SUPPORTING INFORMATION for: Gene duplication and divergence produce diverse MHC genotypes without disassortative mating

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SUPPLEMENTAL LITERATURE REVIEW

MHC-Disassortative Mating and the Monophyly of MHC Loci Assayed

Based on our empirical data and our phylogenetic model, our study ultimately proposes that MHC-based mating preferences can be shaped by the divergence of duplicated MHC loci. Species vary in their number of MHC loci and in the extent of differentiation between those loci. These differences between species have consequences for natural selection and for sexual selection, but they also pose methodological problems for genotyping and consequently for thinking clearly about how MHC variation might affect the evolution of mating preferences. Here, we summarize the locus-specificity of MHC data from studies of MHC-disassortative mating in other species.

In humans and mice, an extraordinary amount is known about MHC structure and function, including the documentation of gene-specific clades of allele sequences of peptide binding grooves within MHC Class I and Class II in humans and within MHC Class II in mice (Gu & Nei 1999). In humans and in mice there are examples and counterexamples of disassortative mating preferences, along with some disagreements about the relevance of studying mate choice in these species (Havlicek & Roberts 2009; Jordan & Bruford 1998; Penn & Potts 1998; Piertney & Oliver 2006). In the other leading model system of MHC architecture, the chicken (Jacob *et al.* 2000; Kaufman *et al.* 1999; Salomonsen *et al.* 2005), junglefowl show interesting evidence of cryptic choice for MHC-dissimilar mates (Gillingham *et al.* 2009; Løvlie *et al.* 2013) and largely locus-specific divergence of alleles between the major and minor Class I genes (BF locus) but phylogenetic comingling of alleles between the major and minor Class II genes (BLB locus) (Worley *et al.* 2008). Despite locus-specific genotype data in those junglefowl mate choice studies, inheritance of unbroken MHC haplotypes in fowl makes it hard to interpret these studies in relation to our model.

In wilder species of animals, details of MHC structure and function are generally much less clear, and tests of MHC-disassortative mating use widely varying kinds of MHC data. In addition to variation in lab methods (RFLP; DGGE, SSCP, or RSCA, with or without sequencing of exemplars; cloning and Sanger sequencing; high throughput sequencing), studies vary in the number of loci assayed and in whether those assays are locus-specific. Among studies that test for MHC-disassortative mating, 14 studies[1] – predominantly of salmonid fishes but also 2 other fish species, 1 amphibian, 1 bird, and 3 mammals – examined exactly one MHC locus, with 7 of those datasets finding evidence for MHC-disassortative mating. In contrast, the vast and increasing majority of studies that test for MHC-disassortative mating use PCR primers that unresolvably amplify multiple loci. In 27 datasets of 21 species[2] – including 2 fish species, 2 reptiles, 10 birds, and 7 mammals – researchers generated data simultaneously from multiple MHC loci with lab methods that prevented the assignment of alleles to loci. Roughly half of these studies found evidence of disassortative mating, and half did not. Unfortunately, most such studies can only hypothesize about the number of loci being amplified, and

many also lack confirmation of expression. Aside from the present work on Leach's storm-petrels, only one study has examined multiple MHC loci with locus-specific data (Huchard *et al.* 2013).

Two points arise from this overview of the literature. First, our study is fairly unique among wild animal studies, in testing MHC-disassortative mating with locus-specific data from multiple MHC loci. Second, the current state of the field makes it hard to test the generality of our model. Outside of salmonid fishes, most systems for studying MHC-disassortative mate choice do indeed have multiple loci. However, the extent of divergence between the alleles of duplicate loci cannot be assessed in those wild animal studies, because the lab methods used cannot assign alleles to particular loci. This situation was actually forecast and lamented a decade ago (Piertney & Oliver 2006) and will likely be exacerbated by continued advances in high throughput sequencing.

[1]Tests of MHC-disassortative mating with single-locus MHC data: (Agbali *et al.* 2010; Bahr *et al.* 2012; Bos *et al.* 2009; Cutrera *et al.* 2012; Evans *et al.* 2012; Evans *et al.* 2013; Forsberg *et al.* 2007; Knafler *et al.* 2012; Landry *et al.* 2001; Lenz *et al.* 2013; Neff *et al.* 2008; Sommer 2005; Yeates *et al.* 2009)

[2] Tests of MHC-disassortative mating that simultaneously screen multiple MHC loci without being able to assign putative alleles to loci: (Aeschlimann *et al.* 2003; Baratti *et al.* 2012; Bichet *et al.* 2014; Bonneaud *et al.* 2006; Eizaguirre *et al.* 2009; Ekblom *et al.* 2004; Freeman-Gallant *et al.* 2003; Huchard *et al.* 2010; Juola & Dearborn 2012; Kalbe *et al.* 2009; Kuduk *et al.* 2014; McCairns *et al.* 2011; Miller *et al.* 2009; Olsson *et al.* 2003; Radwan *et al.* 2008; Reusch *et al.* 2001; Richardson *et al.* 2005; Roth *et al.* 2014; Schwensow *et al.* 2008a; Schwensow *et al.* 2008b; Sepil *et al.* 2015; Setchell *et al.* 2010; Sin *et al.* 2015; Strandh *et al.* 2012; Westerdahl 2004; Winternitz *et al.* 2015)

SUPPLEMENTAL METHODS

Details of Sex Identification

Because storm-petrels are sexually monomorphic, we assessed the sex of all 222 birds with PCR primers 2550F and 2718R (Fridolfsson & Ellegren 1999), using 20 µl reactions of 2.0 µl 10x ABI Amplitaq Gold 360 buffer, 2.0 µl 25mM $MgCl_2$, 2.0 µl 2mM dNTPs, 0.8 µl 10µM each primer, 1.0 µl ABI 360 G-C enhancer, 7.3 µl water, 0.1 µl Amplitaq Gold polymerase, and 4.0 µl 20 ng/µl DNA. Reactions were conducted on a BioRad C1000 thermocycler as follows: 95°C for 10 min; 35 cycles of 95°C for 30 s, 49°C for 60 s, and 72°C for 90 s; and a final 5 min extension at 72°C. In this species, amplicons from the Z and W chromosomes differ by 200 bp and are easy to resolve on 1.5% agarose.

Details of MHC High Throughput Sequencing, Data Processing, and Copy Number Variation

Our nested PCR and sequencing have been described in detail (Dearborn *et al.* 2015), but we recap here several points that affect data quality. Barcoded versions of forward and reverse PCR primers for the inner PCR were used in unique combinations for each bird, such that sequence data from a library of pooled amplicons could later be demultiplexed. Any two barcodes differed in at least three positions, reducing the possibility that sequencing error would cause an amplicon sequence to be assigned to the wrong bird. On each PCR plate, negative controls were also subjected to amplification and sequencing protocols. To reduce the risk of chimera formation, we used a

minimum number of PCR cycles in the outer and inner PCR reactions, a long extension step to avoid
88 incomplete synthesis, and a hot-start polymerase that lacks proof-reading capability.

