Strong selection on male plumage in a hybrid zone between a hybrid bird species and one of its parents

STRONG SELECTION ON MALE PLUMAGE IN A HYBRID ZONE BETWEEN A HYBRID BIRD SPECIES AND ONE OF ITS PARENTS

Richard. Ian Bailey[1,2], Maria Rundhaugen Tesaker[1,2], Cassandra Nicole Trier[1] and Glenn-Peter Sætre[1,3]

[1] Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, P. O. Box 1066 Blindern, N-0316 Oslo, Norway.

[2]joint first authors

[3]Corresponding author; Tel. +47-22857291; Fax. +47-22 85 40 01.

E-mails: r.i.bailey@ibv.uio.no, mariatesaker@gmail.com, c.n.trier@ibv.uio.no, g.p.satre@ibv.uio.no

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Homoploid hybrid speciation (HHS) requires reproductive barriers between hybrid and parent species, despite incomplete reproductive isolation (RI) between the parents. Novel secondary sexual trait values in hybrids may cause prezygotic isolation from both parents, while signals inherited by the hybrid from one parent species may cause prezygotic isolation with the other. Here we investigate whether differences in male plumage function as a premating barrier between the hybrid Italian sparrow and one of its parent species, the house sparrow, in a narrow Alpine hybrid zone. Italian sparrow male plumage is a composite mosaic of the parental traits, with its head plumage most similar to its other parent, the Spanish sparrow. We use geographical cline analysis to examine selection on three plumage traits, 75 nuclear SNPs and hybrid indices based on these SNPs. Several SNPs showed evidence of restricted introgression in the Alps, supporting earlier findings. Crown colour exhibited the narrowest plumage cline, representing a 37% (range 4-65%) drop in fitness. The cline was too narrow to be due to neutral introgression. Only crown colour was significantly bimodal in the hybrid zone. Bimodality may be due to RI or a major QTL, although fitness estimates suggest that selection contributes to the pattern. We discuss the implications with respect to HHS and the species status of the Italian sparrow.

KEY WORDS: Hybrid speciation, cline analysis, bimodality, Italian sparrow, house sparrow

Introduction

Homoploid hybrid speciation (HHS) is the process by which a new species originates through hybridization between two parent species, without a change in the number of chromosome sets (Mallet 2007). Although hybridization is common it is by no means obvious that individuals of mixed ancestry would remain intra-fertile and viable, and yet develop reproductive barriers against both parents. Hence HHS is thought to be rare in nature. Nevertheless, an increasing number of studies report well-supported examples of HHS from a range of different taxa (e.g. Rieseberg et al. 2003; Howarth and Baum 2005; James and Abbott 2005; Schwartz et al. 2005; Gompert et al. 2006; Nolte et al. 2006; Elgvin et al. 2011; Hermansen et al. 2011; Kunte et al. 2011; Stemshorn et al. 2011). Reported mechanisms of reproductive isolation (RI) between hybrid and parent taxa include both mosaics of parent-parent isolating mechanisms and mechanisms evolved *de novo* in the hybrids (Rieseberg 1997; Hermansen et al. 2014). These include novel chromosomal structural rearrangements induced by hybridization, and the inheritance of subsets of structural rearrangements from each parent species (Rieseberg et al. 1995; Rieseberg et al. 2003; Lai et al. 2005). Transgressive segregation leading to extreme phenotypes in hybrids, facilitating adaptation to novel or extreme habitats and hence allowing hybrids to escape competition and gene flow from the parents, is thought to be an important contributor to HHS (Rieseberg et al. 1999; Gross and Rieseberg 2005, Gompert et al. 2006; Mallet 2007).

Sexual isolation and other premating barriers are thought to be primary mechanisms of RI between non-hybrid taxa (Seehausen et al. 2014). However, the potential role of sexual barriers in isolating hybrid taxa from their parents has been little explored (but see Mavarez et al. 2006; Melo et al. 2009 on hybrid trait speciation in *Heliconius* butterflies). Sexual isolation involves traits and preferences that reduce interspecific sexual attraction, thus impeding courtship and mating between species. Structural or behavioural signals such as colour,

displays, song or pheromones are used to identify potential mates (Baker and Baker 1990; Seehausen and van Alphen 1998; Smadja and Ganem 2002). Transgressive segregation, whereby individual signal trait values fall outside the range of the parents, or sorting and mosaicism of multiple traits, may result in novel sexual signals in hybrids. Provided that discriminatory mate preferences for such novel signals or signal combinations develop, they would function as sexual barriers, reducing gene exchange between the hybrid taxon and its parents.

Hybrid zones, areas where genetically divergent populations meet, interbreed and produce hybrids, are valuable for studying speciation and RI (Hewitt 1988; Harrison 1993). The semi-permeable nature of hybrid zones allows gene flow for neutral and beneficial traits but restricts movement of traits selected against, thus allowing for identification of traits and underlying genes contributing to RI. Barton and colleagues (Barton and Hewitt 1985; 1989; Barton and Gale, 1993) argue that most hybrid zones are “tension zones” in which the hybrid zone constitutes a cline maintained by a balance between dispersal of parental types into the zone and selection against hybrids. In the simplest form of geographical cline analysis, clines may vary along a geographical transect in two parameters - width and position (centre), which can be used to infer if there is selection against hybrids, and to compare the strength and direction of selection among traits or loci (e.g. Nurnberger et al. 1995; Gay et al. 2008; Teeter et al. 2008; Bailey et al. 2012). When all clines are narrow and exhibit approximately the same widths (concordant clines) and centre positions (coincident clines) this indicates that selection is strong and pervasive enough across the genome to maintain genome-wide linkage disequilibria (e.g. Szymura and Barton 1986), or alternatively, it indicates recent secondary contact followed by neutral introgression (Barton and Gale 1993). In contrast, variation in cline widths and centres indicates that traits are reacting at least partially independently to selective pressures (Nurnberger et al. 1995). The width of non-neutral clines is determined by

the strength of selection against hybrids relative to average lifetime dispersal; stronger selection produces a narrower cline, and non-coincident cline centres may indicate differential introgression into the other species' range and hence, in the case of sexual signals, asymmetric mate preferences (e.g. Brumfield et al. 2001). Selection against intermediate phenotypes is also expected to produce hybrid zones in which local populations show a bimodal phenotype distribution, and bimodality is likely to be particularly strong in the presence of positive assortative mating (Jiggins and Mallet 2000). Indeed, according to the genotypic cluster definition of species (Mallet 1995), sympatric bimodality of diagnostic genotypes and phenotypes is the primary indicator of speciation having occurred.

The Italian sparrow (*Passer italiae* Vieillot) is a homoploid hybrid species that originated through hybridization between house sparrows (*P. domesticus* L.) and Spanish sparrows (*P. hispaniolensis* Temminck) (Elgvin et al. 2011; Hermansen et al. 2011; Trier et al. 2014; Sangster et al. 2015). It occupies the whole Italian peninsula and some Mediterranean islands (Summers-Smith 1988; Figure 1). Analyses of parent species-informative microsatellites, SNPs and DNA sequence data indicate that Italian sparrows consist of a mosaic genome, and SNP markers reveal a broad cline in hybrid index from about 80% house sparrow in northern Italy to about 30% in Sicily (Elgvin et al. 2011; Hermansen et al. 2011; Trier et al. 2014). Male Italian sparrow secondary sexual plumage is also a mosaic of the characters of the parent species. They have large black bibs, brown crowns, white cheeks and large white eyebrows like Spanish sparrows, while other plumage traits more closely resemble house sparrows (Meise 1936, Summers-Smith 1988; Trier et al. 2014; Figure 1).

