



Triad hybridization via a conduit species

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Introgressive hybridization can affect the evolution of populations in several important ways. It may retard or reverse divergence of species, enable the development of novel traits, enhance the potential for future evolution by elevating levels of standing variation, create new species, and alleviate inbreeding depression in small populations. Most of what is known of contemporary hybridization in nature comes from the study of pairs of species, either coexisting in the same habitat or distributed parapatrically and separated by a hybrid zone. More rarely, three species form an interbreeding complex (triad), reported in vertebrates, insects, and plants. Often, one species acts as a genetic link or conduit for the passage of genes (alleles) between two others that rarely, if ever, hybridize. Demographic and genetic consequences are unknown. Here we report results of a long-term study of interbreeding Darwin's finches on Daphne Major island, Galápagos. *Geospiza fortis* acted as a conduit for the passage of genes between two others that have never been observed to interbreed on Daphne: *Geospiza fuliginosa*, a rare immigrant, and *Geospiza scandens*, a resident. Microsatellite gene flow from *G. fortis* into *G. scandens* increased in frequency during 30 y of favorable ecological conditions, resulting in genetic and morphological convergence. *G. fortis*, *G. scandens*, and the derived dihybrids and trihybrids experienced approximately equal fitness. Especially relevant to young adaptive radiations, where species differ principally in ecology and behavior, these findings illustrate how new combinations of genes created by hybridization among three species can enhance the potential for evolutionary change.

speciation | introgression | trihybrids | fitness | convergence

In the early stages of speciation, nascent species are prone to hybridize and exchange genes through backcrossing and introgression (1–3). The circumstances and consequences of hybridization in animals have received much attention recently because gene exchange can affect the evolution of populations in several important ways: at the most extreme in facilitating the evolution of unisexuality in both vertebrates (4, 5) and invertebrates (6, 7). Hybridization may retard, arrest, or reverse divergence of species (8–11); enable the development of novel morphological and physiological traits (12–15); enhance the potential for future evolution by elevating levels of standing variation (16–20) and by strengthening or relaxing covariances (17, 21); alleviate inbreeding depression in small populations (22–24); and create new species (2, 25–27). Extensive hybridization in the past is known through inferences from phylogenetic reconstructions (3, 15, 28, 29). With regard to contemporary hybridization in nature, most of what is known comes from the study of pairs of species, either coexisting in the same habitat or distributed geographically with little overlap and separated by a hybrid zone (30, 31). In this article, we discuss an additional complexity, the occurrence of hybridization among triplets of species (triads), and consider its context and evolutionary significance.

Triad hybridization has been reported in fish (32–34), frogs (35), lizards (36), birds (17, 37, 38), mammals (39–41), snails (42), butterflies (43), mosquitoes (44), as well as several plant taxa (45, 46). One species acts as a genetic bridge (33, 47, 48) or conduit (41, 49) for the passage of genes (alleles) between two others that, in some cases, rarely if ever hybridize (33, 39, 41). Exchange of genes is generally not studied directly with breeding pairs but is inferred from the presence of individuals of mixed (trisppecies)

ancestry (33, 39, 41) or from the fact that hybridization occurs, or is enhanced, only in the presence of a third species (36, 50).

Triad hybridization, where one species acts as an intermediary for gene exchange between two other noninterbreeding species, raises questions of genome compatibility, selection, fitness, and the evolution of reproductive isolation. Evolutionary effects of bispecies hybridization may be either negated or amplified in trisppecies hybridization. On the one hand, donated alleles may be gradually eliminated by selection and drift, resulting in no net change in genome compositions. In this case, hybridization is likely to be transitory and a prelude to the evolution of stronger reproductive isolation and a decline in gene exchange (51). On the other hand, introgressed alleles may be filtered in transit (52), with some being incorporated into the genomes of the bridging species and in the downstream recipient species, where they affect morphology or physiology and facilitate evolutionary divergence (53–55). Gene dynamics are potentially heterogeneous. Epistasis is expected to be complex because genes from one species function against a genetic background derived in part from two other species. Of interest is the question whether the recipient species receive alleles equally or unequally (filtered) via the intermediary conduit, and whether the conduit species itself donates more or less than it receives. Alternative outcomes depend on the frequency of interbreeding, the direction(s) of exchange, and fitness consequences. With rare exceptions (39, 43), all of these are unknown. Long-term demographic studies are needed to estimate the magnitude of these influences and to document and interpret the significance of triad hybridization.

We report an example from a long-term demographic study of Darwin's finches on the small Galápagos island of Daphne Major (0.34 km²), carried out from 1973 to 2012. On this island, the medium ground finch (*Geospiza fortis*, ~17 g) occasionally breeds

Significance

Introgressive hybridization of pairs of species can affect the evolution of their populations in several important ways. More rarely, three species form an interbreeding complex (triad), but little is known of the consequences. A long-term study of interbreeding Darwin's finches on Daphne Major island, Galápagos, shows that *Geospiza fortis* acts as a conduit for the passage of genes from *Geospiza fuliginosa*, a rare immigrant, to *Geospiza scandens*, a resident. Surprisingly, the species and the derived dihybrids and trihybrids had approximately equal fitness. Gene flow increased in frequency and apparently caused a morphological convergence of the resident species. These findings illustrate how evolutionary change is potentially increased by hybridization among three species and the assembly of novel combinations of genes.

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with the cactus finch (*Geospiza scandens*, ~21 g), a resident species, and the small ground finch (*Geospiza fuliginosa*, ~13 g), a rare immigrant and sister species to *G. fortis* (28, 56). In doing so, *G. fortis* acts as a conduit for the passage of genes between *G. fuliginosa* and *G. scandens*, two species that have never been observed to interbreed on Daphne (Fig. 1). Individuals were initially assigned to species on the basis of morphology (*SI Appendix*, section 1), and hybrids were identified from pedigree data (57). Mixed pairs, first observed in 1976 (57, 58), were generally at frequencies of one to three percent of the breeding populations (56). Offspring of known parents were uniquely marked with leg bands in the years 1976 to 1998, and breeding pairs were identified in every year up to 2012. Questions of paternity were resolved with microsatellite markers when a program of DNA sampling was initiated in 1988 (59). The microsatellite markers were used to identify hybrids throughout the whole study period (*Methods* and *SI Appendix*, sections 2 and 3). Known hybrids were observed to breed from 1983 onward when an El Niño event caused a major change to the environment (56, 60). Hybrids were found to backcross according to the same rules that govern intraspecific mate choice: finches choose mates on the basis of imprinting on paternal song and parental morphology, and occasionally hybridize when they learn the song of another species (61, 62). Frequencies of alleles at 16 unlinked polymorphic loci are used here to assign 3,165 individuals probabilistically (≥ 0.9) to the three species (63), and, if the 0.9 criterion is not met, they are assigned to hybrids (admixtures). We refer to them as primary admixtures when individuals are assigned to two species (dihybrids) and secondary admixtures when they are assigned to all three species (trihybrids).

