704–9709.

Gay L, Crochet PA, Bell DA, Lenormand T (2008) Comparing clines on molecular and phenotypic traits in hybrid zones: a window on tension zone models. *Evolution*, **62**, 2789–2806.

Gill FB, Mostrom AM, Mack AL (1993) Speciation in North American chickadees: I. Patterns of mtDNA divergence. *Evolution*, **47**, 195–212.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate *F*-statistics. *Journal of Heredity*, **86**, 485–486.

Grant PR, Grant BR (1994) Phenotypic and genetic effects of hybridization in Darwin's finches. *Evolution*, **48**, 297–316.

Grant PR, Grant BR, Petren K (2005) Hybridization in the recent past. *American Naturalist*, **166**, 56–67.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportions for multiple alleles. *Biometrics*, **48**, 361–372.

Harr B (2006) Genomic islands of differentiation between house mouse subspecies. *Genome Research*, **16**, 730–737.

Harrison RG (1990) Hybrid zones: windows on the evolutionary process. *Oxford Surveys in Evolutionary Biology*, **7**, 69–128.

Harrison RG, Arnold J (1982) A narrow hybrid zone between closely related cricket species. *Evolution*, **36**, 535–552.

Helbig AJ, Knox AG, Parkin DT, Sangster G, Collinson M (2002) Guidelines for assigning species rank. *Ibis*, **144**, 518–525.

Holm S (1979) A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics*, **6**, 65–70.

Johnson NK, Remsen JV Jr, Cicero C (1999) Resolution of the debate over species concepts in ornithology: a new comprehensive biologic species concept. In: *Proceedings of the 22 International Ornithological Congress, Durban* (eds Adams NJ, Slowtow RH), pp. 1470–1482. Birdlife South Africa, Johannesburg.

Latta RG, Mitton JB (1999) Historical separation and present gene flow through a zone of secondary contact in ponderosa pine. *Evolution*, **53**, 769–776.

Lewis PO, Zaykin D (1999) *Genetic Data Analysis: Computer Program for the Analysis of Allelic Data* (version 1.0). Available at http://www.hydrodictyon.eeb.uconn.edu/people/plewis/software.php.

Lewontin RC, Birch LC (1966) Hybridization as a source of new variation for adaptation to new environments. *Evolution*, **20**, 315–336.

Loader CR (1996) Local likelihood density estimation. *Annals of Statistics*, **24**, 1602–1618.

Macholan M, Munclinger P, Sugerkova M *et al.* (2007) Genetic analysis of autosomal and X-linked markers across a mouse hybrid zone. *Evolution*, **61**, 746–771.

Mallet J (2007) Hybrid speciation. *Nature*, **446**, 279–283.

Martinsen GD, Whitham TG, Turek RJ, Keim P (2001) Hybrid populations selectively filter gene introgression between species. *Evolution*, **55**, 1325–1335.

McDonald DB, Potts WK (1994) Cooperative display and relatedness among males in a lek-mating birds. *Science*, **266**, 1030–1032.

McDonald DB, Clay RP, Brumfield RT, Braun MJ (2001) Sexual selection on plumage and behavior in an avian hybrid zone: experimental tests of male–male interactions. *Evolution*, **55**, 1443–1451.

Mebert K (2008) Good species despite massive hybridization: genetic research on the contact zone between the watersnakes *Nerodia sipedon* and *N. fasciata* in the Carolinas, USA. *Molecular Ecology*, **17**, 1918–1929.

Moore WS (1977) An evaluation of narrow hybrid zones in vertebrates. *Quarterly Review of Biology*, **52**, 263–277.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Parsons TJ, Olson SL, Braun MJ (1993) Unidirectional spread of secondary sexual plumage traits across an avian hybrid zone. *Science*, **260**, 1643–1646.

Payseur BA, Krenz JG, Nachman MW (2004) Differential patterns of introgression across the X chromosome in a hybrid zone between two species of house mice. *Evolution*, **58**, 2064–2078.

Phillips BL, Baird SJE, Moritz C (2004) When vicars meet: a narrow contact zone between morphologically cryptic phylogeographic lineages of the rainforest skink, *Carlia rubrigularis*. *Evolution*, **58**, 1536–1548.

Piálek J, Barton NH (1997) The spread of an advantageous allele across a barrier: the effects of random drift and selection against heterozygotes. *Genetics*, **145**, 493–504.

Price T (2008) *Speciation in Birds*. Roberts and Company, Greenwood Village, CO.

Price TD, Bouvier MM (2002) The evolution of F-1 postzygotic incompatibilities in birds. *Evolution*, **56**, 2083–2089.

de Queiroz K (2005) A unified concept of species and its consequences for the future of taxonomy. *Proceedings of the California Academy of Sciences*, **56**(Suppl. 1), 196–215.

Raymond M, Rousset F (1995) GENEPOP (version 3.1c): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Reynolds J, Weir BS, Cockerham CC (1983) Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, **105**, 767–779.

