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Hybridization following recent secondary contact results in asymmetric genotypic and phenotypic introgression between island species of *Myzomela* honeyeaters

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Hybridization and introgression can have important evolutionary consequences for speciation, especially during early stages of secondary contact when reproductive barriers may be weak. Few studies, however, have quantified dynamics of hybridization and introgression in systems in which recent natural dispersal across a geographic barrier resulted in secondary contact. We investigated patterns of hybridization and introgression between two *Myzomela* honeyeaters (*M. tristrami* and *M. cardinalis*) that recently achieved secondary contact on Makira in the Solomon Islands. Hybridization in this system was hypothesized to be a byproduct of conspecific mate scarcity during early stages of colonization. Our research, however, provides evidence of ongoing hybridization more than a century after secondary contact. Mitochondrial sequencing revealed strongly asymmetric reproductive isolation that is most likely driven by postzygotic incompatibilities rather than prezygotic behavioral barriers. Nuclear introgression was observed from the native species (*M. tristrami*) to the colonizing species (*M. cardinalis*). Nuclear introgression in the reverse direction is almost exclusively limited to birds that are phenotypically *M. tristrami* but possess *M. cardinalis* mitochondrial haplotypes, consistent with introgression of plumage-related alleles into the genomic background of *M. cardinalis*. These results provide unique insight into the dynamics and consequences of hybridization and introgression during early stages of secondary contact.

KEY WORDS: **Hybridization, introgression, meliphagidae, reproductive isolation, speciation.**

Under the predominant mode of speciation, phenotypic divergence between species first evolves in allopatry (Mayr 1942; Coyne and Price 2000; Coyne and Orr 2004). However, unless such divergence drives either complete pre- or postzygotic reproductive isolation, any secondary contact will be followed by hybridization (Harrison 1993; Coyne and Orr 2004; Price 2008). Such hybridization, which is relatively common in nature (Mallet 2005), has important consequences for speciation (Anderson 1949; Harrison 1993; Dowling and Secor 1997; Seehausen 2004; Abbott et al. 2013). Hybridization can potentially break down reproductive barriers (Rhymer and Simberloff 1996; Taylor et al. 2006), promote the evolution of reproductive barriers via reinforcement (Dobzhansky 1940; Ortiz-Barrientos et al. 2009), increase fitness of the hybridizing taxa (i.e., adaptive introgression) (Anderson and Stebbins 1954; Arnold 2004; Seehausen 2004; Heliconius Genome Consortium 2012; Hedrick 2013), or increase biodiversity via homoploid hybrid speciation (DeMarais et al. 1992; Gompert et al. 2006; Brelsford et al. 2011; Hermansen et al. 2011). In this manuscript, we investigate the patterns and potential evolutionary consequences of hybridization and introgression between two species of island birds (*Myzomela cardinalis* and *M. tristrami*) that have recently achieved secondary contact.

Consequences of hybridization for speciation may be particularly strong during the early stages of secondary contact, before selection against hybridization can drive the evolution of strong prezygotic reproductive barriers, and when conspecific mating opportunities are often relatively rare for one species due to unequal dispersal (Wilson and Hedrick 1982; Wirtz 1999). This secondary

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contact generally follows one of two geographic patterns. In the first, changes in climate or other ecological constraints weaken geographic barriers and promote species range expansions that result in secondary contact. When reproductive isolation is incomplete, this secondary contact often results in long-lasting hybrid zones between parapatric species (Harrison 1993; Arnold 1997). In the second pattern, secondary contact results when geographic barriers (e.g., oceans, rivers, mountain ranges) are maintained but do not completely eliminate gene flow resulting from long-distance dispersal (Gill 1970; Grant et al. 1996).

The outcomes of secondary contact resulting from long-distance dispersal across geographic barriers remain relatively understudied, most likely because successful colonization events are rarely observed. Moreover, limited dispersal into the area of sympatry will often cause the consequences of secondary contact events to resolve quickly, both ecologically (e.g., via local extinction or rapid evolution of reproductive isolation) (MacArthur and Wilson 1967; Gill 1970; Mayr and Diamond 2001; Grant and Grant 2014) and on a genomic level (e.g., loss of low-frequency introgressed alleles due to genetic drift). Consequently, most direct knowledge regarding the evolutionary consequences of hybridization following long-distance dispersal has been gleaned from systems where secondary contact resulted from anthropogenic introductions (Rhymer and Simberloff 1996; Mooney and Cleland 2001; Fitzpatrick et al. 2010), which often differ fundamentally from typical natural colonizations (Strauss et al. 2006). A handful of examples of recent secondary contact resulting from natural long-distance dispersal have been identified, primarily in island taxa, including Galapagos finches (Grant and Grant 2009), New Zealand stilts (Steeves et al. 2010), *Ficedula* flycatchers (Qvarnström et al. 2010), and Norfolk Island white-eyes (Gill 1970). Furthermore, several genetic studies have identified historical episodes of gene flow that followed secondary contact between island species (Caraway et al. 2001; Shaw 2002; Cianchi et al. 2003; Larsen et al. 2010; Nunes et al. 2010; Lamichhaney et al. 2015; Lavretsky et al. 2015), suggesting that secondary contact via long-distance dispersal has had important consequences for speciation. Additional studies of secondary contact events on islands will therefore offer further insights into the potential outcomes of this important stage of the speciation process.

The two species of *Myzomela* honeyeaters on Makira in the Solomon Islands are well suited for studying the evolutionary consequences of hybridization following secondary contact. These small, primarily nectivorous birds are members of one of the most species-rich genera of songbirds (Higgins et al. 2008). The all-black, sexually monochromatic Sooty *Myzomela* (*M. tristrami*) (Fig. 1A) is endemic to all elevations and habitats on Makira, as well as to the satellite islands of Santa Catalina and Santa Ana approximately 8 km east of Makira (Fig. 1B). An endemic subspecies of the red-and-black, sexually dichromatic Cardinal *Myzomela* (*M. cardinalis pulcherrima*) (Fig. 1A) is found in coastal and lowland regions of Makira, as well as on the satellite islands of Ugi and Three Sisters approximately 8 km and 20 km north of Makira, respectively (Fig. 1B) (Diamond 2002; Dutson 2011). The two species are sympatric along the coast of Makira and fill similar ecological niches, often feeding from the same flowers and behaving aggressively toward each other (Diamond 2002; J. Sardell, pers. obs.).