90 Amplicons were sequenced in two batches, as part of two different Illumina MiSeq runs using 2x250 bp paired end reads (Jackson Laboratories, Bar Harbor, Maine, and Farncombe Metagenomics
92 Facility, McMaster University). For each run, a PCR-free Illumina sequencing library was prepared with either the KAPA HTP Library Preparation Kit (Kapa Biosystems, Wilmington, MA) or TruSeq DNA
94 PCR-Free Library Preparation Kit (Illumina, San Diego, CA) and an Illumina TruSeq style adapter, with subsequent quality control performed with an Agilent Technologies 2100 Bioanalyzer and with qPCR.
96

Sequences were trimmed to a length of 200 bp using the FASTX package
98 (http://hannonlab.cshl.edu/fastx_toolkit/) based on an average quality curve value of Q30 at 200bp. We filtered out sequences that could not be resolved due to missing primer sequence, unrecognized
100 barcode, sequence ambiguities, or incomplete reads. The trimmed forward and reverse sequences from the paired end reads were assembled with 111 bp overlap for Ocle-DAB1 and 73 bp overlap for
102 Ocle-DAB2.

104 Our primary genotyping algorithm was aimed at identifying single-locus genotypes for the two genes, assuming an absence of copy number variation (see next). Allele coverage within bird and locus
106 should vary since the sequencing results are the product of pooling of many PCR products, with the result that a read-depth cutoff is not the best way to distinguish real alleles from sequencing noise.
108 Instead, our custom genotyping algorithm examined ratios of amplicon read abundance within a bird at each gene: there should be one or two amplicons with clearly high abundance in homozygotes and
110 heterozygotes, respectively, with the remainder being sequencing error and low abundance artefacts. Samples with low coverage or unresolved genotypes were manually inspected, as was a random
112 sample of 20 algorithm-determined genotypes.

114 Three lines of evidence suggest that copy number variation (CNV) does not occur at high frequency in our dataset. First, preliminary Sanger sequencing of uncloned PCR products generally produced either
116 clean sequences with single peaks (i.e. homozygotes) or sequences with some double peaks that could be created by combination of two sequences from homozygotes. Second, in the 22 parent-
118 offspring trios genotyped at both MHC genes, offspring genotypes were consistent with inheritance from their parents. If copy number variation was common, individuals should often have more than
120 two alleles, in which case our non-CNV genotyping algorithm would identify a random subset of the existing alleles. As a result, offspring should periodically have one or more alleles not accounted for
122 by the genotypes of their parents. Our data do not show that pattern. Third, in our repeat PCR and genotyping of both genes in 39 birds (Dearborn *et al.* 2015), 77 of 78 (98.7%) genotypes that were
124 assayed in duplicate yielded identical results. If CNV was widespread, our repeat-genotyping should have had poor success, because individuals with additional gene copies would often have more than
126 two alleles, and the number of reads of these alleles should by chance sort out in a different order of sequencing depth in the two genotyping efforts, resulting in conflicting genotype calls.
128

Nonetheless, the Illumina data do show some birds as having more than two sequences per gene,
130 though with unequal numbers of reads of these sequences within a bird. In case this represents CNV

rather than error, we also defined genotypes and tested for mate choice using a genotyping
132 algorithm that is permissive to the existence of CNV. For each bird, we retained as alleles all sequences that met three criteria: the sequence was also detected as one of the most common two
134 sequences in at least one other bird, the reads of the sequence in the bird being genotyped were more common than reads of sequencing error in the same bird, and the number of reads of that
136 sequence comprised at least 15% of the number of reads of the most common sequence in that bird. This last criterion is a permissive expansion of the following expectation: if CNV has resulted in 3
138 copies of a gene and if a bird's genotype is as skewed as possible – 5 copies of 1 allele and 1 copy of another – the read depth for the rare allele should be 20% of the read depth of the common allele. If
140 the genotype is anything less skewed (e.g., 4 copies of 1 allele, 2 copies of a second allele, and 2 copies of a third allele), there should be a higher ratio of the rarest allele's reads to the most common
142 allele's reads, allowing it to easily pass the 15% cutoff. By these criteria, additional alleles were retained in the genotype calls of 3 of 210 birds (1.4%) at Ocle-DAB1 and in 30 of 210 birds (14.3%) at
144 Ocle-DAB2, resulting in a maximum of 3 and 4 alleles observed per bird at the two genes, respectively.
146

Details of Microsatellite Amplification

148 For paternity analysis and for estimating relatedness between mates, birds were genotyped at 15 microsatellite loci (Table S1) previously developed for either a different population of this species (12
150 loci (Bicknell *et al.* 2011)), a congener (2 loci (Sun *et al.* 2009)), or the zebra finch *Taeniopygia guttata* (1 locus (Dawson *et al.* 2010)). Primers were initially screened for amplification success in our lab,
152 and samples were subsequently sent to Ecogenics (Balgach, Switzerland) for multiplex PCR and fragment analysis. Samples were amplified in 3 multiplex reactions of 4 to 6 loci each, using
154 fluorescently labeled primers. Each 10 µl reaction contained 2-10 ng of genomic DNA, 5 µl HotStarTaq Master Mix (Qiagen 203445), double distilled water, and 0.3 µl of 10µM of each forward
156 and reverse primer. Reactions were conducted on a Techne TC-412 thermocycler as follows: 95°C for 15 min; 35 cycles of 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s; and a final 30 min extension at
158 72°C. Sizing was performed on an Applied Biosystems 3730xl DNA Analyzer, with manual verification of allele calls.
160

Power Analysis for MHC-based Mate Choice

162 We found no evidence of preference for mates that were maximally or intermediately disassortative at MHC, based on randomization tests of means and variances, respectively. Thus, we estimated the
164 statistical power to detect mate choice for amino acid sequence divergence between mates, similar to Lenz et al. (Lenz *et al.* 2013). For the two-tailed randomization test of means, we paired each
166 female with a male chosen randomly without replacement and then increased their MHC divergence away from random by adding a small value, x. For a given value of x, we created 1,000 such sets of 94
168 pairs and estimated power as the percent of the 1,000 iterations that showed significant disassortative mating when compared against the 97.5th percentile of the null distribution used in our
170 original analysis. We then iterated this process over a range of effect sizes by changing the value of x, i.e. by changing the mean MHC divergence between randomly assigned pairs (Figure S5a).
172

For the one-tailed randomization test of variances, the aim of the power analysis was to change the
174 effect size by reducing the among-pair variance in MHC divergence between a female and her mate.

Here, we paired each female with a male chosen randomly without replacement, and then we shifted
176 each pair's female-male MHC divergence towards the mean of all random pairs, by adding or
subtracting a small value, *y*, depending on whether the initial value was below or above the mean.
178 For a given value of *y*, we created 1,000 sets of 94 pairs and estimated power as the percent of the
1,000 iterations that showed significantly smaller variance than the 95th percentile of the null
180 distribution used in our original analysis. We then iterated this process over a range of effect sizes by
changing the value of *y*, thereby changing the variance of randomly assigned pairs around the mean
182 value for randomly assigned pairs (Figure S5b).