Though these plumage traits are secondary sexual male-limited traits, little is known about the selective forces acting on them, either between Italian sparrows and their parents, or between the two parent species. Sympatric Italian and Spanish sparrows in east-central Italy apparently do not exchange genes, probably due to strong premating barriers (Hermansen et al. 2011;

Trier et al. 2014). The isolating mechanisms are currently unknown but, alongside plumage differences, Spanish sparrows are less strongly commensal than Italian sparrows, breed later and favour different habitats, leading to potential spatial and ecological isolation (Hanh Tu 2013). Episodes of migration and gene flow do occur between Spanish sparrows on Sardinia and Italian sparrows on the Italian peninsula (Hermansen et al. 2011; Trier et al. 2014). House and Italian sparrows share a very similar human-commensal niche and life history, and gene flow occurs at their parapatric range boundary in the Alps (Trier et al. 2014).. The most distinct genetic boundaries, involving loci showing the strongest evidence of involvement in RI through narrow genomic clines (Gompert and Buerkle 2011), lie coincident with the observed phenotypic hybrid-parent range boundaries (Trier et al. 2014). Hence, based on its mosaicism and evidence of RI acting at these boundaries, the Italian sparrow meets the criteria of being a homoploid hybrid species (Schumer et al. 2014), and as such represents one of the few documented cases of HHS in animals.

The range boundaries of Italian sparrows were identified primarily through observation of male secondary sexual plumage traits (Summers-Smith 1988). Here, we focus on examination of RI acting on plumage traits shared between Italian and Spanish sparrows in the narrow hybrid zone between house and Italian sparrows in the Alps (Figure 1). This hybrid zone was first identified primarily on the basis of male crown colour (Meise 1936, Summers-Smith 1988) (Appendix S1), and later studies on crown colour corroborated these observations (Hermansen et al. 2011). Other than the presence of this abrupt transition in crown colour and possibly other male plumage traits, nothing is known of the influence male plumage on isolation between house and Italian sparrows. Crown colour is uniformly brown on the Italian peninsula south of the Alps hybrid zone (Summers-Smith 1988; Hermansen et al. 2011), matching the colour of their other parent species, the Spanish sparrow. The extent to which

the other plumage traits are diagnostic between house and Italian sparrows is currently unknown.

The primary purpose of this paper is to examine the role of sexual isolation acting on the subset of plumage traits inherited by the Italian sparrow from Spanish sparrows in the Alpine Italian-house sparrow hybrid zone. We also examine evidence of putative postzygotic isolation from SNP data. While the earlier studies (Trier et al. 2014; Hermansen et al. 2014) focused on genomic cline analysis of SNP data and a search for the geographic locations of genomic subdivision across the whole of Italy, here we are able to employ geographical cline analysis across a narrow hybrid zone, and hence estimate the strength of selection acting at this boundary (Barton and Gale 1993). Using a new set of sparrow samples from the Alps region, we carry out geographical cline analysis on plumage traits and genotypes of a subset of 75 of the same SNPs as used previously (Trier et al. 2014), to investigate if prezygotic and putatively postzygotic isolating mechanisms are acting and are concordant and coincident at this range boundary. We test whether distinct clines exist in the Alps, over and above that expected from the broader cline in hybrid index across Italy. We use the resulting cline widths to estimate the strength of selection maintaining the clines, and the likelihood that the clines can be explained by neutral introgression alone. We also test for trait bimodality across the hybrid zone as a further indicator of involvement in RI, and examine the possible impacts of trait genetic architecture on these results. We discuss the species status of Italian sparrows in light of these results.

Materials and methods

Sampling

A total of 228 male sparrows were caught using mist nets at 42 locations across the Alps in northern Italy and Switzerland during March-July 2012 (Figure 1; Table S1). Only male birds were considered in this study, since the species are sexually dichromatic and only males show clear species differences in plumage traits (Summers-Smith 1988). Birds with house sparrow phenotype (primarily based on crown colour) were caught from north of the main Alpine ridge, Italian sparrow phenotypes from the northern part of the Italian peninsula, close to Lake Como, Lake Lugano and Lake Maggiore, and putative hybrids from the contact zone in the Alps (Figure 1). Plumage measurements were independent of this pre-classification (see below). Permissions for sampling birds were obtained from the appropriate authorities in Italy and Switzerland. We took a 25-50 µl blood sample by puncturing a brachial vein and transferred the blood to a lysis buffer.

DNA isolation and genotyping

DNA was extracted from blood using Qiagen DNeasy 96 Blood and Tissue Kits (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's instructions with the minor adjustment of adding 100µl of blood/buffer in the initial step. A set of 75 species-informative single nucleotide polymorphisms (SNPs) (Table S2) from coding genes were genotyped with the Sequenom MassArray system at CIGENE, Norwegian University of Life Sciences, Ås, Norway, following the protocol in Trier et al. (2014). Species-informative SNPs were those that showed fixed differences in comparisons of transcriptome sequence of six house sparrow versus six Spanish sparrow individuals (see Trier et al. 2014 for transcriptome sequencing methods; Hermansen et al. 2014 for initial identification methods of differentiated SNPs), and

subsequently maintained allele frequency differences after genotyping of larger numbers of parental individuals (though not all fixed; see list of parental allele frequencies in Table S3, Trier et al. 2014 and an alternative list based on different Spanish parental populations in Table S1, Hermansen et al. 2014).

Digital photography

Digital images were taken using a high resolution camera (Nikon D500, 16.2 megapixels) with a Sigma EM-140 DG Macro ring flash and a Sigma 50 mm 1:28 DG Macro lens, mounted in a standardized position. The base was covered with millimetre paper background. All captured males were photographed from three different angles (dorsal, ventral and portrait; Appendix S1), and the images were saved as raw NEF files (Stevens et al. 2007). Light variation among photos was standardized prior to measurement using the Match Colour function of Adobe Photoshop®, (Adobe Systems Inc., 2013), and resulting images were saved as JPEG files. We chose a reference photo (Appendix S1) for Match Colour that contained the entire range of colours of interest. No other features in the images were altered.

Spectrophotometry was performed on specimens from the Natural History Museum in Oslo to test for UV reflectance. Reflectance was measured with an USB 2000 spectrometer (Ocean Optics, Eerbek, Netherlands) connected by a bifurcated fiberoptic probe to a Xenon (PX-2) pulsed light source. Reflectance was calculated relative to a WS-1 white standard (Ocean Optics). Crown and cheek (the eyebrow was too small to make measurements) were measured three times per species, on one specimen from each species (*P. domesticus*, *P. italiae*, *P. hispaniolensis*). UV reflectance would reveal itself by showing a peak in the spectra plots within the range of UV wavelengths (320-400 nm). None of the plumage traits in any of the species showed any UV reflectance (Appendix S1) and so we focused solely on the human-visible spectrum.

We quantified variation in male plumage traits using colour measurements for cheek and crown, and size and colour combined for eyebrow. After filtering for image quality we obtained a full set of plumage measurements for 138 individuals. Sample sizes for each analysis are described below in section 'Geographical cline analysis'.

Crown colour

Dorsal images were used for crown colour measurements (Appendix S1). We aimed to measure the proportion of house sparrow grey (HG) versus Italian sparrow brown (IB) in the crown of each individual. However, initially after the winter moult into breeding plumage, the crown has a thin covering of ambiguous yellow-greyish feather tips (winter plumage; WP), which gradually wears off during the breeding season. Many birds caught early in spring retained variable amounts of WP. We therefore used discriminant function analysis (DFA) to classify all pixels in each crown into the three different crown colour categories – IB, HG and WP. All pixels categorized as WP were removed and the remaining HG and IB pixels were used to calculate percentage HG.

The DFA was performed in R using the linear discriminant analysis (lda) function from the MASS package (Venables and Ripley, 2002). The training set, used as the reference for classifying pixels, was made in Adobe Photoshop using the colour-corrected JPEG files. Eleven house sparrows (from sample sites Andeer, Thusis, Kiesen, Ilanz, Juf and Seedorf; Table S1) and eleven Italian sparrows (from Lecco, Sciranna, Porlezza, Valbrona and Castelveccana) were selected from areas outside the putative hybrid zone. Two photos per individual were selected. Single feather barbs, avoiding shadows and other colours, were chosen using the lasso tool in Photoshop, and the RGB (Red, Green and Blue) levels were recorded using the histogram palette (e.g. Villafuerte and Negro 1998; McGraw et al. 2002; Yam and Papadakis 2004; Setchell et al. 2006; Bergman and Beehner 2008). Each

measurement was repeated three times per photo at randomly selected areas of the crown. Leave-one-out cross validation indicated that DFA correctly classified 99% of HG and IB pixels, and 88% of WP, with an overall average of 96% correct classification.