Secondary contact between the Makira *Myzomela* is recent, with *M. cardinalis* successfully colonizing Makira from Ugi and/or Three Sisters during the early 20th century (Mayr and Diamond 2001; Diamond 2002). Ornithologists collected only *M. tristrami* during several trips to Makira in the 19th century (Ramsay 1883). Two of the eight birds that A.S. Meek (Rothschild and Hartert 1908) collected from west Makira in 1908 were later identified by Diamond (2002) as phenotypically intermediate individuals representing putative hybrids, suggesting that *M. cardinalis* likely had already colonized Makira by the time of Meek's visit. Ernst Mayr and the Whitney South Seas Expedition (Mayr 1945) collected both putative hybrids and *M. cardinalis* from Makira in 1927, although *M. tristrami* was more common in sympatry (Diamond 2002). Finally, Cain and Galbraith (1956) in 1953 and Diamond in the 1970s did not detect any putative hybrids, and found that *M. cardinalis* outnumbered *M. tristrami* in sympatry (Diamond 2002). Based on these collection records, Mayr and Diamond (2001) hypothesized that hybridization in this system only occurs when one species is greatly outnumbered by the other, such as during the earliest stages of secondary contact and along the forefront of *M. cardinalis*'s range expansion around the coast of Makira. In these situations, local scarcity of conspecific mating opportunities among the colonizing species may lead to a relaxation of prezygotic isolation (Mayr and Diamond 2001; Diamond 2002; Price 2008), as predicted by models of female mate choice (Wilson and Hedrick 1982; Real 1990; Wirtz 1999). This phenomenon wherein hybridization is most prominent during early stages of secondary contact has been observed in several other systems (Price 2008), including Galapagos finches (Grant and Grant 2014), white-eyes (Gill 1970), woodpeckers (Short 1969), tits (Vaurie 1957), and butterflies (Cianchi et al. 2003). However, because previous expeditions did not explicitly search for hybrid *Myzomela* on Makira, ongoing hybridization in this system may be more common than currently assumed.

We here present the first investigation of the genetic patterns of hybridization and introgression and their potential consequences for speciation in the Makira *Myzomela*. If introgression is persistent and symmetric, then it may eventually lead to the formation of a single panmictic hybrid swarm (as in Larsen et al. 2010; Lavretsky et al. 2015). Alternatively, asymmetric

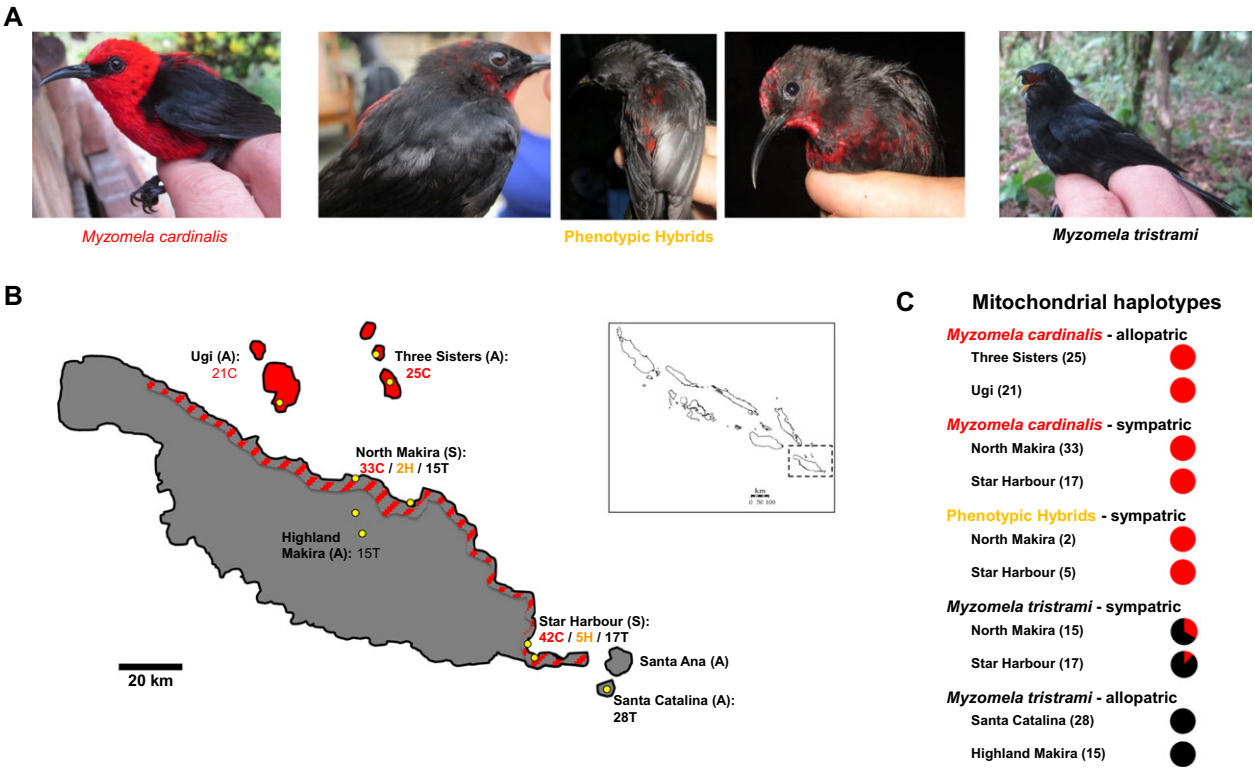

Figure 1. **(A) Representative examples of plumage for, from left to right: adult male *M. cardinalis*, three putative hybrids from the area of sympatry based on intermediate plumage phenotypes, and adult male *M. tristrami*. (B) Map of Makira and satellite islands showing approximate range of: *M. cardinalis* encompassing Ugi, Three Sisters, and coastal Makira; and *M. tristrami* encompassing Makira, Santa Ana, and Santa Catalina. Sampling localities are shown with sample sizes indicated ("C" = *M. cardinalis*, "H" = phenotypic hybrid, "T" = *M. tristrami* based on plumage phenotypes). Allopatric and sympatric populations are denoted by "A" and "S," respectively. Inset in upper right denotes location of Makira within the Solomon Islands. (C) Proportion of individuals in each population possessing mitochondrial *ND2* haplotypes belonging to two highly diverged clades (see Fig. S2 for associated haplotype network). All *M. cardinalis* and all phenotypic hybrids possess "red" haplotypes. All allopatric *M. tristrami* possess "black haplotypes." Sympatric *M. tristrami* populations possess both haplotypes, indicative of mtDNA introgression. Numbers after population names represent number of individuals sampled from that population.**