184 **Details of Phylogenetic Permutation Model of MHC**

Overview – The phylogenetic permutation model tested two possible contributors to amino acid
186 differences between an individual's alleles. The first hypothesis is that MHC-divergent genotypes are
generated by monophyly broadly speaking – that is, because the alleles of the two genes are diverged
188 into locus-specific clades. The second hypothesis is that MHC-diverse genotypes are generated by
particular divergence between the two common alleles in the population (Ocle-DAB1*004 and Ocle-
190 DAB2*0050; Figure 1a).

192 To test these two hypotheses, we used the existing set of alleles from our sample rather than create
sequences de novo via simulated mutation. Thus, we maintained three key aspects of our system:
194 the total number of alleles per locus (11 at Ocle-DAB1 and 13 at Ocle-DAB2), the distribution of allele
frequencies at each locus, and the structure of the phylogeny. Within that framework, we permuted
196 the alleles (and their associated frequencies) across the phylogeny, which changed the distance
between alleles according to their new positions in the phylogeny. The iterative assignment of alleles
198 to new positions in the phylogeny changed iteratively the two factors of interest to us: the extent of
monophyly, and the distance between the two most common alleles. We will discuss these in turn.
200

Monophyly – The phylogenetic analyses show two clades of 11 and 13 alleles, corresponding to Ocle-
202 DAB1 and Ocle-DAB2 (Figures 1a, 2, and S1). When permuting the locations of the alleles within this
phylogeny, the proportion of a gene's alleles that could fall together into one clade (i.e. monophyly)
204 can vary from a low of 0.542 (the smaller clade containing 5 and 6 alleles from Ocle-DAB1 and Ocle-
DAB2 respectively, and the larger clade containing 6 and 7 alleles from Ocle-DAB1 and Ocle-DAB2) to
206 a high of 1 (all 11 Ocle-DAB1 alleles in the smaller clade, and all 13 Ocle-DAB2 alleles in the larger
clade). Including these two extremes, there are 12 possible values for the degree of monophyly in
208 this dataset.

210 *Distance between Common Alleles* – Each gene has a single common allele, Ocle-DAB1*004 and Ocle-
DAB2*0050; these are moderately far apart in the actual phylogeny (Figure 1a, Table S2). In the
212 permutation model, these two alleles could be quite near each other in the phylogeny or could be
very far apart, and this range of possibilities here is largely unconstrained by the degree of
214 monophyly of the full set of alleles; see Figure S5 for examples of permutations that show various
combinations of high and low values for monophyly and high and low values for distance between
216 the common alleles.

218 *Permutation and Output* – We randomly permuted the locations of the alleles in the phylogeny, writing a set of programs in 4th Dimension (4D, Inc; San Jose, CA) to generate stratified permutations
220 with 1,000 independent replicates (varying in distance between the two common alleles) for each of the 12 possible degrees of monophyly (see examples in Figure S5). For each of the 12,000
222 permutations of the alleles in the phylogeny, we recorded three variables as output: the degree of monophyly; the number of amino acid differences between the permuted locations of the two most
224 common alleles, Ocle-DAB1*004 and Ocle-DAB2*0050; and the average MHC diversity in our set of 188 individuals, calculated as the average amino acid differences between the 6 pairwise
226 combinations of an individual's 4 alleles.

228 *Downstream Analysis* – We entered the model's output into regression analyses to test the relative importance of the two hypotheses. Rather than inflate our sample by using a data point from all
230 12,000 permutations, our downstream analysis of model output used the average MHC diversity for each value of the predictor variables. The dependent variable was the extent of MHC diversity within
232 individuals, measured as the average distance between pairwise comparisons of an individual's alleles. Two predictor variables were tested: the extent of monophyly, and the divergence between
234 the most common allele of each gene. The importance of these predictors was tested separately in univariate regressions and then together in a multiple regression. Slopes were calculated as
236 standardized slopes (i.e. the dependent and predictor variables were standardized to a mean of 0 and standard deviation of 1); this has the advantage of allowing the slopes associated with the two
238 predictors to be compared directly, while not altering the significance tests or the model R^2.

240 In the data used in the multiple regression analysis, there was not a problem with multicollinearity, as the degree of monophyly and the distance between the two common alleles were only weakly
242 correlated ($r = 0.096$). Even in the unusual situation of perfect monophyly, the distance between the two common alleles could range from 8 to 22 amino acids (of 89 codons in exon 2). With any of the
244 other degrees of monophyly, the possible range of distances between the two common alleles was even wider, from 1 to 22 amino acids. Consequently, there was ample scope to test for separate
246 effects of monophyly of the genes' alleles and distance between the two common alleles, as reflected in the span of the box plots in Figure 5.
248

250 **SUPPLEMENTAL RESULTS**

Microsatellite Descriptives

252 We resolved 99.4% of 3,330 single-locus microsatellite genotypes. Per-locus genotyping error rate, as estimated by Cervus from 34 mother-offspring pairs, was 0.0100. MICRO-CHECKER found no
254 evidence of stutter-based scoring error, large-allele dropout, or null alleles at any of the 15 loci. The average Oosterhout null allele frequency across all 15 loci was 0.00152. There was no significant
256 genotypic disequilibrium between any pair of microsatellite loci or between microsatellite loci and the MHC genes (all Bonferroni-corrected $p > 0.05$). For the full dataset of 222 birds (188 adults and
258 34 chicks) at 15 loci, N_A ranged from 3 to 40 alleles per locus (mean = 10.0, median = 6; Table S1), with mean H_E across loci of 0.668. In Bonferroni-corrected tests, F_{IS} was not significantly different
260 from zero for any locus. Overall, the microsatellite genotypes appeared suitable for paternity analysis and for estimating relatedness coefficients between mates.

262
Additional permutation tests of microsatellite data for inbreeding/outbreeding were conducted using
264 Moran's I as a relatedness estimator (Hardy & Vekemans 1999), and this produced equivalent results
to those described in the main text (data not shown).
266

Copy Number Variation

268 As detailed above, the evidence for the existence of copy number variation is somewhat mixed. Data
from repeat-genotyping and from parent-offspring analysis suggest that it is rare or absent, but data
270 from Illumina MiSeq catalogs suggest that CNV occurs in 1.4% of birds at Ocle-DAB1 and in 14.3% of
birds at Ocle-DAB2. Here we summarize the minor changes in MHC descriptive statistics when
272 genotype determinations are changed from a single-copy algorithm to a CNV-permissive algorithm.
In our sample of 188 adults and 22 offspring, the average number of alleles per bird at Ocle-DAB1 and
274 Ocle-DAB2 combined changes from 3.33 ± 0.71 to 3.56 ± 0.92 with the inclusion of putative CNV.
Divergence of alleles within individual birds was essentially unaffected: the average difference
276 between each of the unique alleles in an individual changed from 15.0 ± 1.28 to 14.8 ± 1.37 amino
acid differences in the 89 codons of exon 2. Lastly, MHC similarity between mates changed little
278 when including putative CNV alleles: mean allele sharing changed from 41.3% to 41.6%, and the
average number of amino acid differences changed from 12.1 ± 1.59 (range 7.5 to 17.1) to 12.3 ± 1.52
280 (range 7.5 to 17.3). Overall, using a genotyping protocol that accommodates putative CNV has little
impact on MHC diversity and similarity in our population.
282