DFA was then used to classify all individual crown pixels of all birds. We selected the whole crown for pixel classification of the total data set. To ensure a classification of good quality, only pixels with a high posterior probability ($pp > 0.7$) for one of the three categories were retained. The number of pixels in each category per individual was used to calculate percentage HG.

Other plumage traits

The length and maximum height of the eyebrow were measured in the field using a dial calliper to the nearest 0.1 mm. Length was measured along the longest axis, from front to back of the eye. Height was measured as the broadest area perpendicular to the length axis.

Sparrow cheeks and eyebrows consist of neutral colours (grey and white). We therefore measured luminosity (mean and standard deviation) per trait in Photoshop. This measure is sensitive to variation in ambient light. To account for such variation the white millimetre paper was used as a standard reference. Image luminosity measures were obtained by measuring the luminosity of as large an area as possible of white background paper. Linear regression analyses for plumage trait luminosity values against original untransformed image luminosity were performed in R and the residuals used for all further analyses. The sparrow eyebrow is typically split in two; therefore separate luminosity measures were made for the front and rear portions for each bird. All regressions were highly significant (Table S3).

To test for significant differences between parent species in each of the multivariate plumage traits, a permutation test of the Mahalanobis distance between group means was carried out

using the CVA function in the Morpho package (Schlager 2013) in R. We chose a set of parental reference populations (Table S1) for the CVA based on the geographical distances from the main Alpine ridge for each sampling location, measured in Google Earth (Google Inc. 2013) (Figure 1). All 35 sampling locations were plotted into the online map program using their respective latitude and longitude coordinates. The alpine ridge was marked using the *Add a path* function and the distance directly north or south between the alpine ridge and each location was measured in kilometres using the *Ruler* function. Distances south of the Alpine ridge were made negative to indicate direction (Table S1). Sparrows from the southernmost populations relative to the alpine ridge were selected as Italian parental references and those from the northernmost populations as the parental reference for house sparrows. These selections resulted in a parental reference set consisting of 17 Italian and 14 house sparrows.

To convert the multivariate data for each trait into a univariate trait value for all individuals in this study, DFA was performed with the MASS package in R based on the same parental reference set (Table S1), and used to predict scores for all individuals along the primary discriminant axis (LD1). The resulting scores were used in cline analysis. Welch two-sample Student's *t*-tests assuming unequal variances were carried out to estimate differentiation between the parental reference sets for hybrid index and crown colour.

Geographical cline analysis

The primary purpose of this analysis was to estimate the strength of selection maintaining clines in sexual signals and postzygotic incompatibilities across the Alps hybrid zone, and to subsequently test whether any plumage traits were under significantly stronger selection than a genome-wide average. For the genome-wide average, cline fitting was carried out on a

hybrid index based on the aforementioned 75 SNPs, ranging from 0 (pure house sparrow) to 1 (pure Spanish sparrow), produced using the Introgress 1.2.3 package in R (Gompert and Buerkle 2012). For this hybrid index we used the same set of 138 individuals as for plumage traits for direct comparison. More data were available for individual SNPs, and hence we used a larger data set (Table S1). Using the same larger SNP data set, we also fitted a cline to a hybrid index based on 4 unlinked SNPs ("4-SNP HI") that were strongly clinal based on our results and had an estimated allele frequency change > 0.2, in order to compare a hybrid index of putatively selected loci with plumage traits. The chosen SNPs were CHD1Z, RPS4, CLIP2 and NCOA3 (Table S2). Whereas to calculate the genome-wide HI we used allopatric house and Spanish sparrow population samples as the parental reference and hence were able to use Introgress software, for the 4-SNP HI we used estimates of parapatric house and Italian sparrow parental allele frequencies from the cline fits (see below). The 4-SNP HI calculation was carried out in Excel using a modification of the method from Buerkle (2005). Rather than estimating a single global hybrid index by maximum likelihood, we estimated the ML hybrid index for each locus followed by calculating a weighted average across loci for the global value. The formula for each locus is therefore:

(1) $$p_{ij} = h_i r_{ij} + (1 - h_i) s_{ij}$$

Where p_{ij} is the probability of the focal individual possessing a copy of the *j*th allele at the *i*th locus, h_i is the locus-specific hybrid index, r_{ij} is the allele frequency in the reference parental species, and s_{ij} is the allele frequency in the other species. The log-likelihood of *h* for each locus is then:

(2) $$L(h_i|\text{genotype}) =$$

$$\ln(p_{ij}^2) \qquad \text{Homozygous focal species allele}$$

	$\ln(2p_{ij}p_{ik})$	Heterozygote
	$\ln(p_{ik}^2)$	Homozygous other allele

Where genotype = 0 (homozygote house), 1 (heterozygote) or 2 (homozygote Italian), *j* represents the allele with higher frequency in the focal parent (Italian sparrows in this case), and *k* the allele with higher frequency in the other species. The likelihood is maximized over h_i = [0,1]. The global hybrid index is then:

$$(\sum_{i=1}^{n} h_i w_i)/n \quad (3)$$

Where *n* is the number of loci, and the relative weight of the *i*th locus is $w_i = \frac{d_{ij}}{\bar{d}_j}$, $d_{ij}$is the parental difference in allele frequency, and $\bar{d}_j$ is the mean parental difference in allele frequency across loci. The weight is added because loci with bigger differences in parental allele frequency are more informative with respect to parent of origin. Alternatively h_i can be estimated without using likelihood as:

$$-\left(\frac{s_{ij}}{d_{ij}}\right) + ((\frac{genotype}{2})/d_{ij}) \quad (4)$$

With the constraint h_i = [0,1].

The sample locations were collapsed into a one-dimensional transect, ordered according to distance from the alpine ridge (Table S1). Cline analysis was performed in R using the software package HZAR (Derryberry et al. 2013). A standard two-parameter cline model fits a tanh curve to trait or genetic variation with respect to geographic distance along a one-dimensional transect (e.g. Gay et al. 2008; Teeter et al. 2008; Bailey et al. 2012). The estimated parameters are cline centre and width, defined as the inverse of the maximum cline gradient (Barton and Gale 1993). For quantitative traits, HZAR also fits three further parameters; parental trait variances and increase in trait variance over the parental average at

the cline centre (see also Bailey et al. 2012). After testing, the data were deemed insufficient to test the more complex 6 cline-parameter model (e.g. Gay et al. 2008), with exponential tails of introgression surrounding the central cline. We used 2-unit likelihood support limits as a measure of confidence in the parameter estimates (e.g. Barton and Gale 1993 p. 22), and to compare parameter estimates among the SNPs and trait variables. For simplicity we assumed non-overlap of support limits to indicate significant differences in parameter estimates. For genetic data, we did not adjust effective sample sizes due to Hardy Weinberg disequilibrium (HWD; e.g. Gay et al. 2008). None of the SNP loci were fixed at both ends of the sampling area, so copies of the same allele could be derived from two different sources. Hence adjustments based on HWD may produce misleading results because we could not be sure that matching alleles were identical by descent.

We wished to test whether there was a distinct cline in the Alps versus a broader cline across the Italian peninsula (or no cline at all), as exists for SNP hybrid index (Trier et al. 2014). Therefore we did not use fixed parental values but instead allowed HZAR to estimate them as further parameters in the model. We set the maximum cline width to 200 km – 70 km wider than the sampling area - and used the 2-unit support limits for cline width to determine whether the cline in the Alps was significantly narrower than 200 km. The Monte Carlo Markov Chain (MCMC) was constrained to search within reasonable limits for each parameter, to improve search efficiency. For allele frequency data, HZAR also tests against a null model of no change in allele frequency by calculating the likelihood of a linear regression with slope = 0 and comparing with the cline fit using AICc.