introgression from *M. tristrami* to *M. cardinalis* is predicted under Mayr and Diamond's (2001) hypothesis that hybridization in this system is driven by reduced choosiness (i.e., propensity to hybridize) among female *M. cardinalis* during the early stages of colonization. Asymmetric introgression can also result from asymmetric hybrid viability (Bolnick et al. 2008; Ellison and Burton 2008; Ellison et al. 2008; Werren et al. 2010; Trier et al. 2014), fixed differences in mate choice preferences between populations (Kaneshiro 1976, 1980), or a passive "wave-front" process driven by relative abundance, demography, and dispersal (Currat et al. 2008; Excoffier et al. 2009; Steeves et al. 2010; Rheindt and Edwards 2011). The direction and degree of any asymmetric introgression are important because they influence the potential for adaptive alleles to introgress between species, a mechanism that has been proposed to be an important potential source of genetic variation, particularly in small isolated populations (Anderson and Stebbins 1954; Arnold 2004; Seehausen 2004; Grant et al. 2005; Hedrick 2013). Accordingly, determining the magnitude and direction of introgression in the Makira *Myzomela* will offer important insight into the potential for hybridization to promote adaptation in this system, as well as providing another example of the potential outcomes of hybridization during the early stages of secondary contact resulting from long-distance dispersal.

Methods

SAMPLING

We conducted fieldwork at six sites in Makira-Ulawa province, Solomon Islands between May and July in 2012 and 2013. Two sites, Ugi and Three Sisters, contained allopatric populations of *M. cardinalis*; two sites, Santa Catalina and highland Makira,

contained allopatric populations of *M. tristrami*; and two sites, North Makira and Star Harbour, contained populations of both species, with the former site representing an older area of sympatry relative to the latter (Fig. 1B). At each site, we erected mist nets in areas where *Myzomela* were observed, often at flowering trees, and collected a blood sample from the brachial vein of each captured individual. Additionally, we collected blood samples from chicks from four observed nests; each nest contained two chicks, and to avoid potential impacts of relatedness bias, our analysis includes genetic sequence data for only one randomly selected chick from each pair. No adults were captured in the vicinity of any of these nests, so it is unlikely that the parents are included in our dataset. Finally, six samples of *M. cardinalis* and one sample of *M. tristrami* were taken from birds that were incidentally captured in 2008 while mist netting for an unrelated study at the same localities. In total, samples comprise 121 *M. c. pulcherrima*, 75 *M. tristrami*, and seven individuals with intermediate plumage. Additionally, during 2012, we collected three blood samples of another *M. cardinalis* subspecies, *M. c. sanctaecrucis*, from Santa Cruz (a.k.a. Nendo) in the Solomon Island province of Temotu, located approximately 370 km east of Makira.

Collected blood was preserved in lysis buffer (Longmire et al. 1997) and stored at −20°C upon return from the field. Genomic DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen) and DNA concentrations were measured using a Qubit 2.0 fluorometer (Life Technologies).

Birds were assigned to species based on plumage phenotypes observed in the field and recorded in photos (Fig. 1A). Adult *M. tristrami* are entirely black, whereas juveniles are black above and gray below with yellow bills. Male *M. cardinalis* possess bright red heads, breasts, and backs, whereas females are slightly duller with more black on the underparts. Juvenile *M. cardinalis* are highly variable in terms of plumage, ranging from duller versions of male plumage patterns to predominantly gray and olive with patches of red-tinged feathers, but can be distinguished by their yellow nares. Birds possessing abnormal combinations of red and black plumage (e.g., a black body and red back or a black head with patches of red feathers) were classified as phenotypic hybrids (Fig. 1A).

SEQUENCING

We obtained sequence data for one mitochondrial gene and six nuclear markers from each sampled individual. For mitochondrial DNA ("mtDNA"), we used PCR to amplify 1016 bp of NADH dehydrogenase subunit 2 (*ND2*) using the primers H6313 and L5216 and an annealing temperature of 57°C (Sorenson et al. 1999). For nuclear DNA, we amplified the following markers using the primers and protocols cited: Beta-fibrinogen intron 5 (*FGB*), 411 bp (Kimball et al. 2009); myoglobin intron 2 (*MYO*), 647 bp (Slade et al. 1993; Heslewood et al. 1998); rhodopsin intron 1 (*RHO*), 702 bp (Primmer et al. 2002); transforming growth factor β-2 intron 5 (*TGF*β*2*), 523 bp (Primmer et al. 2002); glyceraldehyde 3-phosphate dehydrogenase intron 11 (*GAPDH*), 273 bp (Primmer et al. 2002); and *15246*, 430 bp (Backström et al. 2008). Successful amplification was confirmed by electrophoresing 3 μl of PCR product in a 1% agarose gel. PCR products were prepared for sequencing using manufacturer's protocols for ExoSAP-IT purification (USB Corp.), BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems), and Sephadex purification (Sigma-Aldrich). Sanger sequencing was performed at the Molecular Core Facility of the University of Miami Department of Biology.

Forward and reverse sequence reads were aligned in Sequencher version 4.8 (Gene Codes, Ann Arbor, MI), and all chromatograms were reviewed to visually identify heterozygous sites and errors in base calling. Allelic haplotypes for the nuclear markers were inferred using the PHASE version 2.1 algorithm (Stephens et al. 2001) from DnaSP version 5.10.1 (Librado and Rozas 2009), with the presence of heterozygous indels in certain sequences allowing for the visual determination of specific haplotype sequences from chromatogram data. For most nuclear markers, PHASE was able to assign haplotypes with greater than 95% confidence to more than 98% of all individuals, with nearly all low-confidence assignments representing singleton polymorphisms. However, due to high levels of polymorphism and recombination, PHASE results for *TGF*β*2* were characterized by high degrees of uncertainty. Accordingly, we used custom-designed allele-specific primers (Pettersson et al. 2003) to identify allelic phases for nearly all individuals with ambiguous haplotypes, resulting in the same level of confidence as for the other markers. Haplotype networks for each marker were constructed in TCS (Clement et al. 2000).