We also tested for MHC-disassortative mating using these genotypes that allowed for copy number
284 variation. This approach to determining genotypes necessarily resulted in different birds having
different numbers of alleles, and an inability to determine the number of copies of each allele.
286 Therefore we collapsed each bird's genotype to a simple list, in this case yielding one to four unique
alleles per bird at each of Ocle-DAB1 and Ocle-DAB2 and a total of 2 to 6 alleles per bird. We then
288 conducted mate choice randomization tests based on four different metrics of MHC divergence. In
these analyses, there was no evidence for non-random mating with respect to mean MHC
290 divergence, and the trends for variance were in the wrong direction (i.e., towards more variance from
one mated pair to another, rather than towards all mated pairs exhibiting a similar level of male-
292 female MHC dissimilarity). This was true of (1) allele sharing ($p = 0.836$ for mean, $p = 0.768$ for
variance), (2) p-distance at all 89 codons of exon 2 ($p = 0.778$ for mean, $p = 0.012$ for variance), (3) p-
294 distance at the 22 codons showing evidence of positive selection ($p = 0.694$ for mean, $p = 0.004$ for
variance), and (4) functional distance between alleles using all codons of exon 2 ($p = 0.922$ for mean,
296 $p = 0.026$ for variance). The continued lack of evidence for disassortative mating is consistent with
the observation that the measures of MHC similarity for the 8,836 possible male-female
298 combinations were highly correlated between the data that were based on single-locus genotype
calls and data based on allele lists that included the putative CNV alleles: allele sharing ($r = 0.9456$, p
300 < 0.0001), p-distance at all 89 codons ($r = 0.8955$, $p < 0.0001$), p-distance at positively selected codons
($r = 0.8992$, $p < 0.0001$), and functional distance ($r = 0.8781$, $p < 0.0001$). Overall, even if some
302 amount of copy number variation exists, its inclusion has no apparent impact on mate choice
patterns in our dataset.
304

Additional Analyses of MHC Mating Patterns

To confirm the results of the permutation tests of MHC-random versus MHC-disassortative mating, we used several supplementary analyses beyond those detailed in the main text of the manuscript. First, in our analysis of p-distances between amino acid sequences of mates' alleles, we used additional approaches to choose codons at which variation between alleles might be functionally important. In addition to using all 89 codons of exon 2 and only the 33 putative peptide binding sites as described in the main text, we also analyzed mate choice by examining (a) only the 19 sites that are most likely to be functionally polymorphic as determined by weak or no clustering on the Gonnet PAM 250 matrix (as calculated in Clustal Omega (Sievers *et al.* 2011)), or (b) only the 22 sites that show evidence of positive selection (Ka>Ks, as calculated with the Selecton server (Doron-Faigenboim *et al.* 2005; Stern *et al.* 2007). However, there was still no evidence of maximally or intermediately disassortative mating, either at the sites showing individual signature of positive selection ($p = 0.944$ for means, $p = 0.970$ for variances) or the sites at which allelic polymorphisms included marked differences in the physicochemical properties of the amino acids ($p = 0.992$ for means, $p = 0.933$ for variances).

Second, because the well characterized MHC Class II B of chickens has been shown to include a dominantly expressed major gene and a poorly expressed minor gene (Jacob *et al.* 2000), we also analyzed our two genes separately in case a less-expressed gene might experience less selection and thus create noise that would obscure a mate choice pattern at the more-expressed gene. We know that both genes in storm-petrels are expressed (Dearborn *et al.* 2015), but we do not have data on whether they are expressed equally. Nonetheless, evidence of random mating still held when looking at the two genes individually, based on allele sharing (Ocle-DAB1: $p = 0.874$ for means, $p = 0.238$ for variances; Ocle-DAB2: $p = 0.832$ for means, $p = 0.841$ for variances) or amino acid sequence divergence (Ocle-DAB1: $p = 0.832$ for means, $p = 0.953$ for variances; Ocle-DAB2: $p = 0.798$ for means, $p = 0.654$ for variances).

Third, we considered the possibility that birds can only detect an allele's presence, and not its number of copies, when assessing the MHC alleles of potential mates. To mimic this perspective, we collapsed each bird's genotype to a simple list of the two or three or four unique alleles of that individual's two genes. Note that this parallels the data obtained in studies that simultaneously amplify multiple loci with a single primer pair. This approach, too, led to a conclusion of random mating based on allele sharing ($p = 0.704$ for means, $p = 0.692$ for variances) or amino acid sequence divergence ($p = 0.634$ for means, $p = 0.988$ for variances).

Thus, all analyses of mating patterns showed random assortment with respect to MHC rather than maximally or intermediately disassortative mating.

SUPPLEMENTAL TABLES AND FIGURES

Table S1. Microsatellite variability for 188 adults and 34 offspring at 15 loci, computed with FSTAT 2.9.3.2 (Goudet 2002). To correct for multiple tests, *p*-values for F_{IS} tests should be compared against Bonferroni-adjusted alpha of 0.00333. Loci Ole03 – Ole25 are from (Bicknell *et al.* 2011), Oc63 – Oc87B are from (Sun *et al.* 2009), and TG04-004 is from (Dawson *et al.* 2010).

Locus	Motif	N birds	N_A	H_O	H_E	F_{IS}	*P* for $H_O > H_E$	*P* for $H_O < H_E$
Ole03	tetra-nucleotide	220	22	0.882	0.887	0.006	0.671	0.423
Ole05	penta-nucleotide	220	17	0.796	0.798	0.003	0.585	0.502
Ole07	di-nucleotide	221	3	0.471	0.446	-0.055	0.223	0.831
Ole13	di-nucleotide	221	3	0.348	0.352	0.009	0.608	0.476
Ole14	di-nucleotide	222	3	0.527	0.541	0.026	0.741	0.319
Ole17	di-nucleotide, compound	221	6	0.475	0.48	0.011	0.624	0.458
Ole18	di-nucleotide	221	5	0.72	0.724	0.006	0.591	0.475
Ole21	di-/mono-/tetra-nucleotide	221	40	0.946	0.944	-0.002	0.516	0.596
Ole22	di-nucleotide	221	3	0.353	0.343	-0.03	0.348	0.733
Ole23	di-nucleotide	222	13	0.865	0.872	0.008	0.66	0.42
Ole24	tetra-nucleotide	221	8	0.769	0.764	-0.007	0.481	0.586
Ole25	tetra-nucleotide, compound	221	7	0.787	0.808	0.026	0.804	0.241
Oc63	di-nucleotide	215	6	0.633	0.645	0.02	0.674	0.376
Oc87B	di-nucleotide	221	9	0.769	0.759	-0.014	0.361	0.703
TG04-004	di-nucleotide, compound	221	5	0.615	0.655	0.06	0.926	0.096

N_A = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity

Table S2. Clustal Omega (1.2.1) amino acid alignment of exon 2 alleles at Locus 1 and Locus 2, with allele frequencies from 188 adults. Alleles reported here for the first time – i.e. not found in (Dearborn *et al.* 2015) – are marked with + . Note that in both loci there are alleles with a 3-bp deletion at codon 73. Codons at putative peptide binding sites are shaded in gray.