For quantitative traits and hybrid indices, neutral transformations were made where necessary to increase trait variance above 1 (by multiplying all values by a constant) to improve maximum likelihood model fitting, and to render all trait values positive (by adding a constant).

Selection and neutral introgression

Clines can be the result of neutral introgression after secondary contact. Such clines are predicted to become wider over time (Barton and Gale 1993):

$$w = 2.51\sigma\sqrt{T} \quad (5)$$

Where w = cline width, T = number of generations since secondary contact, and σ = average lifetime dispersal. It is likely that the Italian and house sparrows came into contact some thousands of generations ago (Hermansen et al. 2011; Sætre et al. 2012). Hence, if the neutral model provides much shorter time estimates selection can be inferred.

The strength of selection against hybrids can be calculated from cline width (Barton and Gale 1993). We used the formula for selection against heterozygotes (Bazykin 1969; Barton and Gale 1993):

$$s = \left(\frac{2\sigma}{w}\right)^2 \quad (6)$$

Where s = a standard selection coefficient representing the reduction in fitness at the cline centre.

We used an average lifetime dispersal distance of 2 km based on a review of natal dispersal distances (Anderson 2006). As this estimate does not include long-range dispersal and thus may be an underestimate, we also attempted two indirect methods to estimate dispersal. First, we estimated admixture linkage disequilibrium for each population as average pairwise disequilibrium ($\overline{D}$) among the same 4 unlinked SNPs as used for the 4-SNP HI. Pairwise disequilibrium values were scaled and weighted according to the difference in parental mean allele frequency as estimated from cline fits (see Nurnberger et al. 1995 Appendix 1). Finally,

we estimated the covariance between crown colour and the 4-SNP HI for each population with sample size > 2 with a full set of SNP and crown colour measurements (Barton and Gale 1993; Nurnberger et al. 1995).

We used the best estimates of σ and the maximum likelihood and upper and lower support limits of cline width for each variable to estimate the number of generations of neutral introgression since secondary contact, and the strength of selection required to maintain the cline width.

Bimodality

Mixed populations may be bimodal in genotype or phenotype distributions, indicating RI, or may be unimodal indicating more extensive mixing. To test for bimodality in plumage traits and hybrid index we first increased sample sizes by pooling sets of five populations, adjacent according to distance from the Alpine ridge, in sliding windows south-north, sliding one population per window. Resulting histograms of trait values for each window were divided into 10 bins of equal width, and were then fit to either one or two normal distributions using iterative optimization. The bimodal model included 5 parameters: Italian sparrow mean and standard deviation, house sparrow mean and standard deviation, and the proportion of the data under each curve. The unimodal distribution involved 2 parameters: mean and standard deviation. For any given set of parameter values the probability density under each normal distribution was split into bins with the same width and position as in the histogram. The total probability density present within those bins was then standardized to sum to the sample size, followed by calculation of negative log likelihood with Poisson error distribution. A standard likelihood ratio test was used to compare the 5-parameter with the 2-parameter model for each window, with 3 degrees of freedom.

Bimodality may also be a result of dominance if only one locus is involved, or if dominance at multiple loci is biased towards one or other parent taxon (Nurnberger et al. 1995). Our data do not allow direct testing of dominance. To estimate the number of additively acting genes involved in each trait we used a version of the Castle-Wright estimator (Castle 1921; Lande 1981):

(7) $$N = \frac{\Delta z^2}{8(V_H - \overline{V_{dom} V_{it}})}$$

Where N = effective number of fixed additively acting loci distinguishing the two taxa, Δz = parental difference in trait value (best cline fit estimate), V_H = trait variance at the cline centre, and $\overline{V_{dom} V_{it}}$ = average of the parental trait variances (see also Barton and Gale 1993). We used the ML and upper and lower 2-unit support limits for $(V_H - \overline{V_{dom} V_{it}})$ estimated by HZAR. A value $N < 1$ would indicate a single locus either with heterozygote disadvantage or some degree of dominance.

Results

Species differences

Permutation tests from CVA and Student's *t*-tests on the univariate crown colour and hybrid index indicated that hybrid index and all plumage traits differed significantly between house and Italian sparrows (Table 1). However, only crown colour values were completely non-overlapping (Table 2). While no individuals had a hybrid index above 0.34 - indicating a bias towards the house sparrow parental genome - Italian sparrow values were highly variable, as might be expected for a hybrid species. For cheek colour, luminosity standard deviation loaded more strongly on the primary axis of differentiation than luminosity mean (Table 1), indicating that house sparrows had higher intra-cheek contrast. For eyebrow, height loaded most strongly (Italian sparrows had taller eyebrows), followed by rear eyebrow luminosity standard deviation (more variation in house sparrows) and eyebrow length (Italians had longer eyebrows). The LD1 scores used in cline analysis had identical trait loadings to CVA.

Cline analysis

The support limits for cline centre and width for crown colour were narrower than those for any other variable, indicating that this trait fit most closely to the cline model (Figure 2, Appendix S2). The ML cline width for crown colour of 16.1 km (support limits 12.1 – 20.4 km) was narrower than the other plumage traits, and significantly narrower than both the genome-wide and the 4-SNP hybrid indices, indicating strong selection on this trait. Support limits for the other plumage traits were wide and overlapped with crown colour. While this precluded statistically significant differences, it indicated that the other traits did not fit well to the cline model. Cline width support limits for cheek and eyebrow reached the maximum possible 200 km. Hence for these traits there was no clear indication of a distinct cline

restricted to the Alps hybrid zone. The 4-SNP HI was significantly shifted north relative to crown colour.

For the SNPs, only SNX2 was estimated to be nearly fixed at both ends of the cline (Appendix S2); however this produced a very wide estimated cline (Figure 2). No other SNPs changed more than 0.62 in allele frequency. The cline width for ZCCHC6, a Z-linked marker, was significantly narrower than crown colour. The best estimate was an almost instantaneous shift just south of the Alpine ridge. However, the allele frequency change was only 0.22. Previously, genomic cline analysis combined with Geneland (Guillot et al. 2005) geographic analyses across the whole Italian peninsula identified SNPs within the genes CHD1Z, CETN3 and RPS4 as candidate house-Italian sparrow RI loci, forming boundaries in the Alps coincident with previously identified phenotypic clines (Trier et al. 2014; Hermansen et al. 2014). With respect to these three SNPs, crown colour cline width was significantly narrower than the two Z-linked markers CHD1Z and CETN3, and overlapped with RPS4 (Chr. 4A). Of the strongly clinal SNPs, ZCCHC6, REEP5, GSTK1, RPS4 and CLIP2 were significantly shifted north relative to crown colour.

Selection and neutral introgression

Using the published lifetime dispersal estimate of 2 km and the ML and support limits for crown colour cline width, the estimated number of generations required to produce the crown cline through neutral introgression was 10.3 (5.8 - 16.6) (Appendix S2). The estimated strength of selection maintaining the cline was 0.062 (0.038 - 0.109), representing a 6.2% drop in fitness at the cline centre.

We consider 2 km likely to be an underestimate of average lifetime dispersal, and hence also used indirect dispersal estimation methods. Taking the largest population $\overline{D}$ value of 0.031

from mean pairwise *D* (Figure 3a) and using the ML and support limits for 4-SNP HI cline width, lifetime dispersal was estimated as 4.89 (2.68 – 12.22) km. Using 4.89 km produced an estimate of 1.7 (1.0 – 2.8) generations of neutral introgression and a 36.9% (22.9 – 65.1) drop in fitness.