ESTIMATION OF DIVERGENCE TIMES

Time in allopatry is believed to be important for the evolution of reproductive isolation (Coyne and Orr 2004; Price 2008), so we conducted a phylogenetic analysis to estimate the age of the most recent common ancestor of *M. tristrami* and *M. cardinalis*, as well as the date that the two species colonized Makira and its offshore satellite islands, respectively. We constructed a mitochondrial gene tree for 820 bp of *ND2* using sequences of two randomly selected individuals from each of our two allopatric study populations of *M. tristrami* and *M. c. pulcherrima*, three samples of *M. c. sanctaecrucis*, and sequences for other species available on GenBank (Driskell and Christidis 2004; Smith and Filardi 2007; Gardner et al. 2010; Toon et al. 2010; Nyári and Joseph 2013; Andersen et al. 2014). In total, this dataset includes 36 sequences (Table S1), representing 13 of the 31 currently recognized species of *Myzomela* (including all Solomon Island taxa), at least three subspecies of *M. cardinalis* (the sampling location

of one sequence available on GenBank is not provided in the original study), and the known sister species to this genus, *Sugomel nigrum* (Joseph et al. 2014).

Using MrModeltest (Nylander 2004), we identified GTR + Γ + I as the most appropriate substitution model for Bayesian phylogenetic analyses, based on Akaike Information Criterion (AIC). We then used BEAST version 1.5.4 (Drummond and Rambaut 2007) to perform a Bayesian Markov chain Monte Carlo analysis to construct the phylogeny and estimate divergence times for the taxa of interest. To obtain a substitution rate prior, we adjusted the molecular substitution rate for avian cytochrome B (*CytB*) (2.1% per million years) (Weir and Schluter 2008) to reflect the relative rate of molecular evolution for *ND2* and *CytB* sequences in the five species of *Myzomela* (*M. cardinalis, M. erythrocephala*, *M. obscura*, *M. rosenbergii*, and *M. sanguinolenta*) for which sequences of both mtDNA genes from the same individual were available on GenBank (Driskell and Christidis 2004). The mean pairwise differences per site for *ND2* between these species is 1.3× that of *CytB* (range: 0.95×–1.56×), resulting in an estimated substitution rate of 2.7% per million years for *ND2*. Analyses were run for 10 million generations applying a Yule tree prior under a relaxed tree framework, sampling every 100 iterations, for a total of 100,000 trees. Adequate convergence of the posterior was confirmed using TRACER version 1.6. After applying a 20% burn-in, the consensus majority-rule tree was constructed in TreeAnnotator version 1.5.4 and used to estimate the mean and 95% confidence interval divergence times for *M. tristrami* and the clade containing *M. cardinalis*, as well as the Makira and Santa Cruz subspecies of *M. cardinalis*.

CONFIRMATION AND QUANTIFICATION OF HYBRIDIZATION AND INTROGRESSION

Natural selection and genetic drift will facilitate evolution of population-specific alleles, particularly between small isolated populations such as those on oceanic islands that have also undergone one or more founder events associated with island colonization prior to secondary contact (Hedrick 2011). We therefore identified species-specific mitochondrial and nuclear haplotypes as those that are exclusively present in allopatric populations of one species. Presence of such species-specific alleles in sympatric heterospecifics is considered to be evidence for introgression. This approach does not account for the possibility of introgression of alleles into allopatric populations, conservatively assuming instead that sharing of any alleles in allopatry is due to incomplete lineage sorting rather than past or present gene flow. Moreover, robust sample sizes are important for this approach, as false identification of introgression may occur if alleles that are shared by both species due to incomplete lineage sorting go undetected in allopatric populations of one species. To estimate the potential effect of sample size on our analysis, we used a maximum-likelihood approach to calculate the 95% likelihood-based confidence limit for the true frequency of an allele that was not detected among our allelic sample size (e.g., twice the number of sampled individuals) in each population (Whitlock and Schluter 2015).

Extensive sharing of nuclear haplotypes may result in a failure to detect actual hybrids using our approach. To test our ability to identify hybrids and backcrosses accurately, we used a custom script developed in R (R Core Team 2014) that simulates the outcomes of hybrid pairings based on actual genotypes of allopatric birds. For each simulated hybridization event, alleles for the six nuclear markers were chosen at random from randomly selected parental genotypes. Simulated offspring were then assigned to the following categories based on the presence of species-specific alleles: (1) hybrid if possessing at least one *M. tristrami* specific and at least one *M. cardinalis* specific allele; (2) *M. tristrami* if possessing at least one *M. tristrami* specific allele but no *M. cardinalis* specific alleles; (3) *M. cardinalis* if possessing at least one *M. cardinalis* specific allele but no *M. tristrami* specific alleles; and (4) ambiguous if possessing no species-specific alleles. A total of 100,000 F_1 and F_2 hybrid simulations were performed for each of the four possible hybrid pairings of the sampled allopatric populations. A similar approach was then used to backcross each simulated hybrid with randomly chosen individuals from each of the two allopatric parental populations. Simulated backcrosses were assigned to populations based on presence and absence of species-specific alleles as described above.

We used DnaSP to calculate pairwise F_{ST} and Φ_{ST} values between populations for each marker to test for evidence that heterospecific populations are more similar in sympatry than allopatry, as expected under introgression in sympatry. Mean F_{ST} values across all six nuclear markers were computed for each pairwise comparison, and a two-tailed paired *t*-test with Bonferroni correction was used to test for significant differences between cross-species F_{ST} values for allopatric populations versus populations from within the area of sympatry. Finally, we used STRUCTURE version 2.3.4 (Pritchard et al. 2000) to perform a Bayesian population assignment analysis, based on haplotype data, a model run of 1.5 million iterations, a burn-in of 200,000, and $k = 2$ *a priori* populations.

Results

SAMPLING AND SPECIES DISTRIBUTIONS

Myzomela tristrami and *M. cardinalis* continue to coexist on coastal Makira. *Myzomela cardinalis* represented 64% of total captures (30/47) in North Makira and 50% (13/26) of total captures in Star Harbour at sites where both species were observed multiple times. The presence of *M. cardinalis* at Star Harbour indicates that the area of sympatry has expanded recently into

the eastern portion of Makira, as the species was absent from Star Harbour when Diamond visited during the 1970s (Diamond 2002). The current relative species abundances in sympatry are similar to those observed over the past 60 years (Diamond 2002). In most habitats immediately adjacent to the sea (e.g., coastal mangroves and coconuts, beach vegetation), *M. cardinalis* was abundant (i.e., several individuals observed during every visit) but *M. tristrami* was rare (i.e., observed during multiple visits, but never captured during netting and representing less than 5% of all *Myzomela* detected). These areas were not large (approximately 10–200 m wide), and *M. tristrami* was abundant in similar seaside habitats on Santa Catalina where *M. cardinalis* was absent. *Myzomela cardinalis* was never observed in primary rainforest or isolated secondary habitats (e.g., gardens and villages) in interior Makira, but is common (i.e., reliably observed during every visit) in secondary habitats at 255 m in elevation behind the town of Kirakira (North Makira) more than 1.3 km from the coast, and was observed more than 3.5 km from the coast in contiguous secondary habitat along the Ravo River. However, a more robust assessment of habitat preferences is needed to quantify whether the species indeed sort ecologically.