We present quantitative estimates of the frequencies of di- and trihybrids and introgression of neutral microsatellite markers. We show that, as expected, birds with genomic admixtures are intermediate in morphology between the means of the parental

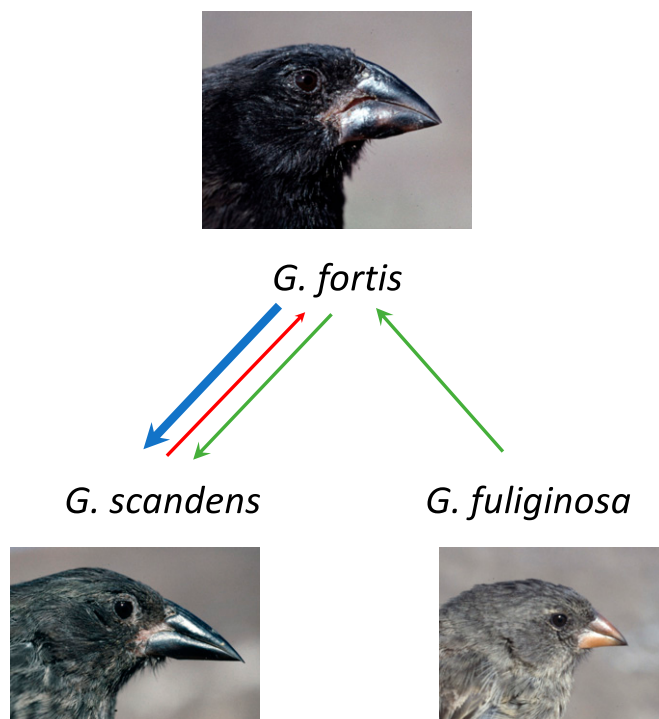


Fig. 1. Gene exchange among three species of Darwin's finches. The green lines indicate an indirect transfer from *G. fuliginosa* to *G. scandens* via *G. fortis* acting as a conduit. *G. fuliginosa* is too rare to form an independent breeding population. The principal flow of genes is from *G. fortis* to *G. scandens* (blue line).

Table 1. Numbers of sampled *G. fuliginosa*, *G. fortis*, *G. scandens*, and pairwise admixtures produced each year

| Year | <i>fuliginosa</i> | <i>fortis</i> | <i>scandens</i> | Ff + fF | FS | SF | Sf + fS |
|--------|-------------------|---------------|-----------------|---------|----|----|---------|
| 1975 | — | — | 2 | — | — | — | — |
| 1978 | — | 7 | 3 | — | 1 | — | 1 |
| 1979 | — | 1 | 1 | — | — | 1 | — |
| 1981 | — | 9 | 3 | 2 | — | 1 | 1 |
| 1983 | 1 | 45 | 18 | 2 | 2 | 1 | 1 |
| 1984 | 1 | 24 | 2 | 3 | — | — | — |
| 1986 | — | 1 | — | — | — | — | — |
| 1987 | 6 | 283 | 57 | 21 | 7 | 2 | 3 |
| 1990 | — | 27 | 4 | 3 | — | — | — |
| 1991 | 5 | 648 | 127 | 41 | 11 | 4 | 2 |
| 1992 | 7 | 123 | 46 | 15 | 2 | 3 | 1 |
| 1993 | 7 | 126 | 102 | 5 | 5 | 5 | — |
| 1997 | — | 6 | 13 | — | — | 1 | — |
| 1998 | 1 | 140 | 98 | 8 | 6 | 6 | 3 |
| 2000 | — | — | 15 | 1 | 1 | 2 | 1 |
| 2001 | 1 | 53 | 4 | 5 | 1 | — | — |
| 2002 | 3 | 68 | 66 | 17 | 4 | 6 | 1 |
| 2005 | 7 | 93 | 27 | 10 | 5 | 6 | 1 |
| 2007 | 1 | 31 | 13 | 5 | 8 | 11 | 3 |
| 2008 | 3 | 75 | 82 | 4 | 3 | 14 | 3 |
| 2009 | 2 | 53 | 41 | 8 | 5 | 2 | 1 |
| 2010 | 8 | 74 | 59 | 6 | 3 | 9 | 3 |
| 2011 | 8 | 14 | 26 | 4 | 4 | 6 | 3 |
| Totals | 61 | 1,901 | 809 | 160 | 68 | 80 | 28 |

Abbreviations: letters designating hybrids refer to the species *G. fuliginosa* (f), *G. fortis* (F), and *G. scandens* (S). The first letter of admixtures denotes the species with majority assignment probability.

species that gave rise to them, and with elevated variances. The main finding is that di- and trihybrids survived surprisingly well, approximately as well as *G. fortis* and *G. scandens* over the first 6 to 10 y of life. High survival, even of trihybrids, may be characteristic of relatively young adaptive radiations in fluctuating environments.

Results

Frequencies of Primary Admixtures. *G. scandens* × *G. fortis* (SF) and *G. fortis* × *G. scandens* (FS) hybrids were produced in every well-sampled year from 1978 onward (Table 1): the first letter denotes the species with majority assignment probability. *G. fortis* × *G. fuliginosa* (Ff) hybrids were produced from 1981 onward, and in greater numbers. A small number of admixed individuals ($n = 27$) were assigned to *G. scandens* and *G. fuliginosa* (Sf and fS) but not to *G. fortis*. Most (0.74) occurred after 1998 when the detailed breeding study ceased. They may have been the undetected product of interbreeding of the two species, which was never observed in 1976 to 1998 but may have occurred through extrapair mating (61). Alternatively, and perhaps more likely, they were the product of interbreeding of *G. scandens* and Ff, or some more complicated pairing such as SF × fF.