Covariances between crown colour and 4-SNP HI produced similarly mixed but qualitatively different estimates of $\overline{D}$ (Figure 3b), with the strongest positive and negative values being from two populations just a few km apart along the same valley, close to the crown cline centre. The estimates were larger, however, with a maximum positive $\overline{D}$ of 0.126. Given the small sample sizes and large variation between nearby populations, we did not use these measures to estimate dispersal.

Bimodality

For crown colour almost all sliding windows containing at least one designated hybrid population were significantly bimodal (Table S4; Figure 4). The only exceptions were windows containing house sparrows combined with any of the three populations closest to the Alpine ridge on its southern slopes, which clustered with house sparrows. None of the windows containing only populations classified as house or Italian sparrow were significantly bimodal. No windows were bimodal for cheek or eyebrow (Table S4). For hybrid index, only 3 windows – 2 containing only designated Italian sparrow populations and 1 containing only house sparrows – were significantly bimodal.

The estimated number of loci controlling the difference in crown colour was 0.30 (0.14 – 0.49). Therefore the best estimate was 1 locus with heterozygote deficit or partial dominance.

Discussion

Of the male plumage traits, crown colour most strongly distinguished Italian and house sparrows either side of the Alps hybrid zone, confirming it as the clearest diagnostic plumage trait between the hybrid species and its parent. Crown colour was strongly clinal in the region just south of the main Alpine ridge, with a cline width of 16 km giving an estimated drop in fitness at the cline centre of 37% (range 4 - 65%, depending on the dispersal estimate). The crown colour cline was significantly narrower than clines for both a genome-wide hybrid index and a hybrid index based on 4 unlinked SNPs each showing strongly significant clines, further supporting its role in RI in this region. The cline was also significantly narrower than those for CHD1Z and CETN3, two Z-linked loci previously identified as candidate postzygotic RI markers (Trier et al. 2014). The crown cline was significantly shifted south relative to several individual SNP clines and the 4-SNP hybrid index. Crown colour was significantly bimodal within the hybrid zone, indicating either strong disruptive selection on a polygenic trait, heterozygote disadvantage in a single-locus additive trait, or partial dominance, most likely also of a single locus. Other plumage traits showed weaker clines, and no distinct cline associated with the Alps could be clearly discerned.

Evidence for putative postzygotic isolation between house and Italian sparrows appeared to be weaker than that for prezygotic isolation acting on crown colour, and only 19 out of 75 SNP loci fit the cline model better than a model of no change in allele frequency. However, a number of those 19 SNPs also had no clearly distinct cline associated with the Alps contact zone. Although the Alps represents the clearest boundary for several loci (Trier et al. 2014), the SNPs, which were originally chosen to distinguish house from Spanish sparrows, showed relatively small allele frequency shifts, which may indicate that they are linked neutral markers rather than the cause of RI. On the other hand, some of the loci were estimated to

have near zero Spanish sparrow allele frequency in parapatric house sparrows, as is observed in allopatric populations (Trier et al. 2014), suggesting that there may be asymmetric selection against introgression of the Spanish allele into house sparrow populations. Identification of markers more clearly distinguishing Italian from house sparrows is now required to clarify the role these and other loci play in isolation in this hybrid zone.

No previous evidence exists of the role of male plumage in isolating sparrow taxa, and it is unknown whether selection acts via male-male interactions and/or female choice. Furthermore, the inheritance mechanisms of female choice are unknown. Recombination breaks down associations between signal, choice and postzygotic isolation loci, and can lead to a collapse of selective pressures on signal traits (Felsenstein 1981). Crown colour bimodality in the hybrid zone appears to be strong enough to maintain these associations above a threshold level, and hence maintain selection towards bimodality. This process would be assisted by mechanisms reducing recombination between loci affecting the three sets of traits. Reduced recombination due to strong physical linkage (e.g. Kronforst et al. 2006; Shaw & Lesnick 2009; Merrill et al. 2011), fixed chromosomal inversions (Kulathinal et al. 2009), pleiotropic effects of single loci on multiple aspects of RI, or imprinting on parental phenotypes (and hence non-genetic inheritance of choice; Verzijden et al. 2012) may achieve this.

We suggest that selection against intermediate crown colour, possibly via female choice, maintains bimodality in mixed populations. An alternative explanation may be partial dominance at a single locus. The estimate of 0.3 loci involved in crown colour differences suggests that this is possible, as it indicates a single locus with a deficit of intermediates. However, this estimate is based on the increase in trait variance at the centre of the hybrid zone, which could also be explained by disruptive selection on a polygenic trait. The presence

of a variety of mixed crowns in the hybrid zone (e.g. Appendix S1) indicates that the trait is not strictly controlled by a single dominant QTL, and one major QTL combined with several minor QTL may be more likely. One published study (Macke 1965) and observations of 5 aviary-reared F1 house-Spanish sparrow hybrids (F. Eroukhmanoff, pers. comm.) indicate F1 males have brown crowns (but see Alonso 1984 for one apparently black-crowned F1 individual), and hence brown may be dominant over house sparrow grey. However, in the Alps hybrid zone the crown colour cline is shifted south, favouring grey crowns, relative to several clinal SNPs; the opposite direction to that expected if brown crowns were dominant. Trait-genotype association studies are required to distinguish the different possibilities for the genetic architecture of this trait, and hence more clearly examine the role of disruptive selection in maintaining bimodality.

Steep character clines can arise even without selection acting on the trait in question if the two species involved have met recently, as neutral diffusion takes time to flatten the cline (Endler 1977; Hewitt 1988; Barton and Gale 1993). We consider this scenario highly unlikely for crown colour in the Alps hybrid zone. The two species are estimated to have come into secondary contact only 2-34 years ago based on neutral introgression with a 2 year generation time. Earlier observations of hybrids between Italian and house sparrow prove contact between them for at least 125 years (Wallis 1887), although this itself is almost certainly a large underestimate. The sparrows most likely came in contact some thousands of years ago (Hermansen et al. 2011; Sætre et al. 2012). If we assume 2000 years since contact, the crown colour cline should have been approximately 150 - 400 km wide today after one thousand generations of free diffusion. Hence, even when using highly conservative estimates of dispersal and time since contact, the most plausible explanation is that the crown colour cline is maintained as narrow by selection against hybrids in the contact zone.

548 The historical scenario leading to the formation of the sparrow hybrid zone in the Alps is not
549 known. Trier et al. (2014) suggested genes incompatible between parental species may have
550 moved from where hybridization originally took place when the Italian sparrow was formed,
551 and only later came to rest at the two current hybrid-parent species range boundaries,
552 including the Alps. The broad cline across the Italian peninsula in hybrid index (Trier et al.
553 2014) is consistent with this scenario, and further studies have shown that the candidate
554 hybrid-parent RI loci are indeed also candidate incompatibilities between the two parent
555 species (Hermansen et al. 2014). If this were how the Alps hybrid zone was formed, rather
556 than through secondary contact, we would expect the movement of incompatibilities to have
557 left behind broad clines in neutral alleles (Currat et al. 2008). The expected time since contact
558 would then be overestimated from cline widths, not underestimated. Hence, if crown colour
559 had been neutral under this scenario we would have expected it to possess a broad, shallow
560 cline. Selection against intermediate crown colour is therefore the most plausible explanation
561 for the narrow crown colour cline.

562 Previously identified plumage differences between Italian sparrows and house and Spanish
563 sparrows (Summers-Smith 1988; Hermansen et al. 2011) combined with evidence that
564 postzygotic reproductive barriers are strongest at the Italian sparrow range boundaries (Trier
565 et al. 2014) and a lack of hybridization in sympatry between Italian and Spanish sparrows
566 (Hermansen et al. 2011; Trier et al. 2014) provide a strong argument that the Italian sparrow
567 is a distinct homoploid hybrid species (Sangster et al. 2015). Additional evidence presented
568 here, of RI acting on a diagnostic male plumage trait at one of the range boundaries,
569 strengthens that conclusion. Unlike strongly divergent taxa that form hybrid zones, such as
570 the fire-bellied toads *Bombina bombina* and *B. variegata* (Nurnberger et al. 1995), the trait
571 and genetic clines between Italian and house sparrows do not all appear to be identical in
572 width (concordant) or location (coincident). Concordance and coincidence would be expected

573 if divergence and RI were strong enough to maintain genome-wide linkage disequilibria
574 (Nurnberger et al. 1995). The different isolating factors may therefore be evolving quite
575 independently. This is not surprising, given that the genome-wide hybrid index of Italian
576 sparrows in this region indicates that they are already about 80% identical to house sparrows.
577 Hence genome-wide disequilibria are unlikely, and in general may be less likely in hybrid
578 zones between hybrid and parent taxa than between two non-hybrid taxa.