Seven putative hybrids were captured on Makira in 2012 and 2013: two in North Makira and five in Star Harbour. These birds possessed intermediate plumage, generally having much more extensive black plumage than typical *M. cardinalis* but with red or reddish patches of carotenoid-based plumage atypical of *M. tristrami* (Fig. 1A). No such intermediate birds were observed in allopatric populations, suggesting that they represent true hybrids and not rare color morphs.

One phenotypic hybrid was observed brooding two nearly fledgling age chicks in Star Harbour during 2012. Molecular sexing (Han et al. 2009) confirmed that this individual was a female, and both chicks shared mitochondrial haplotypes and at least one nuclear allele of each of the six sequenced nuclear markers with the female, suggesting that they were indeed its offspring. Both chicks possessed plumage typical of *M. cardinalis* suggesting that they were the product of a backcross between the hybrid and a male *M. cardinalis*. Although anecdotal, this observation demonstrates that this system is not characterized by complete hybrid infertility.

DIVERGENCE TIME ESTIMATES

The mitochondrial divergence time analysis dated the most recent common ancestor of *M. cardinalis* and *M. tristrami* at a mean estimate of 5.8 million years (95% CI: 3.1–9.3 mya) (Fig. S1). This estimate is statistically comparable to the 7 million years that has been cited as the average divergence time for avian species pairs exhibiting nearly complete hybrid infertility (Price 2008). The estimated age of the most recent common ancestor of the Makira and Santa Cruz subspecies (i.e., the likely source population for *M. cardinalis*'s colonization of Ugi and Three Sisters; Mayr and Diamond 2001) was 1.2 million years (95% CI: 0.24–2.7 mya) (Fig. S1), which is within the estimated age of both islands (the Pliocene for Makira, Ugi, and Three Sisters; Coulson and Vedder 1986, and the early Miocene for Santa Cruz; Luyendyk et al. 1974).

MITOCHONDRIAL INTROGRESSION

Sequencing revealed two highly diverged clades of species-specific *ND2* haplotypes within the Makira *Myzomela* (Fig. S2). Haplotype diversity was much greater among *M. tristrami* than *M. cardinalis* (Table S2). All allopatric individuals of both species possessed species-specific mitochondrial haplotypes, as did all *M. cardinalis* from the area of sympatry (Fig. 1C). However, all seven phenotypic hybrids and 22% (7/32) of *M. tristrami* from the area of sympatry possessed mitochondrial haplotypes belonging to the *M. cardinalis* clade, including 33% (5/15) of *M. tristrami* from North Makira and 12% (2/17) from Star Harbour, indicative of mitochondrial introgression from *M. cardinalis* to *M. tristrami.* Finally, two low-frequency mitochondrial haplotypes were present in populations of *M. cardinalis* from Makira and Three Sisters but not from Ugi, suggesting that at least some colonizing individuals originated from the allopatric population on Three Sisters.

Pairwise Φ_{ST} values for *ND2* were consistent with asymmetrical mitochondrial introgression (Table S5): Φ_{ST} values for populations of *M. tristrami* from within the area of sympatry versus *M. cardinalis* populations (ranging from 0.61 to 0.84) were uniformly lower than Φ_{ST} values for allopatric populations (0.97–0.98). Further, pairwise Φ_{ST} values of *M. tristrami* from within the area of sympatry versus *M. cardinalis* in the older sympatric population, North Makira (0.62–0.63) were uniformly lower than in Star Harbour (0.84–0.86). In contrast, pairwise Φ_{ST} values for populations of *M. cardinalis* from within the area of sympatry versus *M. tristrami* populations were essentially equivalent to pairwise Φ_{ST} values for allopatric populations of *M. cardinalis* versus *M. tristrami*.

NUCLEAR INTROGRESSION

In contrast to mtDNA, none of the haplotype networks for the six nuclear markers showed obvious species-specific haplotype clusters (Fig. S3). Indeed, most haplotypes (mean = 76.4% of total sequences, ranging from 42.4% for *TGFβ2* to 98.8% for *FGB*) were shared by at least one allopatric individual of each species. These observed patterns were consistent either with high levels of incomplete lineage sorting among ancestral populations or extensive historical introgression between species. As with mtDNA, nuclear haplotype diversity was again greater among *M. tristrami* than *M. cardinalis*, consistent with a larger effective population size for the former species. Nuclear markers provided

no insight into the source population of the initial *M. cardinalis* colonization event, as all of the *M. cardinalis* specific haplotypes that were detected in sympatric populations were also found in both allopatric populations of *M. cardinalis* (i.e., Ugi and Three Sisters).

Despite the relatively high frequency of haplotype sharing between species, sufficient interspecific genetic variation existed across all markers to identify most individuals with hybrid ancestry. One hundred percent (43/43) of allopatric *M. tristrami* and 98% (44/45) of allopatric *M. cardinalis* possessed at least one species-specific nuclear haplotype (Table 1). In total, 20% (15/75) of *M. cardinalis* from within the area of sympatry possessed at least one heterospecific nuclear haplotype, including 15% (5/33) from North Makira and 24% (10/42) from Star Harbour. Simulations predicted that *M. tristrami* specific haplotypes should be present in 68–71% of hybrid backcrosses with *M. cardinalis* (Table S3), suggesting that these observations underestimate introgression into *M. cardinalis*.