Hybrid production increased in frequency, especially after 2000 (Fig. 2). All slopes of regressions of numbers on year are positive. The Ff relationship is not significant, either with ($F_{1,16} = 1.61$, $P = 0.2228$) or without the outlier in 2002 ($F_{1,15} = 1.36$, $P = 0.2617$), and nor is the relationship for Sf and fS combined ($F_{1,16} = 2.20$, $P = 0.1572$). The FS relationship is significant with the two outliers in 2007 and 2011 included ($F_{1,16} = 7.48$, $P = 0.0147$) or excluded ($F_{1,14} = 4.64$, $P = 0.0491$), and the SF relationship is also significant without the strong outlier in 2007 ($F_{1,15} = 15.00$, $P = 0.0015$).

To quantify the trends, we calculated means after deleting the conspicuous outliers. Since the remaining frequencies vary relatively

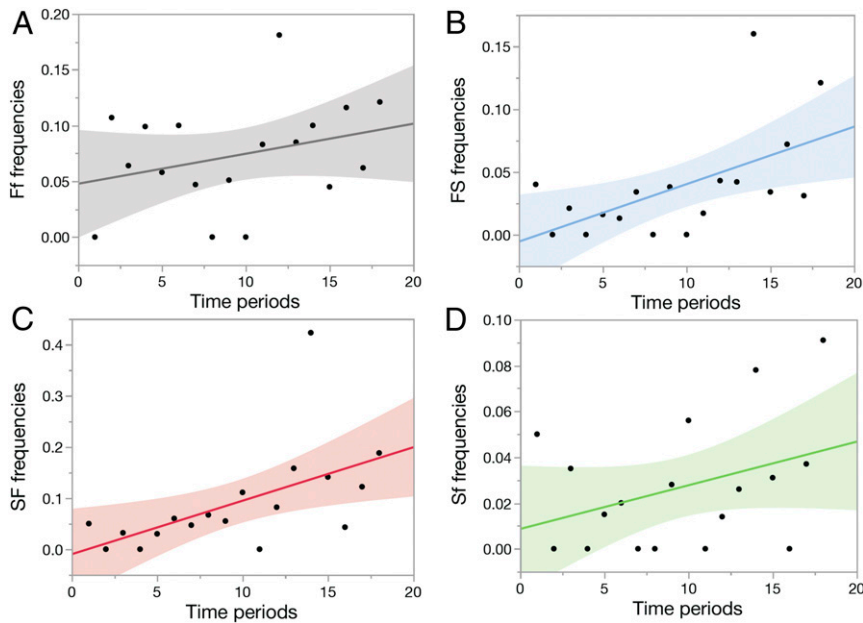


Fig. 2. Frequencies of hybrids produced in each year of breeding. Hybrids are expressed as frequencies of the offspring produced in each of the *G. fortis* groups (*G. fortis*, Ff [A], FS [B]) and *G. scandens* groups (*G. scandens*, SF [C], Sf [D], fs). Regressions are significant for FS and SF frequencies (see *Frequencies of Primary Admixtures*).

little and are normally distributed, we did not transform them. Frequencies are much higher in Ff (0.073 ± 0.011 SE) and SF (0.089 ± 0.023) than FS (0.037 ± 0.010) and Sf (0.028 ± 0.007 ; $n = 18$ for each group). Sf frequencies are notably lower than Ff ($P = 0.0160$) and SF ($P = 0.0327$) frequencies by Kramer HSD tests. The inequalities imply that alleles were transferred twice as frequently from *G. fuliginosa* to *G. fortis* (Ff) and from *G. fortis* to *G. scandens* (SF) as in the reverse direction (FS).

Secondary Admixtures. Secondary admixtures occur through interbreeding of *G. scandens* and Ff hybrids or backcrosses. Frequencies were uniformly low (Table 2), generally much lower than the frequencies of primary admixtures (Table 1). Allele transfer from *G. fuliginosa* to *G. scandens* via members of the *G. fortis* population (including hybrids) is indicated by the breeding of trispecific hybrids. Three of these hybrids with a majority assignment to *G. fortis* or *G. fuliginosa* produced offspring by breeding with *G. scandens*, and four others did so by breeding with *G. fortis*. In the four cases where fathers of the trihybrids were known (three *G. fortis* and one *G. scandens*), they all followed the mating rule of pairing according to paternal song type. As with the dihybrids, gene exchange involving trihybrids was bidirectional between *G. fortis* and *G. scandens*.

Introgression. As a result of an exchange of alleles, *G. scandens* became progressively more similar genetically to *G. fortis* across five periods of breeding (Fig. 3). The pattern of genetic convergence has been reported before (56), but without statistical analysis. Fourteen of the 16 slopes for individual loci are negative, which is a significant departure from the null hypothesis expectation of eight (two-tailed binomial test, $P = 0.004$). Moreover, three of the slopes for individual loci are significant (Fig. 3).

We tested the null hypothesis that *G. fortis* and *G. scandens* contributed equally to genetic convergence. We did this by comparing the average Nei's D_A value, a measure of genetic distance, between the earliest *G. scandens* and latest *G. fortis* samples with the average D_A value between the earliest *G. fortis* and latest *G. scandens* samples. They should be equal according to the null hypothesis. The power of the test for this type of comparison with small samples is low; nevertheless, the difference is

close to being significant: paired $t_{15} = 1.95$, $P = 0.07$ with arc sign-transformed data. Late *G. scandens* are more similar to early *G. fortis* (average $D_A = 0.185$) than late *G. fortis* are to early *G. scandens* (average $D_A = 0.241$). Thus, *G. scandens* appear to have converged genetically more than *G. fortis*, consistent with the inference from the higher frequency of SF than FS, and consistent with the morphological trends (51). Furthermore, *G. scandens* gained 16 alleles present in the earliest *G. fortis* sample but not in the earliest *G. scandens* sample, whereas *G. fortis* gained only three alleles from *G. scandens*. However, the difference is partly explained by the fact that *G. fortis* had more alleles ($n = 190$) to donate than *G. scandens* ($n = 159$).