579 Despite the apparent independence of different traits and genes, a number of clines are
580 situated in broadly the same location, with cline centres about 0-30 km south of the main
581 Alpine ridge. Clines often coincide with barriers to dispersal (Barton and Hewitt 1985), and
582 this may be a major reason why this hybrid zone sits in its current location. It remains
583 important to discover whether any interactions are occurring between different isolating
584 mechanisms in this hybrid zone, and particularly between male crown colour and as yet
585 unidentified female choice and postzygotic isolating mechanisms.

586 In this study, we have shown that male crown colour differences between the hybrid Italian
587 sparrow - which inherited its crown colour from Spanish sparrows - and its parent species the
588 house sparrow, are maintained in the form of a narrow cline, indicating strong sexual isolation
589 based on this trait. Evidence is now building that isolating mechanisms inherited from the
590 parent species can have important effects on RI between hybrid and parent (see also Trier et
591 al. 2014; Hermansen et al. 2014). It remains to be seen how important transgressive traits and
592 novel mutations are in this system, relative to these pre-existing isolating mechanisms.

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Figure legends

Figure 1. Map of sampling locations across the house sparrow/Italian sparrow hybrid zone in Italy and Switzerland. Yellow dots indicate populations with Italian sparrow phenotypes, dark green dots indicate populations with Italian sparrow phenotypes and pale green dots indicate populations with mixed and hybrid phenotypes. The orange line indicates the location of the Alpine ridge. The insert shows the location of the study area in Europe (red rectangle) along with the distribution of allopatric house sparrows in green, house-Spanish sparrow sympatric regions in green with red diagonals, allopatric Spanish sparrows on the island of Sardinia, and the small sympatric Italian-Spanish sparrow region on the Gargano peninsula (both red). Italian sparrow distributions on the mainland and the island of Corsica are marked in yellow, and the Alps house-Italian sparrow hybrid zone in greenish-yellow. Sparrow heads are representative of the change in male plumage phenotype across the hybrid zone.

Figure 2. Maximum likelihood cline centres and widths (symbols) and upper and lower 2-unit likelihood support limits (error bars). (A) plumage traits and the two hybrid indices (triangles). (B) SNPs with the biggest improvement in AICc of the cline model over the null model, plus crown color for comparison. SNPs used in the 4-SNP hybrid index are represented as triangles. CETN3 is omitted for clarity. Negative centre values are south of the Alpine ridge.

Figure 3. Per-population estimates of admixture linkage disequilibrium (*D*). (A) Average pairwise *D* based on 4 unlinked SNPs: CHD1Z, RPS4, CLIP2 and NCOA3, for samples

with 5+ individuals. (B) Estimate based on covariance between the 4-SNP hybrid index (same 4 SNPs as above) and crown color for samples with 3+ individuals.

Figure 4. Mean ± standard deviation (SD) of crown % grey, based on results of bimodality tests on sliding windows of 5 populations per window. Windows that were not significantly bimodal are represented either by the Italian sparrow mean and SD (red dots) or house sparrow mean and SD (blue dots). Significantly bimodal windows are represented by both house and Italian sparrow estimates. Windows within the grey shaded area contain at least one population pre-classified as within the hybrid zone.

Table 1. Test statistics for parental differentiation of plumage traits and hybrid index. All P values are significant.

Multivariate trait	Canonical variates		Mahalanobis distance	
	Trait	**CV**	**Distance**	**P value**
Cheek	Mean luminosity	0.01	1.52	0.019
	SD luminosity	-0.18		
Eyebrow	Eyebrow length	0.14	1.88	0.019
	Eyebrow height	1.10		
	Rear mean luminosity	0.03		
	Front mean luminosity	0.01		
	Rear SD luminosity	-0.22		
	Front SD luminosity	-0.09		
Univariate trait		**t value**	**df**	**P value**
Crown colour	-	29.87	20.4	2.2e-16
Hybrid index	-	-8.14	29.0	5.7e-09

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Table 2. Parental trait scores with range in brackets. Cheek and eyebrow are house-Italian linear discriminant axis scores (LD1) **for these multivariate traits. 'Hybrid index' refers to the 75-SNP hybrid index estimated using Introgress software.**

Species	Proportion grey	Hybrid index	Cheek	Eyebrow
Italian	0.04 (0, 0.21)	0.2 (0.09, 0.3)	0.69 (-1.73, 1.89)	0.85 (-0.75, 2.45)
House	0.94 (0.7, 1)	0.04 (0, 0.18)	-0.84 (-3.03, 0.58)	-1.03 (-2.68, 0.88)

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Supporting Information

Table S1. Sample population details.

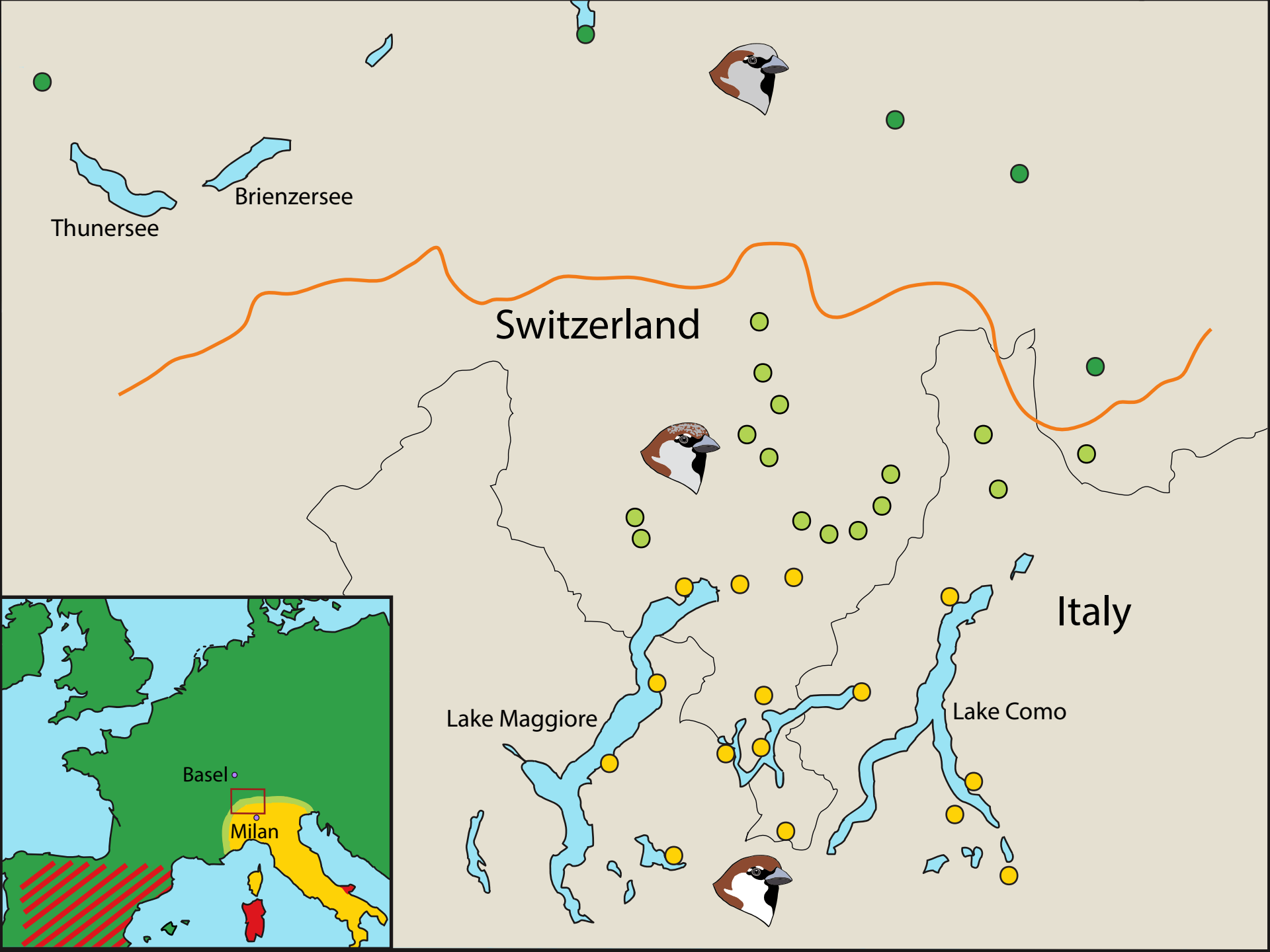
Table S2. Details of SNPs used in the analyses.