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Ocle-DAB1              5    10   15   20   25   30   35   40   45   50   55   60   65   70   75   80   85
Allele   Freq      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
  004    0.431     FFQDMFKAECYFTNGTERVRLLARYIYNRQQDVHFDSDVGFFVADTPLGEPDAKYWNSQPDLLEDRRASVDTFCRHNYGVWTPFTVERR
  028    0.152     Y..E................F.D........RA........Y.........S.........I..RK......................K
  055    0.141     ...E................F.D........RA........Y...................I...E..A....................
  080    0.080     Y..E................F.D........F.........Y.........S.........I..RK......................K
  113    0.066     Y..E............K...F.D........RA........Y.........S.........I..RK......................K
  090    0.048     Y...................F.D........F.........Y...................I...E..A...................K
  079    0.043     Y..E...S.........Q..HVT...H....F.........Y.........I..D.....AI..QT..EM...................
  060    0.021     Y..E................F.D........RA........Y.........S.........I..RK......-...............K
  149    0.011     ...................................................I..D.....AI..QT..EM...................
+ 428    0.005     Y..E................F.D........RA........Y.........S.........I..RK..A...................K
+ 644    0.003     ...E................F.D........RA........Y...................I...E..A...................K
                   :**:***:********::** : ***:**** .********:********* ** ***** :**  ** :** ***************:

Ocle-DAB2              5    10   15   20   25   30   35   40   45   50   55   60   65   70   75   80   85
Allele   Freq      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
  0050   0.569     FFQWIGKAECQYLNGTERVRLLVRYIHNRQQFVHFDSDVGFYVADTPLGEPDAKYWNSQPDLLEQRRAEVDTYCRHNYGVSTPFIVERR
  0054   0.125     V..RMF................A.S.Y..............................................................
  0131   0.096     V..RMF..............Y.A.S.Y........................I..D......I......A...........V...T....
  0074   0.061     ...EMF................A.S.Y........................I..D.............S...-...............K
  0176   0.048     V..EM.....H.F.........A.S.Y........................I..D......I......A...........V...T....
  0539   0.035     .........................................F...................I...K......-.......A...T....
  0046   0.019     V..............................D.........F.........I..D......I..................V...T....
  0193   0.016     V..RMF................A.S.Y........................I..D......I......A...........A...T....
  1158   0.016     V..EM.....H.F.......F.D...Y....D.............................I...K......-.......A...T....
  0249   0.008     ...EMF................A.S.Y..............................................................
  0791   0.003     ...EMF................A.S.Y........................I..D..................................
  1553   0.003     ....................Y.E............................I..D......I..................V...T....
+ 0132   0.003     V..EM..S..H.F.......F.D...Y....RA........F...................I..DE..A...F.......V...T....
                   .** : *:**:*:******* * * *:**** .********:********* ** ******:**:.** *** ******* *** ***:
```

* = fully conserved residue at that locus