In contrast to these results, the genetic distance between *G. fortis* and *G. fuliginosa* remained unchanged across the five periods ($F = 1.40$, $P = 0.3224$), consistent with constant frequencies of Ff hybrids (as detailed earlier). We interpret the constancy as a steady-state gene flow, with input of *G. fuliginosa* alleles into *G. fortis* through repeated immigration and hybridization roughly balanced by output as the backcrosses to *G. fortis* across generations

Table 2. Numbers of trihybrids produced each year

| Year | FfS | fFS | FSf | fSF | Sff | SfF |
|--------|-----|-----|-----|-----|-----|-----|
| 1987 | 3 | 1 | 4 | 1 | 1 | — |
| 1991 | 1 | 1 | 1 | — | — | — |
| 1992 | 1 | — | 1 | 1 | — | — |
| 1993 | 2 | — | — | 1 | — | — |
| 1997 | — | — | — | — | 1 | — |
| 1998 | — | — | 1 | — | 1 | — |
| 2002 | 1 | — | — | 1 | — | — |
| 2005 | 2 | — | 1 | — | 3 | 1 |
| 2007 | 1 | 2 | — | — | — | 1 |
| 2008 | 1 | — | 1 | — | — | 1 |
| 2009 | 1 | — | — | — | 1 | 1 |
| 2010 | 3 | — | 1 | 1 | 3 | 1 |
| Totals | 16 | 4 | 10 | 5 | 10 | 5 |

Abbreviations: letters designating hybrids refer to the species *G. fuliginosa* (f), *G. fortis* (F), and *G. scandens* (S), and the descending order of assignment probability to the three species.

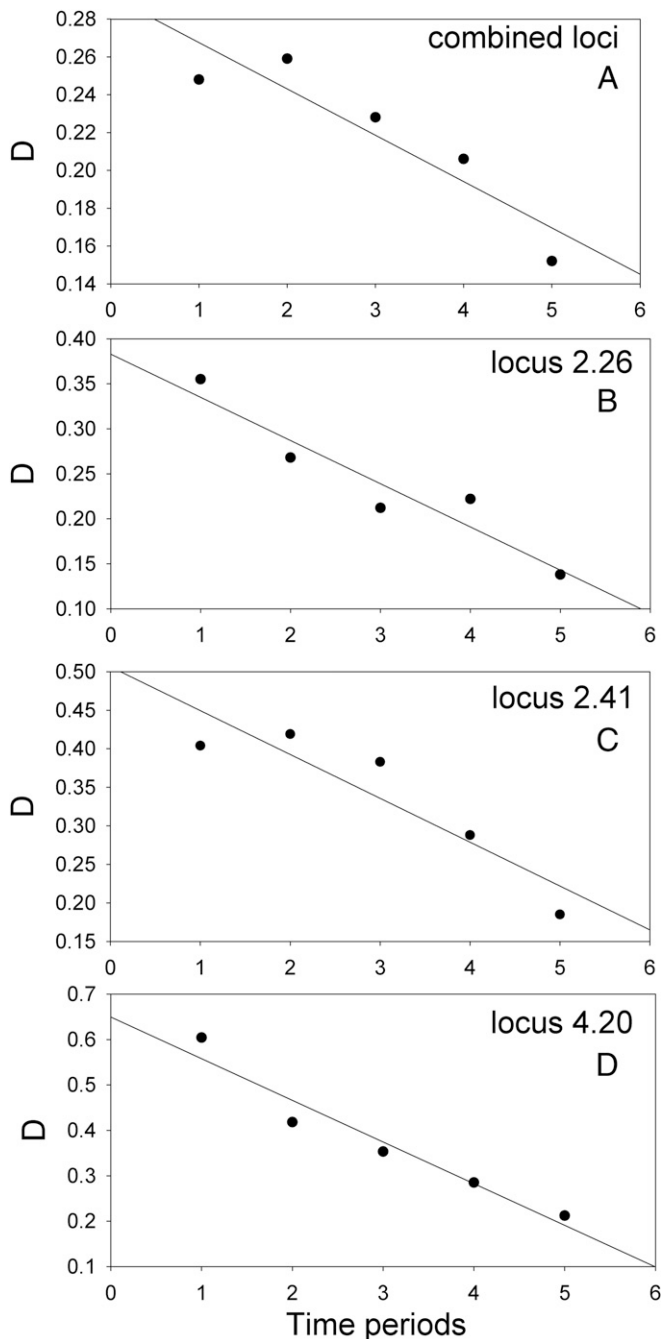


Fig. 3. Genetic convergence. (A) *G. scandens* became progressively more similar genetically to *G. fortis* across five periods (1975 to 1987, 1990 to 1993, 1997 to 1998, 2000 to 2002, 2005 to 2011) separated by years of little or no breeding. The regression relationship of annual mean D_A values on time for the combined data are significant ($F_{1,4} = 15.30$, $P = 0.0297$, R^2 adj = 0.78), and has a negative slope of -0.024 ± 0.006 (A). (B–D) Significant regression relationships at three of the individual loci: locus 2.26 ($F = 27.92$, $P = 0.0132$, $b = -0.048 \pm 0.009$ SE, R^2 adj = 0.87), locus 241 ($F = 15.06$, $P = 0.0303$, $b = -0.057 \pm 0.015$, R^2 adj = 0.78), and locus 420 ($F = 47.13$, $P = 0.0063$, $b = -0.092 \pm 0.013$, R^2 adj = 0.92).

become increasingly indistinguishable from *G. fortis* itself. Input exceeds output in *G. fortis* and *G. scandens*. The net result is a predominant flow of genes from *G. fuliginosa* and *G. fortis* to *G. scandens*, with some reverse flow to *G. fortis*, as depicted in Fig. 1. Unequal exchange of genes has also been reported for two species of tree finches on Floreana island (*Camarhynchus* spp.), predominantly from a rare to a common species (64).

Phenotypic Consequences of Hybridization. Morphological traits are highly heritable in *G. fortis* and *G. scandens* (65, 66). Therefore hybrids, both dihybrids and trihybrids, are predicted to have intermediate means and increased phenotypic variances in the absence of selection (17). We used principal components analysis (PCA; *SI Appendix*, section 4) to test these predictions.