Nuclear introgression from *M. cardinalis* into *M. tristrami* was restricted almost exclusively to admixed individuals possessing *M. cardinalis* mtDNA. Among sympatric *M. tristrami* possessing conspecific mtDNA haplotypes, only 4% (1/25) possessed an *M. cardinalis* specific nuclear haplotype (Table 1), even though our simulations predicted that 46–65% of hybrid backcrosses with *M. tristrami* should possess at least one *M. cardinalis* specific haplotype (Table S3). Among the seven birds that were phenotypically *M. tristrami* but possessed *M. cardinalis* mtDNA haplotypes ("mitochondrial hybrids"), three possessed no heterospecific nuclear haplotypes, three possessed species-specific nuclear haplotypes for both species, and one was classified as genotypically *M. cardinalis* (Table 1). Similarly, for the seven "phenotypic hybrids" (birds with intermediate plumage), three were classified as hybrids, two were classified as *M. cardinalis*, and two were classified as *M. tristrami*, based on species-specific nuclear haplotypes (Table 1). Simulations predicted that, under this approach, 71–83% of true F_1 hybrids should be identified as genetically hybrid, 13–24% as genetically *M. tristrami*, and only 2–6% as genetically *M. cardinalis* (Table S3).

Maximum-likelihood analysis indicated that sample sizes were likely to be robust enough to minimize the chance of false mischaracterization of alleles as "species-specific." Based on our sample sizes, the 95% confidence interval for the true allele frequency of a nuclear allele that went undetected was between 0.030 and 0.064 for our populations (Table S4), while the maximum-likelihood estimate for each population was zero. As such, any undetected alleles likely represent truly rare variants in the population. Our smallest sample sizes and greatest confidence interval estimates were in the sympatric populations of *M. tristrami* and the allopatric population of *M. tristrami* from Highland Makira. Under our approach for identifying hybrids, failure to detect alleles in the latter population would result in a potential for mischaracterizing shared nuclear alleles as "*M cardinalis* specific" and, as a result, falsely identifying putative introgression from *M. cardinalis* to *M. tristrami*. However, we note that introgression in this direction was separately confirmed via mitochondrial sequencing, and in all but one case, nuclear introgression was restricted to known mitochondrial hybrids.

Mean F_{ST} values between populations of sympatric *M. tristrami* and allopatric *M. cardinalis* (ranging from 0.09 to 0.14) were lower than mean F_{ST} values of allopatric *M. tristrami* versus allopatric *M. cardinalis* (0.15–0.24) (Table S5), as expected with introgression of *M. cardinalis* alleles into *M. tristrami* in sympatry. Likewise, mean F_{ST} values of populations of sympatric *M. cardinalis* versus allopatric *M. tristrami* from Highland Makira (0.12–0.13) were marginally lower than mean F_{ST} values of allopatric *M. cardinalis* versus the same *M. tristrami* (0.14–0.16), consistent with introgression. However, none of these differences were statistically significant (uncorrected *P*-values: 0.13–0.79) as the observed patterns were not consistent for all six nuclear markers (Tables S6–S8), which is to be expected if rates of introgression varied across the genome.

The STRUCTURE analysis (Fig. 2) also revealed evidence for nuclear introgression in this system. As expected, STRUCTURE identified two species-specific genotypic clusters, with all but one allopatric individual assigned to the conspecific cluster with a posterior probability ("p.p.") >90%, with 91% (29/32) of *M. tristrami* and 96% (44/46) of allopatric *M. cardinalis* assigned with >95% p.p. The exception was an allopatric adult male *M. cardinalis* from Ugi that STRUCTURE assigned with intermediate probability (p.p.: 59% *M. tristrami*, 41% *M. cardinalis*), even though all of its alleles were shared by at least one other allopatric *M. cardinalis*. Still, we could not dismiss the possibility of hybrid ancestry in this individual, as Ugi is only 8 km from mainland Makira, and there is a published report of vagrant *M. tristrami* from Ugi (Dutson 2001). No *M. tristrami*, however, were sighted during any of our visits to Ugi.

Among sympatric *M. cardinalis*, STRUCTURE assigned nearly all individuals to the correct cluster, but with slightly lower confidence than for allopatric individuals, consistent with low levels of introgression from *M. tristrami*. Although 93% (70/75) of sympatric *M. cardinalis* were assigned to the conspecific cluster with >90% p.p., only 83% (62/75) were assigned with >95% p.p., significantly lower than for allopatric populations (two-tailed *Z*-test: 2.10, $P = 0.036$) and consistent with backcrossing. Five *M. cardinalis* from the area of sympatry possessed intermediate genetic structure consistent with hybrid ancestry (p.p. of assigning to the conspecific cluster = 68.3–88.6%). Among sympatric *M. tristrami*, 92% (23/25) of individuals with conspecific mitochondrial haplotypes were assigned to the correct cluster with >95% p.p., statistically equivalent to the assignment probabilities for

Table 1. Percent of sampled *Myzomela* individuals assigned to genotypes based on presence/absence of species-specific alleles for six nuclear markers.

Population	Number individuals	Percent of individuals assigned to genotype			
		M. cardinalis	Hybrid	*M. tristrami*	Ambiguous
M. cardinalis					
Three Sisters (A)	25	0.96	0.00	0.00	0.04
Ugi (A)	21	1.00	0.00	0.00	0.00
North Makira (S)	33	0.79	0.06	0.09	0.06
Star Harbour (S)	42	0.71	0.21	0.02	0.05
Phenotypic hybrids (all possess *M. cardinalis* mtDNA haplotypes)					
North Makira (S)	2	0.50	0.00	0.50	0.00
Star Harbour (S)	5	0.20	0.60	0.20	0.00
Mitochondrial hybrids (*M. tristrami* possessing *M. cardinalis* mtDNA haplotypes)					
North Makira (S)	5	0.20	0.60	0.20	0.00
Star Harbour (S)	2	0.00	0.00	1.00	0.00
M. tristrami (possessing conspecific mtDNA haplotypes)					
North Makira (S)	10	0.00	0.10	0.90	0.00
Star Harbour (S)	15	0.00	0.00	1.00	0.00
Santa Catalina (A)	28	0.00	0.00	1.00	0.00
Highland Makira (A)	15	0.00	0.00	1.00	0.00

Individuals assigned to a given species genotype possess at least one species-specific allele for that species and none for the other. Individuals assigned as "hybrids" possess at least one species-specific allele for each species, and individuals assigned as "ambiguous" possess no species-specific alleles. Populations are specified in the table as allopatric ("A") or sympatric ("S").