: = conservation between groups of strongly similar properties, scoring > 0.5 in Gonnet PAM 250 matrix

. = conservation between groups of weakly similar properties, scoring ≤ 0.5 in Gonnet PAM 250 matrix.

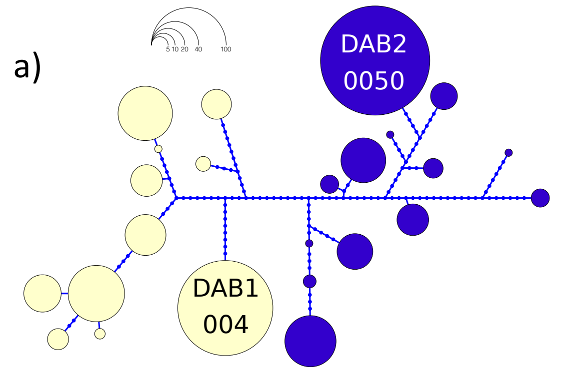
Figure S1. Illustration of the phylogeny permutation model. Yellow = Ocle-DAB1 alleles; dark blue = Ocle-DAB2 alleles.

a) Original DNA network.

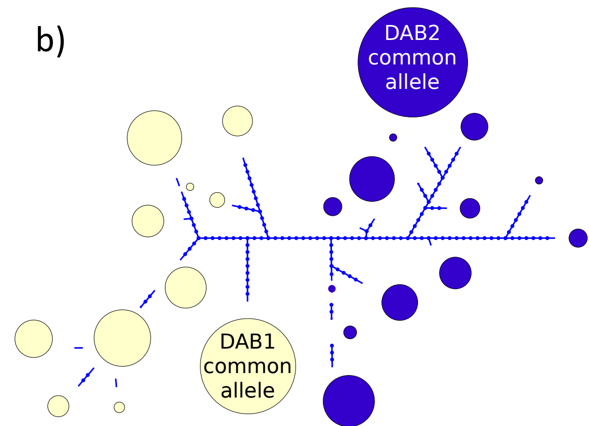
b) Alleles (and their associated frequencies) detached from original locations and ready for permutation.

c) Four of the 6.204 x 10^{23} possible permutations of the 24 alleles within the phylogeny. The four examples illustrate low versus high values of monophyly and small versus large distance between the two most common alleles of Ocle-DAB1 and Ocle-DAB2.

a)



b)

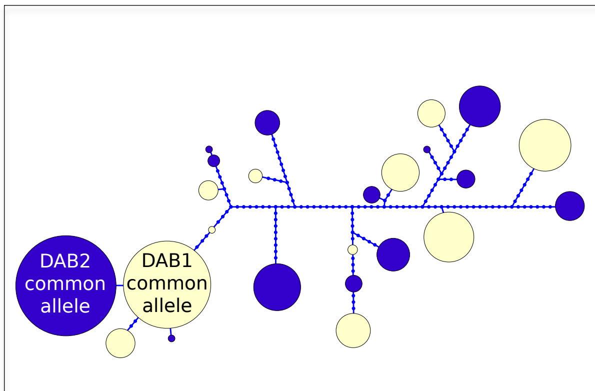


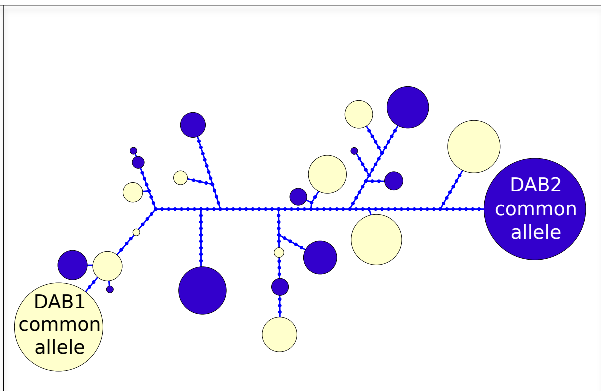
c)

Small **Distance**
Between Common Alleles

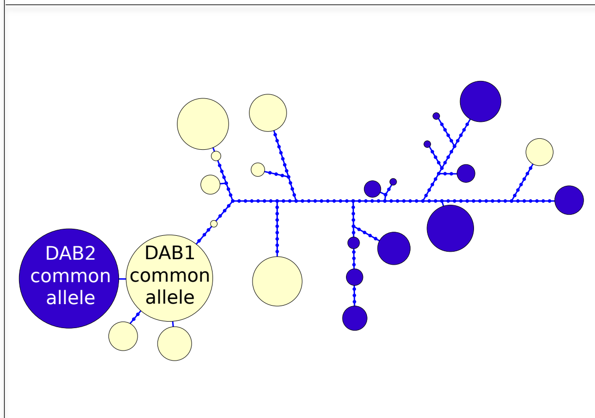
Large **Distance**
Between Common Alleles

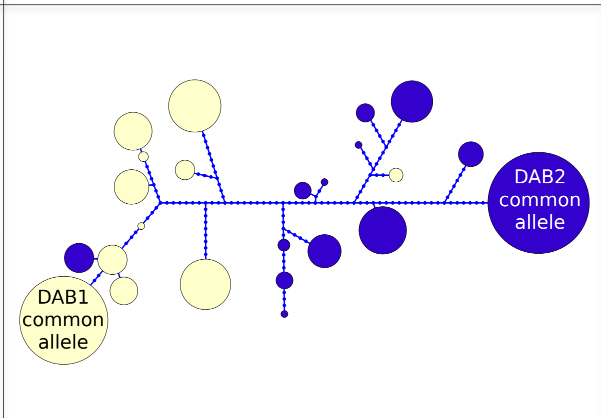
Low Monophyly





High Monophyly





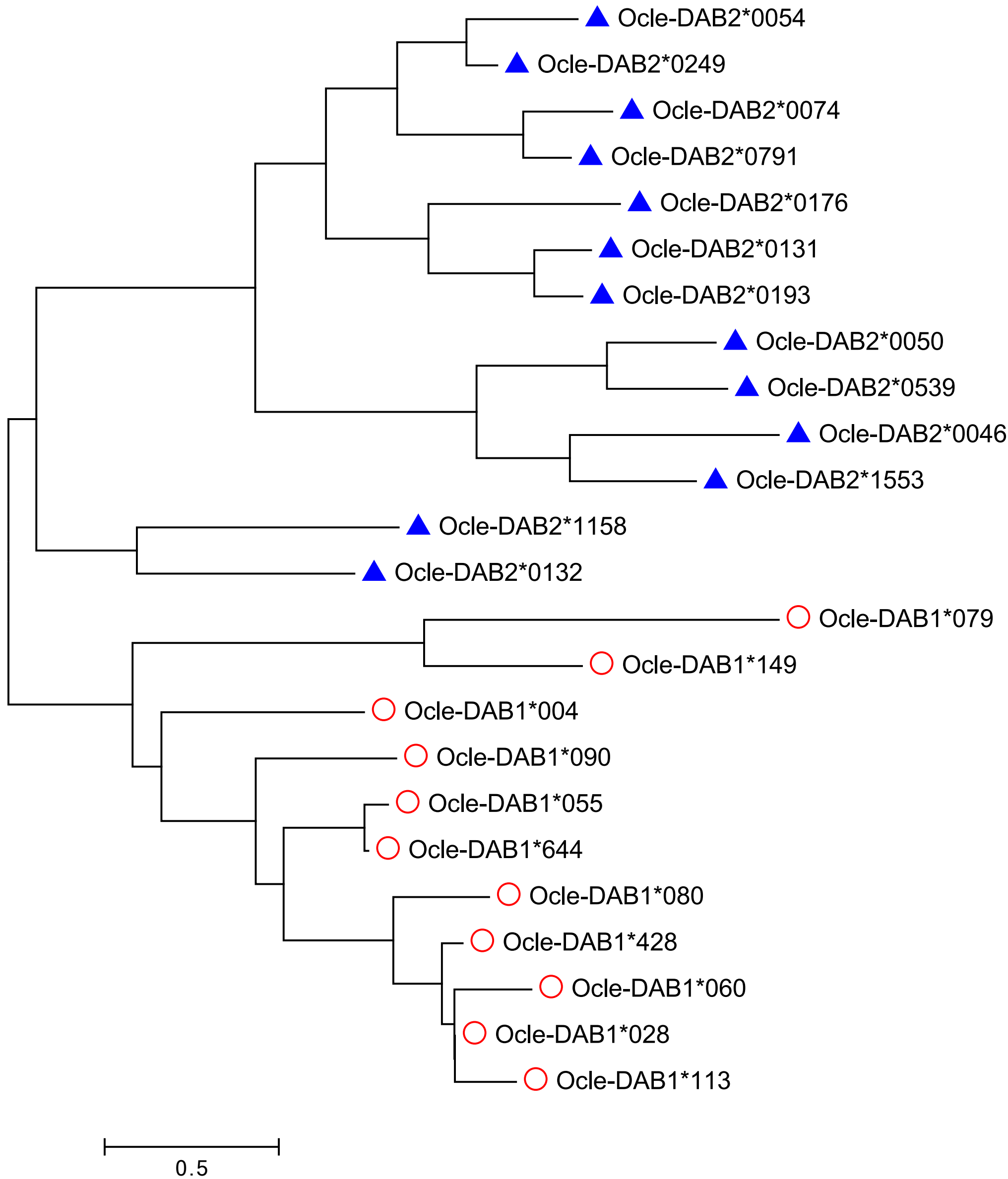


Figure S2. Monophyly based on functional distance between alleles. Neighbor Joining tree was made from a distance matrix of functional divergence between exon 2 alleles, based on physicochemical properties of amino acid polymorphisms. Alleles are marked with open circles for Ocle-DAB1 and solid triangles for Ocle-DAB2.

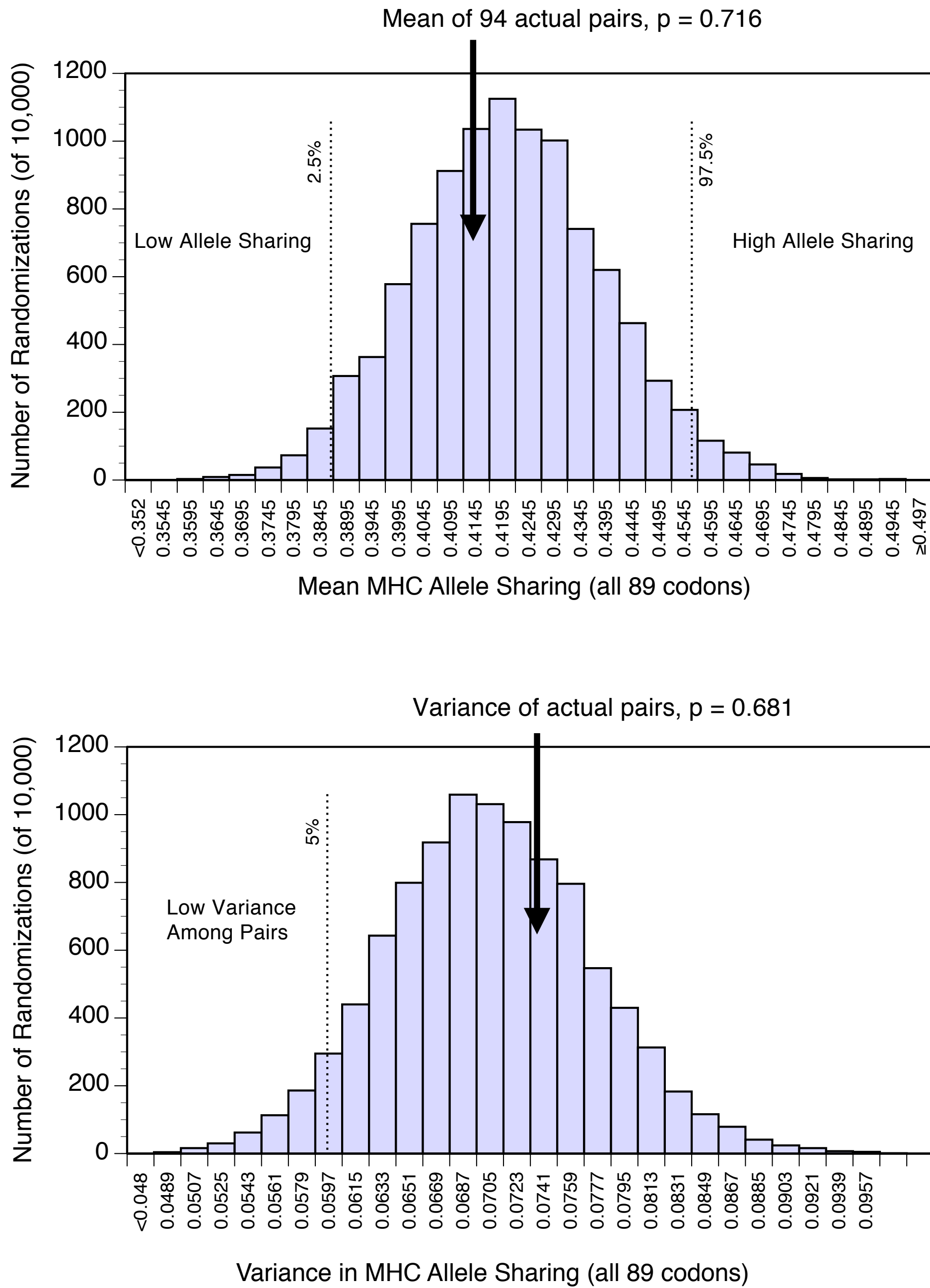


Figure S3. Allele sharing between actual mates (n=94 pairs) and randomized mates at two MHC Class II B loci, using all 89 codons of exon 2. Distribution from 10,000 permutations is shown in shaded bars; value from actual pairs is shown with arrow.