Mean measurements match expectations. The hybrid groups are larger in size than smaller species (*G. fuliginosa* and *G. fortis*) and smaller than the larger species (*G. scandens*; Table 3). Beak shape means are similarly intermediate. For each of these traits, 14 comparisons were made between a group of hybrids and a species that contributed to them. Most differences in body size and beak shape are significant by Tukey's HSD tests (Table 3).

The expectation of larger variances of the hybrid groups is realized in all 42 comparisons, and 35 of them are significant by one-tailed F tests (Table 4). Dihybrids differ from the species in variances of beak traits more than do the trihybrids, although not in variances of body size. Average ratios of variances for the dihybrids are 2.00 (body size), 3.24 (beak size), and 3.86 (beak shape), and, for the trihybrids, they are 2.65, 3.04, and 3.09 in the same sequence. These are notably large values.

Transgressive Segregation. Some interbreeding populations of other species produce hybrids that are beyond the range of the combined phenotypic variation of the parental species, a phenomenon known as transgressive segregation and attributed to a variety of genetic and developmental causes (67–71). To detect transgressives, we compared admixed individuals (hybrids) with 99% ellipses around the joint beak shape–body size means of each of the three species, *G. fortis*, *G. fuliginosa*, and *G. scandens* (Fig. 4). The boundaries are conservative because some individuals assigned to a species by the ≥ 0.9 probability criterion may in fact be advanced-generation backcrosses, and, additionally, the sample size of one of the species, *G. fuliginosa*, is small. We found a single fS individual beyond the range of both parental species (Fig. 4B). However, this example is anomalous and should be discounted because admixture does not reflect ancestry: although they were not genotyped, both parent individuals were *G. fortis* morphologically. A few other individuals lay within the range of parental group phenotypes but just outside the ellipses. Noteworthy are three trihybrids that are smaller or larger than one of the parental species but with the same beak

Table 3. Comparison of morphological means from PCA between species and hybrids by Tukey HSD tests, contrasting size and shape of species and hybrids

| Species | Hybrid | Body size | | Beak size | | Beak shape | |
|-------------------|---------|-----------|---------|-----------|---------|------------|---------|
| | | P | Largest | P | Largest | P | Pointed |
| <i>fuliginosa</i> | Ff | 0.0001 | Hybrid | 0.0001 | Hybrid | 0.0001 | Species |
| <i>fortis</i> | Ff | 0.2723 | — | 0.0001 | Species | 0.0028 | Hybrid |
| <i>fuliginosa</i> | Sf | 0.0001 | Hybrid | 0.0001 | Hybrid | 0.4205 | — |
| <i>fortis</i> | FS | 0.9629 | — | 1.0000 | — | 0.0001 | Hybrid |
| <i>fortis</i> | Sf | 0.0001 | Hybrid | 0.9995 | — | 0.0001 | Hybrid |
| <i>scandens</i> | FS | 0.0001 | Species | 0.0619 | — | 0.0001 | Species |
| <i>scandens</i> | Sf | 0.0001 | Species | 0.0681 | — | 0.0001 | Species |
| <i>scandens</i> | Sf | 0.0002 | Species | 0.8883 | — | 0.0001 | Species |
| <i>fortis</i> | FfS+fFS | 0.0040 | Hybrid | 1.0000 | — | 0.0001 | Hybrid |
| <i>fortis</i> | FSf+fSF | 0.1922 | — | 0.9961 | — | 0.0003 | Hybrid |
| <i>fortis</i> | SfF+SfF | 0.0165 | Hybrid | 0.9998 | — | 0.0001 | Hybrid |
| <i>scandens</i> | FfS+fFS | 0.0001 | Species | 0.8100 | — | 0.0001 | Species |
| <i>scandens</i> | FSf+fSF | 0.0001 | Species | 0.1342 | — | 0.0001 | Species |
| <i>scandens</i> | SfF+SfF | 0.0001 | Species | 0.9138 | — | 0.0001 | Species |

Abbreviations: Symbols of the hybrids as in Tables 1 and 2. SE, SEM. PCA is principal components analysis. Sf and fS are combined as Sf. Sample sizes as used in Table 4.

Table 4. Ratios of variances of hybrids to species

| Hybrid | Species | df | Body size | Beak size | Beak shape |
|-----------|-------------------|---------|-----------|-----------|------------|
| Ff | <i>fuliginosa</i> | 112,17 | 2.76* | 8.45**** | 2.84* |
| Ff | <i>fortis</i> | 112,733 | 2.63**** | 2.63**** | 1.38*** |
| fS | <i>fuliginosa</i> | 22,17 | 2.80* | 5.81**** | 12.81**** |
| FS | <i>fortis</i> | 31,733 | 1.75* | 1.06 | 2.63**** |
| SF | <i>fortis</i> | 58,733 | 2.10**** | 1.12 | 2.80**** |
| FS | <i>scandens</i> | 31,200 | 1.06 | 1.83*** | 2.26**** |
| SF | <i>scandens</i> | 58,200 | 1.28 | 1.94**** | 1.75**** |
| Sf | <i>scandens</i> | 23,200 | 1.62* | 3.04**** | 3.44**** |
| FfS + fFS | <i>fortis</i> | 16,733 | 1.74* | 1.47 | 4.12**** |
| FSf + fSF | <i>fortis</i> | 12,733 | 4.85**** | 3.60**** | 3.48**** |
| SFf + SfF | <i>fortis</i> | 14,733 | 3.31**** | 1.60 | 3.83**** |
| FfS + fFS | <i>scandens</i> | 16,200 | 1.06 | 2.54**** | 2.57**** |
| FSf + fSF | <i>scandens</i> | 12,200 | 2.94**** | 6.23**** | 2.17* |
| SFf + SfF | <i>scandens</i> | 14,200 | 2.01* | 2.78**** | 2.38*** |

Symbols of the hybrids as in Tables 1 and 2. Variances are from principal components analyses (PCA). *df* = degrees of freedom. One to four asterisks are one-tailed probabilities from *F* tests in the sequence 0.05, 0.01, 0.005, 0.001.

shape as the species to which they are most genetically affiliated. Six large Ff dihybrids have unusually pointed beaks for birds of their body size. Collectively, these few individuals should be considered allometric transgressives as they are displaced from the principal axes of parental group covariation.