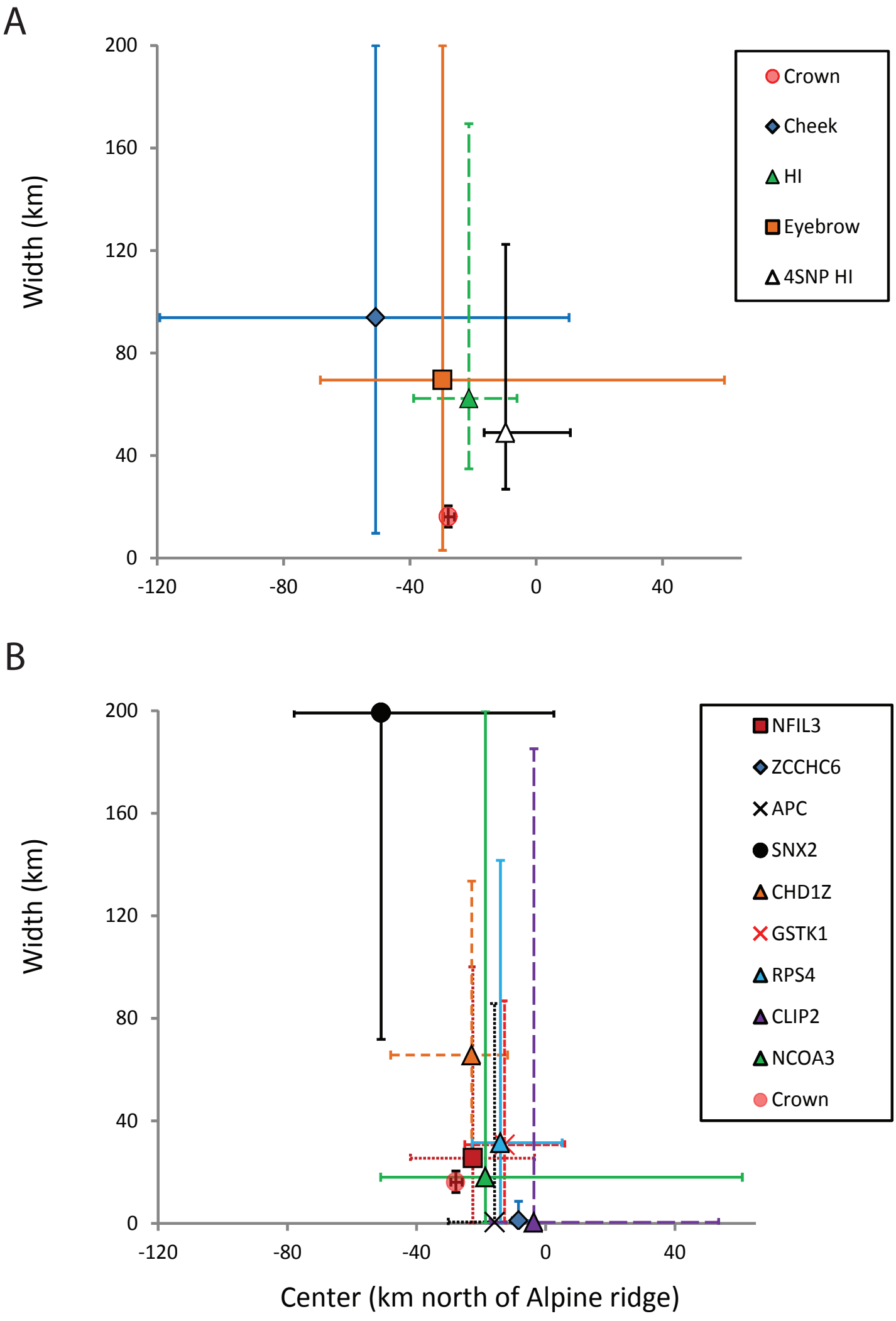
Table S3. Image luminosity regression results.

Table S4. Likelihood ratio test results for bimodality of plumage traits and hybrid index.

Appendix S1. Bird photographs with examples of colour variation; reflectance spectra for crown and cheek.

Appendix S2. Full cline analysis results tables.