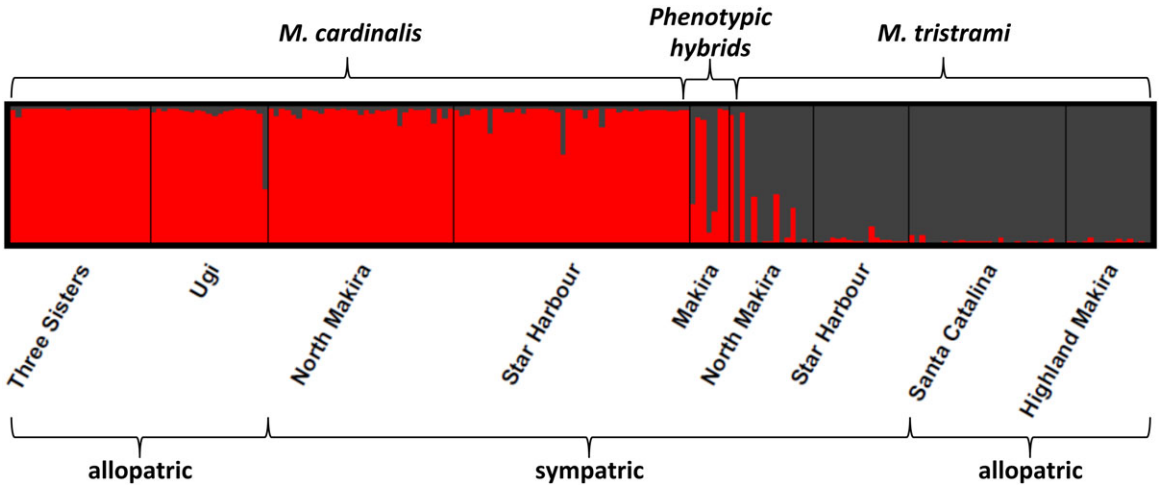

Figure 2. STRUCTURE plot based on haplotype data of six nuclear markers for 203 *Myzomela* from Makira-Ulawa province, assuming two population clusters ($k = 2$). *Y*-axis represents Bayesian posterior probability of assignment to clusters representing *M. cardinalis* (red) or *M. tristrami* (dark gray). Plumage phenotype of individuals is indicated above the plot, sampling location is indicated below the plot, with black bars separating locations.

allopatric populations (two-tailed *Z*-test: 0.18, $P = 0.857$), again consistent with extremely limited nuclear introgression into *M. tristrami* matrilines within the area of sympatry. In contrast, *M. tristrami* possessing heterospecific mitochondrial haplotypes and phenotypic hybrids both varied substantially between individuals in their population assignments (Fig. 2), consistent with genetic admixture.

Discussion

Our study represents the first quantification of hybridization and introgression between the two species of *Myzomela* honeyeaters present on Makira in the Solomon Islands, a rare example of recent secondary contact between congeners in an island radiation (Mayr and Diamond 2001). Hybridization between the native *M. tristrami* and the recent colonizer *M. cardinalis* was previously

noted by Mayr and Diamond (2001), who hypothesized that hybrid pairings in this system were limited to the early stages of colonization when conspecific mating opportunities for *M. cardinalis* were scarce (Mayr and Diamond 2001; Diamond 2002; Price 2008). Our findings indicate that hybridization is ongoing more than a century after the identified colonization event, with one phenotypic hybrid captured per every 4.6 *M. tristrami* (i.e., the less common species) captured in sympatry. This ongoing hybridization occurs even though the Makira *Myzomela* shared a most recent common ancestor between 3.1 and 9.3 million years ago. Despite this divergence, our observation of an intermediate-plumage female with near-fledgling age chicks demonstrates that F_1 hybrid sterility is not complete. As a result, hybridization has led to widespread asymmetric introgression in the Makira *Myzomela*, which provides insight into the dynamics of hybridization and its potential consequences for speciation in this system.

Mitochondrial DNA sequencing indicates that all successful F_1 hybrid pairings in this system involve female *M. cardinalis*, with 33% of phenotypically *M. tristrami* individuals from the oldest portion of the area of sympatry (North Makira) possessing *M. cardinalis* mtDNA haplotypes. This asymmetric introgression from the colonizing to the native species is opposite to the pattern predicted by wavefront models of introgression (Currat et al. 2008) and which is observed in nearly all shifting avian hybrid zones (Rheindt and Edwards 2011). In contrast, mitochondrial introgression in this system is influenced predominantly by asymmetric reproductive isolation, that is, one hybrid pairing is more likely to occur successfully than the reverse. Asymmetric reproductive isolation and mtDNA introgression from *M. cardinalis* to *M. tristrami* is predicted under Mayr and Diamond's (2001) hypothesis that hybridization in this system was driven by a relaxation of mate choice preferences among colonizing *M. cardinalis* females immediately following secondary contact when conspecific mates were rare. However, if differences in mate choice preferences are primarily responsible for the asymmetric patterns of mtDNA introgression observed in this system, we would expect to observe introgression of *M. cardinalis* nuclear alleles into sympatric *M. tristrami* matrilines, as a consequence of pairings of *M. tristrami* females with all-black mitochondrial hybrid males. Yet only one sympatric *M. tristrami* possessing a conspecific mtDNA haplotype exhibited any degree of hybrid ancestry based on nuclear markers.

Instead, observed patterns of highly asymmetric mtDNA introgression likely result from asymmetric postzygotic genetic incompatibilities. Incompatibilities between autosomal and mitochondrial genes involved in the OXPHOS pathway have been hypothesized to be an important driver of postzygotic reproductive isolation between species (Turelli and Moyle 2007; Hill and Johnson 2013), and evidence for a link to asymmetric hybrid viability has been observed in many taxa (Bolnick et al. 2008; Ellison and Burton 2008; Ellison et al. 2008; Trier et al. 2014). Strong postzygotic reproductive isolation in this system is unsurprising as we estimate that the most recent common ancestor for *M. cardinalis* and *M. tristrami* lived between 3.1 and 9.3 million years ago, statistically comparable to the 7 million years that has been cited as the average divergence time for species exhibiting nearly complete hybrid infertility (Price 2008). However, direct quantification of the relative viability of offspring from differential parental combinations as well as direct assays of female mate choice preferences are needed to determine the relative importance of pre- and postzygotic reproductive isolation to the observed patterns of asymmetric mitochondrial introgression between the Makira *Myzomela*.