Fitness. We measured fitness in terms of survival and, independently, the probability of becoming a recruit to the breeding population. Maximum longevities recorded on Daphne were 17 y for both *G. fortis* and *G. scandens* (56). Hybrid longevities are apparently shorter (Fig. 5), but this is likely to be an artifact of their smaller sample sizes. The oldest hybrids are not included in Fig. 5 because they hatched in years before genotyping began. They are approximately the same: 13 y (one Ff, one Sf), 12 y (one SF), and 11 y (one FS).

Overall, di- and trihybrids survived approximately as well as *G. fortis* and *G. scandens* over the first 6 to 10 y of life in the period 1987 to 2010 (Fig. 5). Ff hybrid survival was almost identical to *G. fortis* survival, FS survival was lower after year 2 by up to 10%, and SF survival was higher than *G. scandens* survival until year 5. Dihybrids with admixture of *G. fuliginosa* and *G. scandens* had the highest survival. Relative survival of the trihybrids is heterogeneous. The two *G. fortis* trihybrid groups, FfS and FSf, survived better than *G. fortis*, whereas SFf survival was inferior to that of *G. scandens* by about 10% at each age. Note each group of dihybrid and trihybrids deviate from their respective species in opposite directions and hence their effects tend to cancel each other, resulting in no, or reduced, net advantage or disadvantage of the combined hybrids relative to the species. This is illustrated with *G. fortis* and hybrids in Fig. 5. *G. scandens* combined with hybrids shows the same pattern (SI Appendix, section 5 and Fig. S2).

Recruitment success of the two cohorts with the most complete data did not differ between species and hybrids (Table 5). A possible exception is the low recruitment of FS in the 1991 cohort, but the numbers are small, without statistical evaluation, and, furthermore, this hybrid group of the 1987 cohort experienced the opposite, high success. Together, survival and recruitment of hybrids provide no indication of a fitness disadvantage.

Hybrid Fitness in Relation to Morphology. Despite the high fitness of hybrids, those deviating most strongly in morphology from parental species may lie in a fitness valley between peaks occupied by the species. We tested the hypothesis with survival from year 1 posthatch to year 2 with the only samples sufficient for analysis from the period 2005 to 2012. The hypothesis was supported in the expected direction for the SF hybrids with PC data ($n = 48$, 0.62 survival) on body size ($F = 6.85$, $P = 0.0119$) and beak size ($F = 10.13$, $P = 0.0026$) but not beak shape ($F = 0.02$, $P = 0.8936$). The survival difference is not the consequence of males, the larger sex, surviving better than females, as it also occurs among males alone ($n = 28$, 0.75 survival) in body size ($F = 11.19$, $P = 0.0025$) and beak size ($F = 15.34$, $P = 0.0006$) but

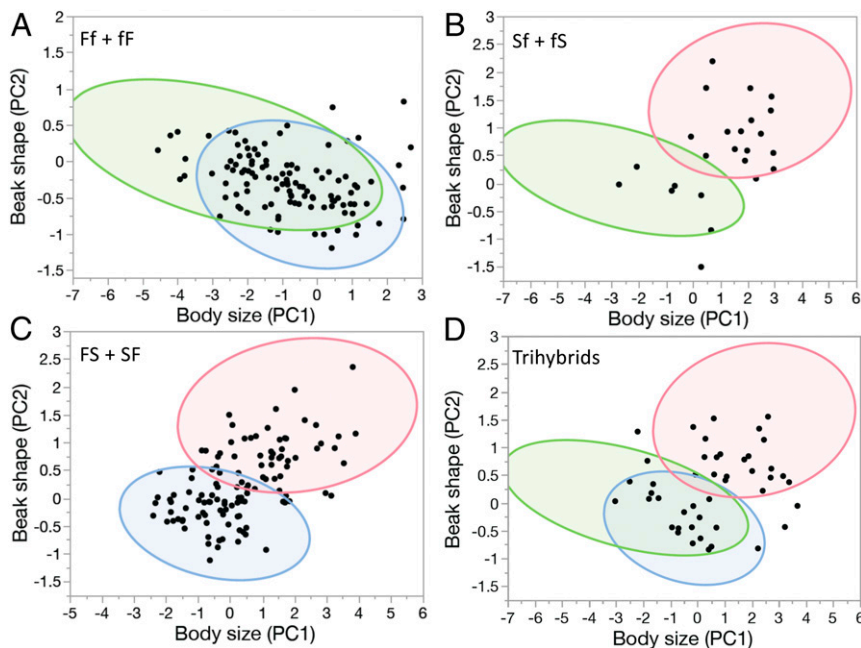


Fig. 4. Beak shape and body size of hybrids (dots) in relation to 99% ellipses around the distributions of *G. fuliginosa* (green), *G. fortis* (blue), and *G. scandens* (pink). (A) *G. fuliginosa* × *G. fortis* dihybrids. (B) *G. fuliginosa* × *G. scandens* dihybrids. (C) *G. fortis* × *G. scandens* dihybrids. (D) Trihybrids: beak pointedness increases from origin to the top, and body size increases from left to right. The smallest trihybrid outlier is fFS, and the other two are FfS.

