

pha, beta, and gamma males are well known to use discrete alternative mating behaviors (7–10). In these species, negative frequency-dependent selection with intransitive fitness interactions among tactics (e.g., rock-paper-scissors) appear to maintain trimorphism within populations (9, 10, 32). It is unknown how male reproductive behavior varies in species with facultative trimorphism, and it will be important to determine whether similar fitness intransitivity of tactics applies in these cases. It is interesting that the smallest male forms (gamma males), at least in the beetle families studied so far, invariably resemble females. This suggests that male reproductive tactics may include a dominant (fight/guard) tactic, a subordinate (sneak) tactic, and a female-mimicry tactic. This is a striking parallel to allelically trimorphic fish (8), isopods (7), and birds (11) and would support predictions from recent rock-paper-scissors models that suggest that many taxa will contain cryptic (undetected) female-mimicking males (10, 32).

Recognizing that there are at least two distinct thresholds also affects studies of the evolution of these developmental mechanisms. It is already clear that there are many developmental routes to the polyphenic regulation of male weapon systems in beetles, and, as these mechanisms are discovered and described, they are routinely compared across species (20–22, 33). We suggest that the most meaningful comparisons will be those that explicitly consider the type of threshold mechanism involved and treat these accordingly as distinct and evolutionarily independent processes. Our findings raise the possibility that horned male majors in species with threshold

mechanism 1 (Fig. 1A, left) are actually, developmentally, beta males, whereas the horned majors in species with threshold mechanism 2 (Fig. 1A, right) are alpha males. Acknowledging this distinction can help us better understand the full complexity of their rich behavioral repertoires, as well as more appropriately study the developmental and genetic architectures of their facultative regulatory mechanisms.

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#### Supporting Online Material

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Materials and Methods  
Figs. S1 and S2

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## Sequential Sympatric Speciation Across Trophic Levels

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A major cause for biodiversity may be biodiversity itself. As new species form, they may create new niches for others to exploit, potentially catalyzing a chain reaction of speciation events across trophic levels. We tested for such sequential radiation in the *Rhagoletis pomonella* (Diptera: Tephritidae) complex, a model for sympatric speciation via host plant shifting. We report that the parasitic wasp *Diachasma alloeum* (Hymenoptera: Braconidae) has formed new incipient species as a result of specializing on diversifying fly hosts, including the recently derived apple-infesting race of *R. pomonella*. Furthermore, we show that traits that differentially adapt *R. pomonella* flies to their host plants have also quickly evolved and serve as ecological barriers to reproduction, isolating the wasps. Speciation therefore cascades as the effects of new niche construction move across trophic levels.

The idea that species induce speciation has been inferred to explain current and past patterns of biodiversity by paleontologists, ecologists, and evolutionary biologists alike (1–3). However, this hypothesis of sequential radiation is difficult to directly test in nature. Examples such as adaptive radiations after mass extinctions (4), species richness in the tropics (1), and the increased diversity of insects having herbivorous

life styles (5, 6) have mainly been investigated on the basis of phylogenetic inference and/or correlative analyses.

Host plant-specific phytophagous insects and their parasites may be good candidates for testing the sequential radiation hypothesis (7). This is because new resource opportunities become available when a plant-eating insect diversifies by shifting and adapting to a novel host plant, with its guild

of associated parasites potentially following suit and speciating in kind. Unfortunately, a lack of historical and biogeographic information concerning host shifting and the absence of a free-living parasite life stage often complicate our understanding of plant-insect-parasite systems. In these cases, cocladogenesis (cospeciation resulting from parallel allopatry of interacting organisms) rather than the cascading effects of shifting host ecology across trophic levels could trigger codiversification (8). One cannot rule out that insect and parasitoids became separated in tandem from other conspecifics and evolved into new species as a consequence of their shared physical isolation.

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The *Rhagoletis pomonella* sibling species complex is a model for speciation in the absence of geographic isolation via host plant shifting. The natural history of these flies, including the recent sympatric host shift of the species *R. pomonella* from hawthorn to introduced, domesticated apple within the past 150 years (9), suggests that they did not evolve as a result of passive cocladogenesis. Therefore, we tested for sequential radiation of its specialist parasitoid wasp, *Diachasma alloenum* [see supporting online material (SOM) text for parallels between fly and wasp biology], by examining whether wasps attacking the ancestral hawthorn and the derived apple races of *R. pomonella*, as well as the closely related sibling species *R. mendax* (host: blueberry, *Vaccinium* spp.) and *R. zephyria* (snowberry, *Symphoricarpos* spp.), display patterns of host-related genetic variation similar to those of the flies. *D. alloenum* only attacks *R. pomonella* complex flies found on these four host plants (10). We also investigated whether wasps differed by the same type of host plant-specific mating and diapause traits that are ecologically isolating the flies.

The pattern of genetic differentiation among *D. alloenum* populations was similar to that for *R. pomonella* flies (11, 12). Mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) sequences displayed only modest host-related differentiation for the wasps (Fig. 1A, figs. S1 and S2, and tables S1 and S2). Therefore, the wasps are apparently of relatively recent origin and do not represent highly genetically diverged cryptic sibling species. However, we did find a mtDNA haplotype in snowberry wasps that was not found in the other wasp populations (fig. S2 and table S2). In addition, a common mtDNA haplotype in apple, hawthorn, and blueberry wasps was not present in the snowberry population. Thus, in a mtDNA genetic distance network, the snowberry wasp was offset from the other taxa (Fig. 1A).