(a) Mean of mated pairs.
(b) Variance among mated pairs.

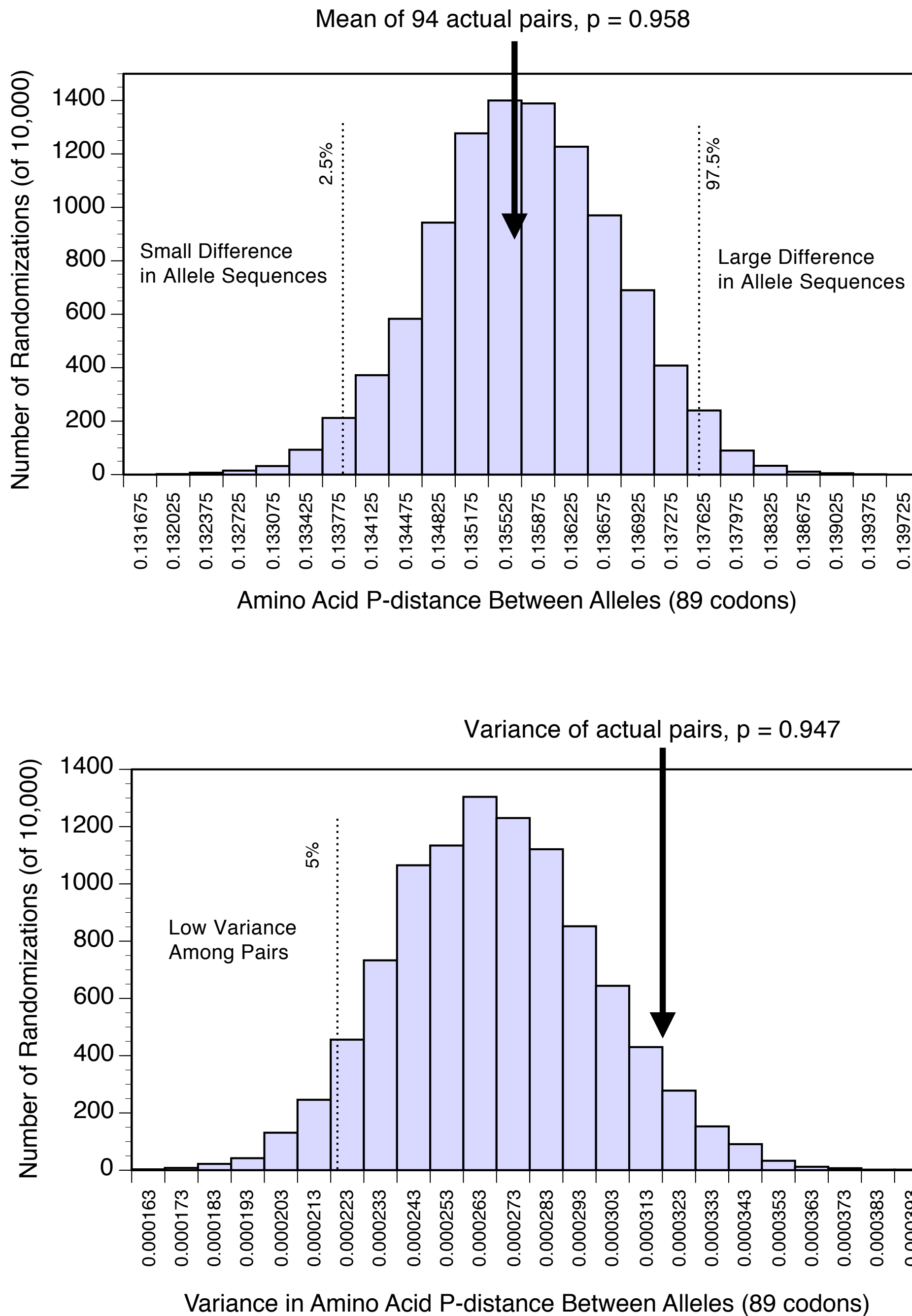


Figure S4. Amino acid sequence p-distances between pairwise comparisons of mates' alleles from two MHC Class II B genes, using all 89 codons of exon 2 from 94 mated pairs. Distribution from 10,000 permutations is shown in shaded bars; value from actual pairs is shown with arrow.
(a) Mean of mated pairs.
(b) Variance among mated pairs.

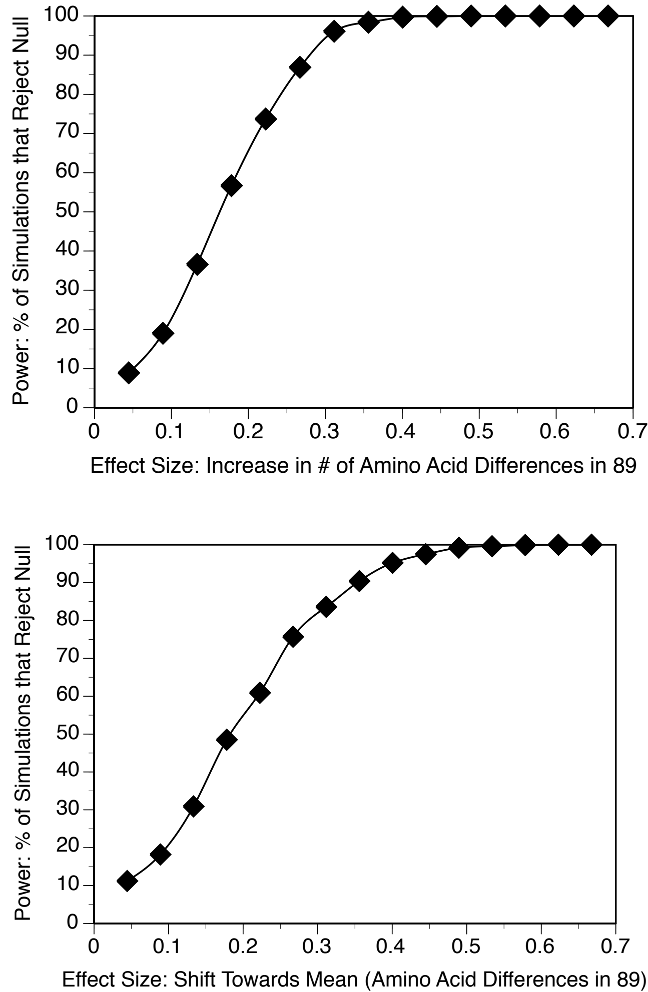


Figure S5. Power analysis of randomization tests of MHC-disassortative mating preferences.
(a) Power to detect different degrees of maximally disassortative mating, where effect size is the amount of disassortative shift applied to randomly assigned pairings of each female with a male. The shift towards disassortative preference is measured as the increase in average number of amino acid differences between mates' 89-codon exon 2 sequences, relative to strictly random mating.
(b) Power to detect different degrees of intermediately disassortative mating, where effect size is the average number of amino acids (in 89 codons) by which MHC divergence of randomly assigned pairs is shifted inward towards the overall mean of random matings.

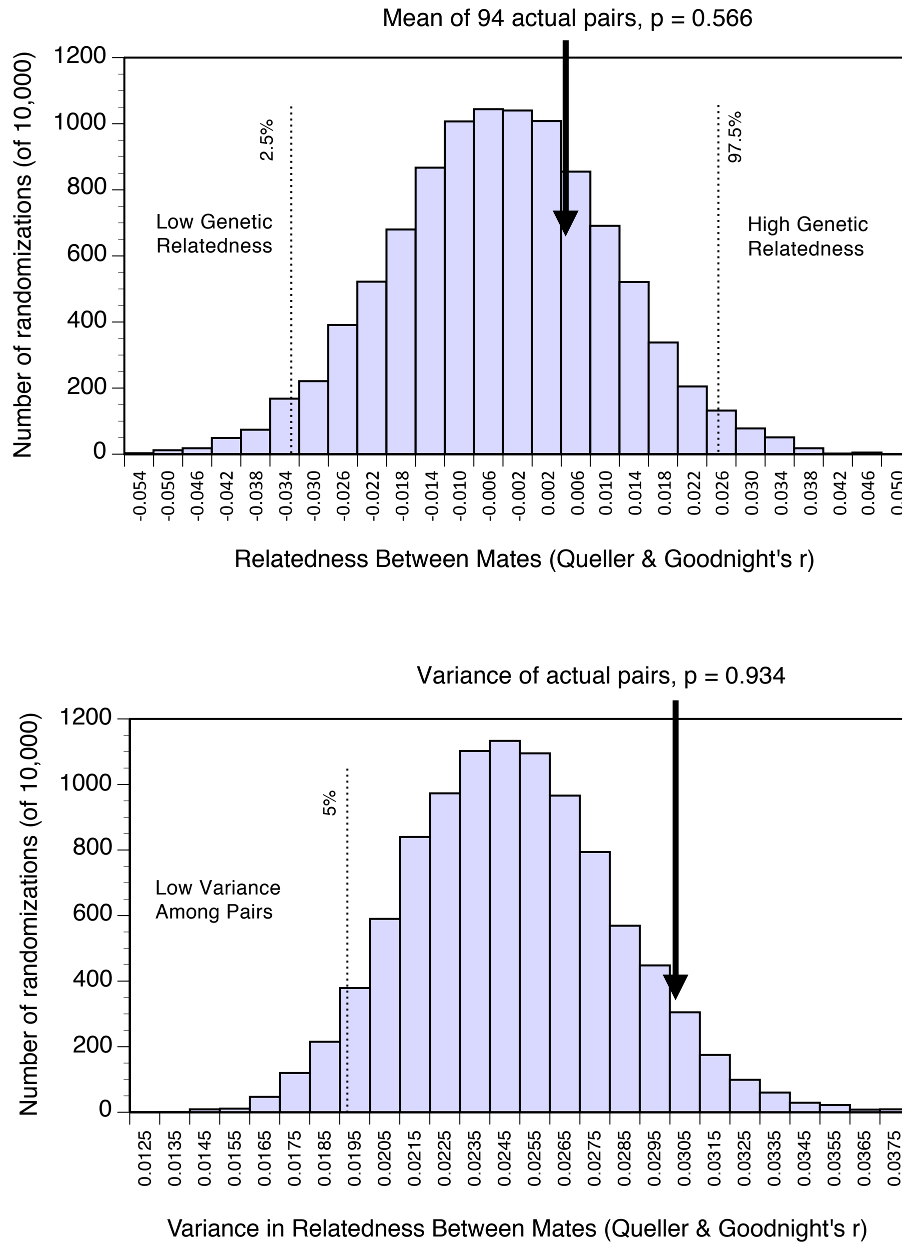


Figure S6. Absence of inbreeding or outbreeding, based on Queller and Goodnight's relatedness coefficient between mates calculated from 15 microsatellite loci. Distribution from 10,000 permutations is shown in shaded bars; value from actual mates (n=94 pairs) is shown with arrow.
(a) Mean of mated pairs.
(b) Variance among mated pairs.
Similar results were obtained using Moran's I as a relatedness estimator.

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