Thunersee
Brienzersee
Switzerland
Italy
Lake Maggiore
Lake Como
Basel
Milan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Width (km)
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40
Crown
Cheek
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Eyebrow
4SNP HI
B
Width (km)
200
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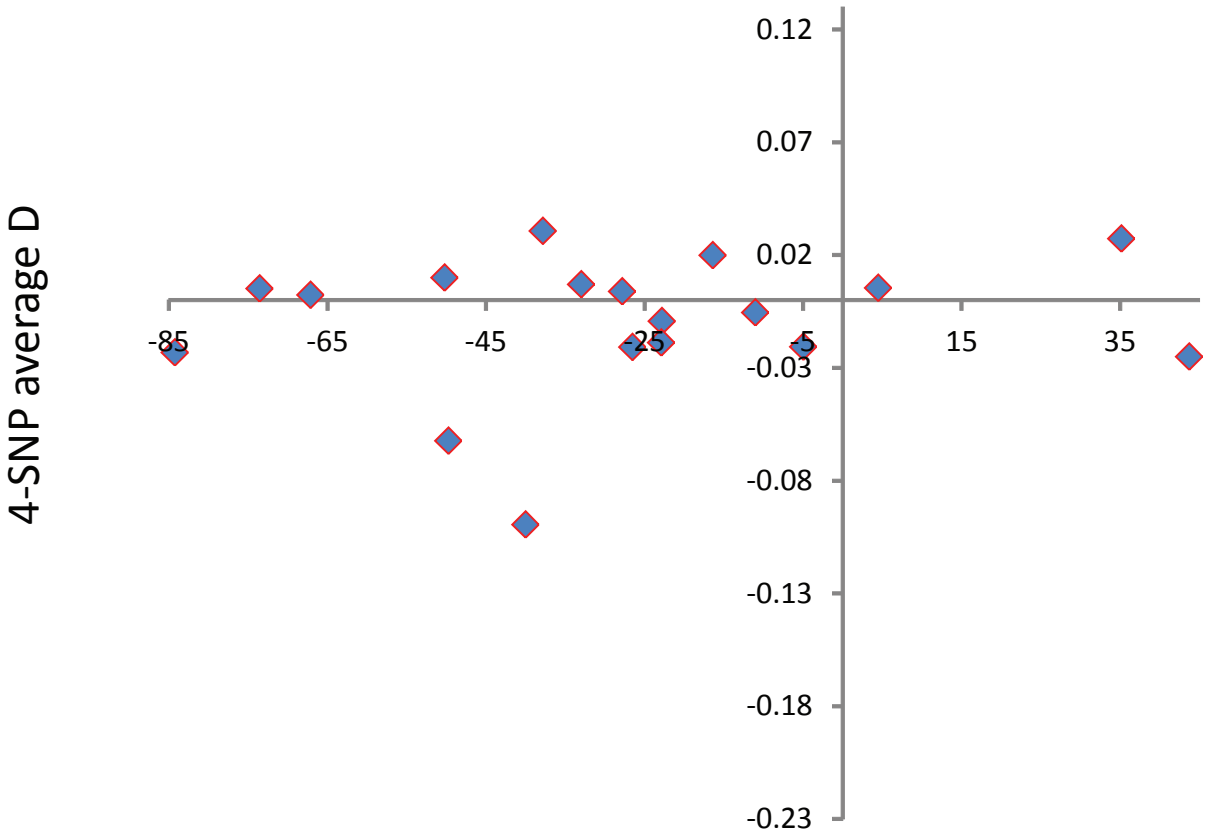
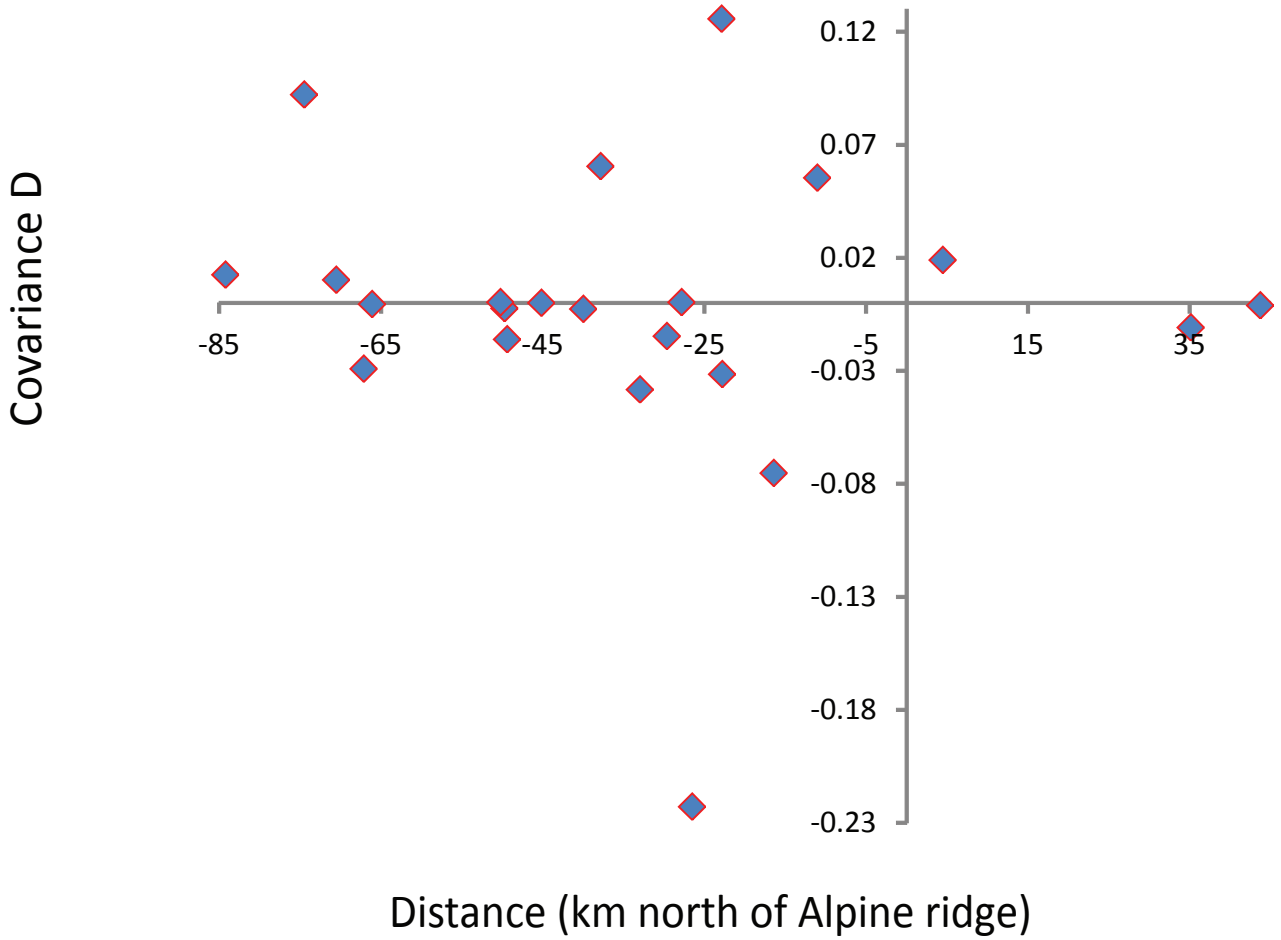
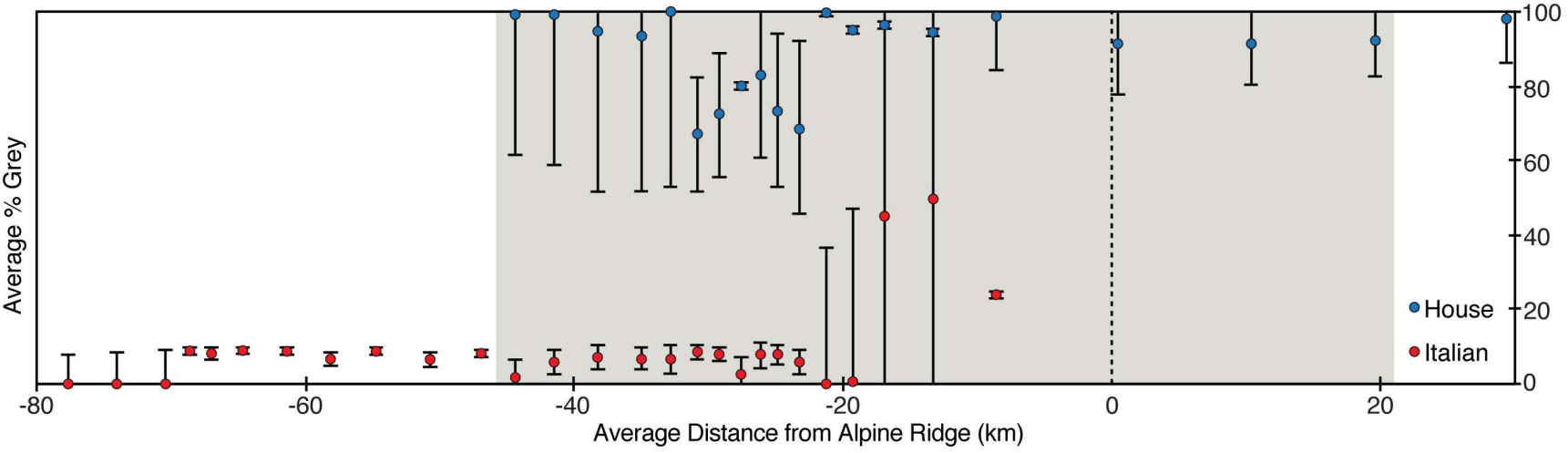
-120
-80
-40
0
40
Center (km north of Alpine ridge)
NFIL3
ZCCHC6
APC
SNX2
CHD1Z
GSTK1
RPS4
CLIP2
NCOA3
Crown

A


4-SNP average D
0.12
0.07
0.02
-0.03
-0.08
-0.13
-0.18
-0.23
-85
-65
-45
-25
-5
15
35

B


Covariance D
0.12
0.07
0.02
-0.03
-0.08
-0.13
-0.18
-0.23
-85
-65
-45
-25
-5
15
35
Distance (km north of Alpine ridge)


Average % Grey
100
80
60
40
20
0
House
Italian
-80
-60
-40
-20
0
20
Average Distance from Alpine Ridge (km)