Rieseberg LH, Burke JM (2001a) A genic view of species integration – commentary. *Journal of Evolutionary Biology*, **14**, 883–886.

Rieseberg LH, Burke JM (2001b) The biological reality of species: gene flow, selection, and collective evolution. *Taxon*, **50**, 47–67.

Rieseberg LH, Whitton J, Gardner K (1999) Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. *Genetics*, **152**, 713–727.

Rieseberg LH, Widmer A, Arntz AM, Burke JM (2002) Directional selection is the primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 12242–12245.

Riginos C, Sukhdeo K, Cunningham CW (2002) Evidence for selection at multiple allozyme loci across a mussel hybrid zone. *Molecular Biology and Evolution*, **19**, 347–351.

Robbins MB, Braun MJ, Tobey EA (1986) Morphological and vocal variation across a contact zone between the chickadees *Parus atricapillus* and *P. carolinensis*. *Auk*, **103**, 655–666.

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. *Genetics*, **140**, 1413–1419.

Sattler GD, Braun MJ (2000) Morphometric variation as an indicator of genetic interactions between black-capped and Carolina Chickadees at a contact zone in the Appalachian mountains. *Auk*, **117**, 427–444.

Schilthuizen M, Hoekstra RF, Gittenberger E (2001) The 'rare allele phenomenon' in a ribosomal spacer. *Molecular Ecology*, **10**, 1341–1345.

Schilthuizen M, Hoekstra RF, Gittenberger E (2004) Hybridization, rare alleles and adaptive radiation. *Trends in Ecology and Evolution*, **19**, 404–405.

Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. *Chromosoma*, **109**, 365–371.

Schluter D (1988) Estimating the form of natural selection on a quantitative trait. *Evolution*, **42**, 849–861.

Seehausen O (2004) Hybridization and adaptive radiation. *Trends in Ecology and Evolution*, **19**, 198–207.

Shorey L, Piertney S, Stone J, Höglund J (2000) Fine-scale genetic structuring on *Manacus manacus* leks. *Nature*, **408**, 352–353.

Stein AC, Uy JA (2006) Unidirectional introgression of a sexually selected trait across an avian hybrid zone: a role for female choice? *Evolution*, **60**, 1476–1485.

Szymura J, Barton NH (1986) Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, near Cracow in southern Poland. *Evolution*, **40**, 1141–1159.

Szymura J, Barton NH (1991) The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*: comparisons between transects and between loci. *Evolution*, **45**, 237–261.

Teeter KC, Payseur BA, Harris LW *et al.* (2008) Genome-wide patterns of gene flow across a house mouse hybrid zone. *Genome Research*, **18**, 67–76.

Tibshirani R (1984) *Local likelihood estimation*. PhD Thesis, Stanford University, California, USA.

Tibshirani R, Hastie T (1987) Local likelihood estimation. *Journal of the American Statistical Association*, **82**, 559–567.

Vallender R, Robertson RJ, Friesen VL, Lovette IJ (2007) Complex hybridization dynamics between golden-winged and blue-winged warblers (*Vermivora chrysoptera* and *Vermivora pinus*) revealed by AFLP, microsatellite, intron and mtDNA markers. *Molecular Ecology*, **16**, 2017–2029.

Via S, West J (2008) The genetic mosaic suggests a new role for hitchhiking in ecological speciation. *Molecular Ecology*, **17**, 4334–4345.

Weir BS (1996) *Genetic Data Analysis II*. Sinauer Associates, Inc., Sunderland, MA.

Wilding CS, Butlin RK, Grahame J (2001) Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *Journal of Evolutionary Biology*, **14**, 611–619.

Yanchukov A, Hofman S, Szymura JM *et al.* (2006) Hybridization of *Bombina bombina* and *B. variegata* (Anura, Discoglossidae) at a sharp ecotone in western Ukraine: comparisons across transects and over time. *Evolution*, **60**, 583–600.

---

T.Y. is an avian evolutionary biologist with special interest in population genetics, speciation, phylogenetics, and life history trait evolution. R.W.J is Professor of Statistics, and his research interests include the development of statistical methods for evolutionary biology and environmental science. R.T.B. is interested in avian population genetics, speciation, biogeography, systematics, genomics, transcriptomics, and behaviour. N.K.B. currently works on mapping and valuation of ecosystem services in regions of conservation priority, and is also interested in conservation genetics and behavioural ecology. M.J.B. is an evolutionary geneticist studying phylogeny, speciation and Neotropical biogeography.

---

## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** List of samples used in this study with their voucher information

**Table S2** Coancestry coefficient (θ) matrix among 12 populations using 13 microsatellite loci (upper diagonal) and 7 genetic markers from Brumfield *et al.* 2001 (lower diagonal)

**Table S3** Pairwise statistical comparisons of cline centres using smoothing splines and bootstrap resampling (upper right), and using stepped cline model and likelihood support limits (lower left)

**Table S4** Pairwise statistical comparisons of cline widths using smoothing splines and bootstrap resampling (upper right), and using stepped cline model and likelihood support limits (lower left)

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.