Asymmetric hybridization has important consequences for biodiversity in this system. Unlike other known examples of secondary contact on small islands, the Makira *Myzomela* appear to exhibit sufficient reproductive isolation to maintain phenotypic differences between species rather than collapsing into a hybrid swarm (in contrast to Larsen et al. 2010; Steeves et al. 2010; Lavretsky et al. 2015). For example, in contrast to trends immediately following secondary contact, relative species abundances have remained relatively stable since the mid-20th century, and sympatric populations continue to exhibit generally strong species-specific genetic structure. However, reproductive isolation in this system is not sufficient to prevent introgression between species (in contrast to Qvarnström et al. 2010; Warren et al. 2011). Instead, nuclear introgression is occurring from the native species, *M. tristrami*, to the colonizer, *M. cardinalis*. Similar patterns of introgression also characterize several other island systems where long-distance dispersal resulted in secondary contact, including New Zealand stilts (Steeves et al. 2010), Galapagos finches (Grant and Grant 2014), and Mediterranean butterflies (Cianchi et al. 2003). If the colonization patterns on Makira are similar to those directly observed in *Geospiza* (Grant and Grant 2014), then all sympatric *M. cardinalis* should be descendants of a small number of individuals that originally colonized Makira and hybridized with *M. tristrami*, and whose offspring assortatively mated among themselves. Indeed, the proportion of *M. cardinalis* possessing heterospecific nuclear alleles is greater in populations that have achieved secondary contact more recently. Based on our simulations (Table S3), however, these proportions are significantly less than those expected under this scenario. Instead, they are more consistent with three backcross events, suggesting one or more of the following: a relatively large founder population of *M. cardinalis*, repeated dispersal of *M. cardinalis* from the satellite islands to Makira, or strong selection for individuals with reduced hybrid ancestry. Therefore, even though reproductive isolation is incomplete, gene flow resulting from secondary contact between these highly diverged island species does not appear to be substantial enough to break down species boundaries. Hybridization

does allow, however, for adaptive alleles to potentially introgress into the colonizing species.

The Makira *Myzomela* also exhibit apparent nuclear introgression from the colonizer, *M. cardinalis*, to the native species, *M. tristrami*, in contrast to the pattern observed in nearly all avian hybrid systems (Rheindt and Edwards 2011). However, the rarity of *M. cardinalis* nuclear haplotypes among individuals descended from *M. tristrami* matrilines is consistent with a high degree of reproductive isolation between female *M. tristrami* and the melanic birds that possess *M. cardinalis* mitochondrial haplotypes. Accordingly, it may be more appropriate to characterize gene flow patterns as an introgression of plumage alleles from the native species to the invading one, which matches the observations from other avian systems. Such introgression would be facilitated if black plumage is favored by sexual selection in *M. cardinalis* (e.g., due to sensory bias). Similar patterns of sexual selection driving asymmetric plumage introgression have been documented in hybrid zones between *Manacus* manakins (Brumfield et al. 2001; Stein and Uy 2006) and between subspecies of red-backed fairy-wrens, *Malurus melanocephalus* (Baldassarre and Webster 2013; Baldassarre et al. 2014). However, the relative rarity of mitochondrial hybrids on Makira and the persistence of presumably costly carotenoid-based red plumage in allopatric *M. cardinalis* suggest that plumage introgression is unlikely to be driven by shared preferences for black mates. Ecological selection could also favor black plumage on Makira, but an opposite pattern in terms of plumage distribution is found in the chestnut-bellied flycatcher (*Monarcha castaneiventris*) complex, which includes melanic populations on Ugi and Three Sisters and more colorful birds on Makira (Uy et al. 2009; Uy and Safran 2013). Plumage introgression would also be facilitated if colonization events resulted in relaxed female preference for plumage in *M. cardinalis* females (Kaneshiro 1980), under which introgression would be driven primarily by genetic drift. Alternatively, black plumage among individuals of hybrid ancestry may be favored by disruptive intrasexual selection if it results in decreased aggression that allows otherwise low-quality males to hold higher quality territories, as demonstrated in other examples of avian plumage polymorphisms (Greene et al. 2000; Vallin et al. 2012). Finally, the alleles for black plumage may be linked to alleles for genes responsible for nonplumage-related locally favored adaptations (e.g., disease resistance, metabolism of local food sources, etc.) (Ducrest et al. 2008). Under this hypothesis, plumage has undergone introgression because the recent history of secondary colonization between species has not provided sufficient opportunity for recombination to break down linkage disequilibrium between these alleles. With the advent of affordable next-generation sequencing, it is now possible to examine genome-wide patterns of introgression and identify genomic regions that show signatures of selection. Such follow-up studies on this system will provide further insight into the evolutionary consequences of hybridization and introgression for the maintenance of biodiversity in island systems.

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DATA ARCHIVING

Genetic sequencing data are archived in NCBI GenBank: accession numbers KU519747—KU521321.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Consensus majority Bayesian phylogenetic tree representing evolutionary history of genus *Myzomela* inferred from a single mitochondrial gene (*ND2*).

Figure S2. Haplotype network for 121 *M. cardinalis*, 75 *M. tristrami*, and seven phenotypic hybrids sequenced at one mitochondrial marker: NADH dehydrogenase subunit 2 (*ND2*).

Figure S3. Haplotype networks for 121 *M. cardinalis*, 75 *M. tristrami*, and seven phenotypic hybrids sequenced at six nuclear markers: (a) beta-fibrinogen intron 5 (FGB); (b) rhodopsin intron 1 (RHO); (c) 15246; (d) glyceraldehyde 3-phosphate dehydrogenase intron 11 (GAPDH); (e) myoglobin intron 2 (MYO); (f) transforming growth factor β-2 intron 5 (TGFβ2).

Table S1. Source of samples used in phylogenetic analysis.

Table S2. Distribution of haplotypes for one mitochondrial and six nuclear markers among allopatric and sympatric populations of *M. cardinalis* and *M. tristrami*.

Table S3. Percent of 100,000 simulated *F*1, *F*2, and backcross pairings that are assigned to specified genotypes based on presence/absence of species-specific alleles for six nuclear markers.

Table S4. Ninety-five percent likelihood-based confidence limits of the true frequency of a nuclear allele that went undetected in each population, based on number of alleles sampled.

Table S5. Cross-population Φ_{ST} and F_{ST} values for ND2 and all nuclear markers for sampled allopatric and sympatric populations of *M. cardinalis* and *M. tristrami*.

Table S6. Cross-population F_{ST} values for FGB and MYO for sampled allopatric and sympatric populations of *M. cardinalis* and *M. tristrami*.

Table S7. Cross-population F_{ST} values for RHO and TGFβ2 for sampled allopatric and sympatric populations of *M. cardinalis* and *M. tristrami*.

Table S8. Cross-population F_{ST} values for GAPDH and 15246 for sampled allopatric and sympatric populations of *M. cardinalis* and *M. tristrami*.