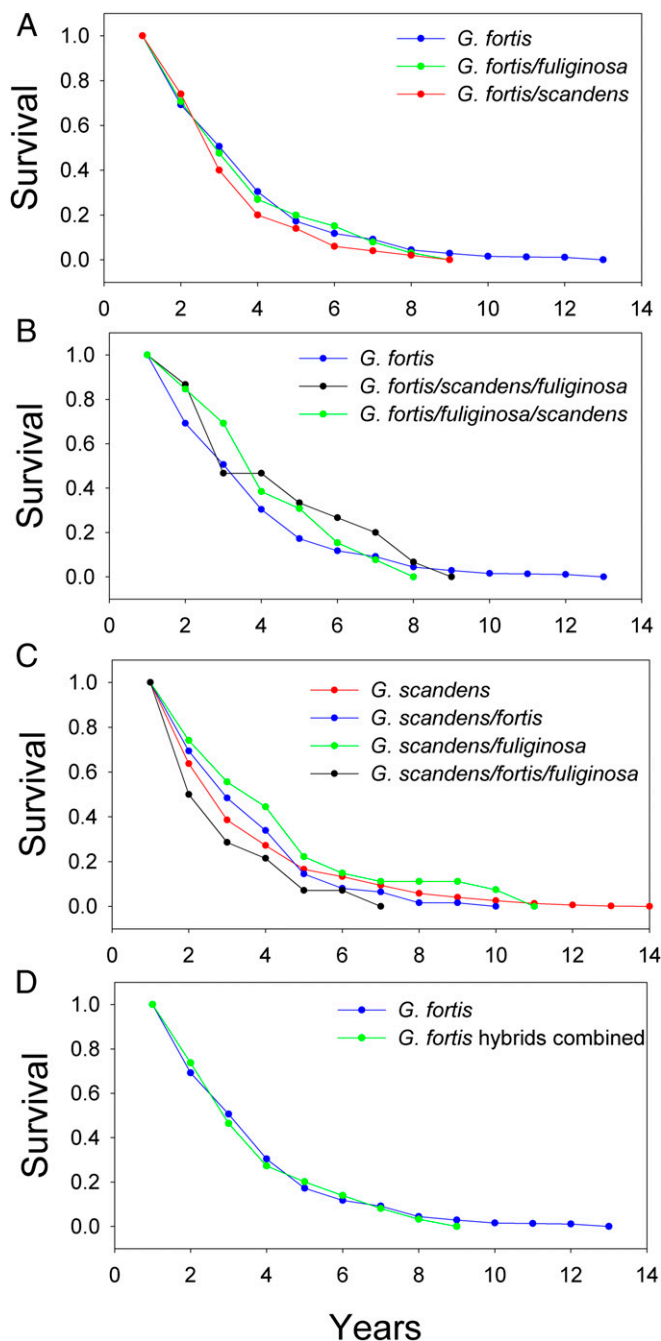


Fig. 5. Survival of di- and trihybrids in relation to *G. fortis* and *G. scandens* from year 1 in the years 1987 to 2010. (A) Dihybrids Ff (green) and FS (red). (B) Trihybrids FSf + FSf (black) and Ffs + fFS (green). (C) Di- and trihybrids SF (blue), Sf (green), and Sff + Sff (black). (D) *G. fortis* compared with dihybrids and trihybrids with predominant assignment to *G. fortis* (Ff, FS, Ffs, fFS, FSf, fSF).

not beak shape ($F = 1.72$, $P = 0.2006$). There are no morphological differences between surviving and nonsurviving FS hybrids ($n = 26$, all $P > 0.30$, 0.50 survival) or Ff hybrids ($n = 35$, all $P > 0.20$, 0.54 survival).

Discussion

The main finding of the study is the role of *G. fortis* as a conduit species, which is demonstrated by the occurrence of two types of hybrids. The first group of hybrids are *G. scandens* × *G. fuliginosa* admixtures (dihybrids), since these two species are not known to

interbreed on Daphne. *G. scandens* and *G. fuliginosa* differ strongly in the traits used in mate choice—beak size and shape, body size, and song (61, 62, 72)—whereas morphological difference between them are smaller on San Cristóbal island, where they do interbreed (personal observations, 1997 and 2018). The second group of hybrids are admixtures of three species (trihybrids).

We used neutral markers, microsatellites, to assign individuals to species or hybrids, using an arbitrary but often used and biologically justified criterion (36, 39, 43, 46) to separate them (*SI Appendix, section 2*). Assignments are subject to error (*SI Appendix, section 2*), and it is possible that the small number of loci results in an inflated number of erroneous assignments to hybrid classes. There are three reasons for believing this is not a source of strong bias. First, in a simulation study, most hybrids (0.85) were correctly identified (63). Second, each hybrid group has morphological features expected from its assigned genetic composition: intermediate means between the parental groups that are known or estimated to give rise to it, and increased variances. This would not be expected if many of the hybrids were not in fact hybrids. Third, there is no evidence of inflation in the samples from 1984 to 1998 when the pedigree allowed identification of first-generation backcrosses. Ten of 1,808 individuals with all four grandparents and both parents known have genomes with mixtures of *G. fortis*, *G. scandens*, and *G. fuliginosa*. Eight of the 1,808 individuals were assigned to trihybrids by microsatellites.

Existence of the two groups raises questions of genetics and fitness. How can a two-way or three-way mixture of genomes of different species function in individual organisms, what combinations of genes are compatible and which ones are not, how stable or transitory are their long-term dynamics, and what factors permit, promote, or hinder their existence? Answers to these questions can help to explain how barriers to interbreeding become strengthened in the process of speciation leading to complete reproductive isolation (1, 2, 15, 73, 74). Similar questions have been addressed in studies of interbreeding populations of Neanderthals, Denisovans, and ancestors of modern humans (75, 76), and the answers may portend future discoveries with finches and other organisms. Introgressive hybridization has been associated with a transfer of adaptive genes, for example those affecting skin color and immune function (75), deleterious genes affecting male fertility (76), and presumably an abundance of selectively neutral genes; in other words, a mixture, subject to repeated selective filtering.

To address these genetic questions, a preferable approach would be to use whole genomes. Nonetheless, microsatellites are

Table 5. Recruitment of hybrids and species from the two cohorts with most data

| Hybrid/species | Bred | Did not breed | Total | Proportion bred |
|--------------------|------|---------------|-------|-----------------|
| 1987 | | | | |
| <i>G. fortis</i> | 136 | 130 | 266 | 0.51 |
| Ff | 10 | 7 | 17 | 0.59 |
| FS | 5 | 2 | 7 | 0.71 |
| Ffs | 5 | 3 | 8 | 0.62 |
| 1991 | | | | |
| <i>G. fortis</i> | 204 | 428 | 632 | 0.32 |
| Ff | 16 | 24 | 40 | 0.40 |
| FS | 3 | 8 | 11 | 0.27 |
| Ffs | 2 | 1 | 3 | 0.67 |
| <i>G. scandens</i> | 30 | 88 | 118 | 0.25 |
| SF | 2 | 2 | 4 | 0.50 |

Symbols of the hybrids as in Tables 1 and 2, except that Ffs combines all trihybrids with an assignment probability greater to *G. fortis* than to *G. scandens*. Other hybrid categories were not present or represented by single individuals.

sufficient to show that, in combination, dihybrids and trihybrids were at no disadvantage in terms of survival or recruitment when compared with the species that gave rise to them during a period of favorable food conditions following the change in ecology caused by the 1982 to 1983 El Niño event. High fitness is surprising, and indicates that selection against novel gene combinations in this group of finches must be weak at most. The only disadvantage we could detect was low survival of the smallest SF hybrids. This should slow the rate of convergence of *G. scandens* on *G. fortis*; however, we doubt if this happens because it is counteracted by relatively high survival of Sf hybrids (Fig. 5), which are also smaller in body size than *G. scandens* (Table 3).

We can identify two factors that help to explain how the genome of one species is apparently tolerant to invasion (77) by two others. The first is the phylogenetic youth of the group. *G. fortis* and *G. fuliginosa* are sister species according to a whole-genome phylogenetic analysis of autosomes (28). They shared a common ancestor ~240,000 y ago, and they shared a common ancestor with *G. scandens* ~260,000 y ago or earlier. These are minimum estimates because allele sharing makes them appear to be more similar and younger than would be revealed by their true history. Their youth means that relatively few genes have diverged in separate species; they mainly affect morphological traits related to feeding and breeding ecology, and they cause no incompatibility and have little or no transgressive effects upon phenotypes. The second factor is the genetic similarity of *G. fuliginosa* and *G. scandens*, as indicated by their microsatellites. Although *G. fortis* and *G. fuliginosa* are the most similar pair of species (Nei's $D = 0.28$), *G. scandens* is more similar to *G. fuliginosa* ($D = 0.46$) than it is to *G. fortis* ($D = 0.76$). *G. scandens* is also more similar to *G. fuliginosa* in beak shape, although not in body size (Fig. 3D).

Triad hybridization, with one species acting as bridge or conduit between two noninterbreeding species, is valuable for what it reveals about the potential for the generation of novel phenotypes. Introgressive hybridization increases the potential by increasing additive genetic variances, altering genetic covariances, and constructing new combinations of interacting genes (12, 15). As expected, we found increased morphological variances in both dihybrids and trihybrids. A previous study provided evidence of relaxed genetic covariances in dihybrids (17), as has also been found in fish (21, 70). Multivariate effects should be greater through hybridization of three species than of two species. We found no clear case of extreme phenotypes beyond the range of variation of both of the contributing parental groups (transgressive segregation); however, three trihybrids displayed allometric transgression, lying outside the region of body size variation but not outside the range of beak shape variation of the parental groups. Some dihybrids displayed similar patterns. The evolutionary potential of allometric transgression has been demonstrated with the origin of a new lineage on Daphne Major. The lineage was initiated by hybridization of an immigrant *Geospiza conirostris* with a resident *G. fortis*, resulting in the formation of a reproductively isolated population that displayed allometric transgression, that is, a deviation from the two species in the relationship between beak size and body size (27, 78). Transgressive morphology is implicated in reproductive isolation because mate choice is based in part on beak and body size (61, 62, 72) and the relationship between the two (79).

Triad hybridization is likely to be more common than is currently recognized by the few cases that have been documented. For example, we would expect it in species-rich adaptive radiations that have diversified relatively recently. Foremost among them are the hundreds of cichlid fish species in the Great Lakes of Africa, for which genome data are becoming rapidly available (78–84), and the many species of *Heliconius* butterflies that participate in mimicry rings (3, 26, 43, 85). Other possible groups are numerous species complexes that are similar morphologically

and genetically and sometimes difficult to resolve taxonomically, such as Cottoid fish in Lake Baikal (86), *Anastrepha* flies (87, 88), ant-nest beetles (89), some groups of mosquitoes (44) and, among plants, Andean lupins (90) and the Hawaiian Silversword Alliance (91).

Since triad hybridization occurs in contemporary time, it must have occurred in the past. Together with hybridization of one species with two others separated in time or in space (a pair of dyads) (92, 93), triad hybridization may be responsible for polytomies in phylogenetic trees. Polytomies occur when branching cannot be resolved into bifurcations because an ancestral species and two descendant species are genetically so similar (94–97). The power to measure divergence is likely to be reduced when the samples are affected by triad hybridization.

Hybridization on Daphne is relevant to a global outlook on the future. The frequency and success of hybridization on the island increased from 1983 onward as a result of a change in vegetation caused by abundant rain associated with an intense and prolonged El Niño event. This major perturbation is a natural analog of an anticipated unnatural, human-caused change in the global environment. Whether or not populations have sufficient genetic variation for adaptive responses is the subject of ongoing critical debate (98–101). One way that variation is enhanced is through interspecific gene exchange (19, 102). Hybridization is believed to be increasing in frequency as a result of anthropogenically caused change to climate and to habitat that reduces population sizes and brings together previously separated species (102–106). Hybridizing species may therefore be disproportionately successful in coping with a changing environment in the future, as in the past (9, 18, 107). Fur seals on Macquarie Island (39) provide a prime example of what might come. The original population became extinct as a result of overexploitation in the 19th century. The island has been recolonized by two interbreeding species, *Arctocephalus gazella* and *Arctocephalus tropicalis*. A ménage à trois was created by a single male of a third species, *Arctocephalus forsteri*, and it led to the production of offspring with genes from all three species. It remains to be seen in this case and more generally if multispecies hybridization has significant consequences in terms of evolution and conservation in a world of increasing habitat destruction and climate change.

Methods

Sampling Design. Field methods have been extensively described in previous publications (56, 57, 63, 65). Birds were captured in mist nets every year from 1973 to 2012. All birds were measured as adults (63), defined as birds in the year following the year of hatching, except in 1983 and 1987, when some birds that hatched that year bred and so are included as adults. The proportion of adults captured, measured, and banded uniquely was ~90% in 1979 and ~98% in 1981 (108). By 1992, all breeding adults had been banded. A large number of nests were followed in the years 1976 to 1978, and, from 1979 to 1998, attempts were made to find all nests on the island and identify their owners (banded or not banded). In later years, the year of hatching was estimated by plumage of birds in the hand (56). Nestlings were banded at day 8. Blood samples were taken from birds captured in mist nets or from nestlings for microsatellite analyses. Sixteen unlinked polymorphic loci were used for parentage and hybrid assignment with the program STRUCTURE, as described previously (63). The Princeton University Animal Care Committee approved the research procedures.

Statistical Analyses. Statistical analyses were performed in JMP (SAS Institute). All tests were two-tailed unless indicated otherwise.

Data Availability. Supplementary Information.

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