Nine of 21 microsatellite loci analyzed (DA003, DA013, DA019, DA150, DA174, DA183, DA192, DA202, and DA205) displayed consistent host-related allele frequency differences between at least two of the wasp populations (table S3 and SOM appendix), much like allozymes for *R. pomonella* flies (13, 14). Locus DA003 showed particularly pronounced frequency differences (see Fig. 2 for graph of combined DA003 196+200+204 allele frequencies). Indeed, allele 196 was common in apple (mean frequency = 0.292;  $n = 291$  total alleles scored), blueberry (0.179,  $n = 246$ ), and snowberry (0.257,  $n = 101$ ) wasp populations but absent from all hawthorn wasps ( $n = 385$  alleles scored; SOM appendix). The lack of allele 196 in hawthorn wasps for locus DA003 suggests that there is little or no effective interhost gene flow from apple, blueberry, or snowberry wasp populations into the hawthorn population. Neighbor-joining trees for the nine loci displaying host-related divergence (Fig. 1B) and the full 21-loci data set (fig. S3) separated hawthorn and blueberry wasp populations at different ends of the networks. The lone snowberry wasp site analyzed was genetically most closely as-

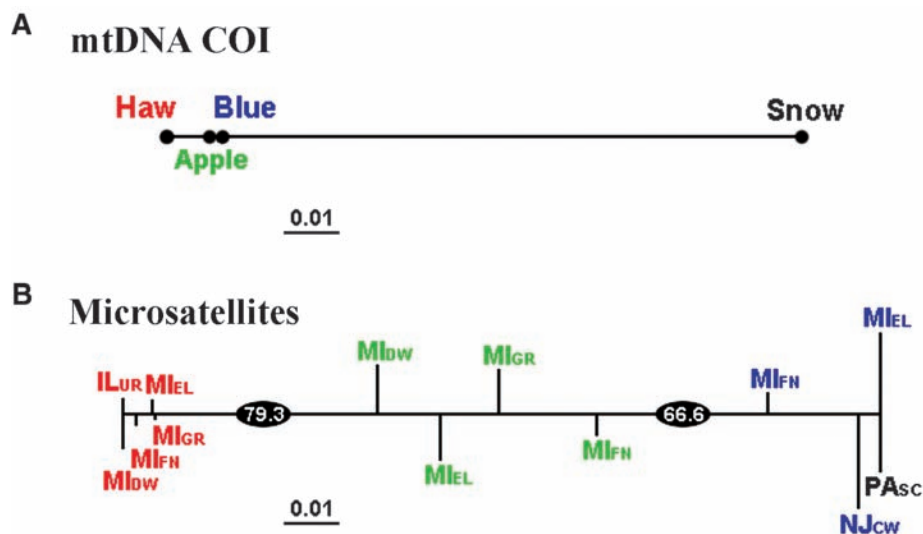
sociated with blueberry wasps, whereas the apple wasps were placed at an intermediate position between blueberry and hawthorn populations (Fig. 1B).

We next investigated whether the same host-related adaptations ecologically isolating *R. pomonella* flies were responsible for genetically differentiating the wasps. Because *Rhagoletis* flies mate on or near the fruit of their respective host plants (15–17), host choice generates prezygotic isolation and facilitates the evolution of host-specific performance traits. A field study of free-living adult *D. alloenum* in a blueberry patch in Fennville, MI, suggested that the wasps also use host fruit as a rendezvous site for mating. Before copulation, all male and female wasps that formed the 24 mating pairs recorded in the study were first observed flying toward blueberries from distances of 1 to 3 m away. In all cases, wasps flew close to blueberries, sometimes making contact with the fruit, before they initiated coupling on nearby host leaves [mean distance mating from fruit =  $7.4 \pm 6.3$  cm (SD),  $n = 24$ , range = 3 to 30 cm]. The average time from first observation to mating was  $172.2 \pm 94.7$  s SD.

*Rhagoletis* flies use the volatile compounds emitted from the surface of ripening fruit as olfactory cues to both find and discriminate among host plants for mating and oviposition (18). We thus tested whether wasps show similar discriminatory behavior for surface host fruit volatiles by using a y-tube olfactometer (fig. S4). We found that naïve adult wasps from apple, hawthorn, and blueberry populations positively oriented to the arm of the y-tube containing their natal fruit odor and were antagonized by nonnatal volatiles (Table 1). There was no qualitative difference between males

and females in their behavioral responses to surface fruit volatiles. However, female hawthorn wasps displayed stronger preferences for natal hawthorn fruit volatiles (Fisher's exact test,  $P = 0.006$ , 1 df) and more pronounced antagonism to nonnatal apple volatiles ( $P = 0.030$ , 1 df) than male hawthorn wasps did. Snowberry wasps showed behavioral specificity for their natal fruit volatiles that was similar to that of the other parasites, but too few snowberry wasps were available for testing to draw definitive conclusions (Table 1). All wasps exhibited behavioral antagonism to the volatiles of flowering dogwood fruit (*Cornus florida*), a host for an undescribed sister taxon to *R. pomonella* that *D. alloenum* does not attack (19). These data suggest that fruit odor discrimination may act in a similar manner in the wasps as in the flies and may generate host-specific mating, resulting in an ecological barrier to gene flow in sympatry. Because we cannot mate or rear *D. alloenum* for multiple generations in the laboratory, it is unknown whether maternal effects and/or larval conditioning may contribute to genetically based fruit volatile discrimination. In the rare instances in which larval experience was inferred to influence an adult insect's preference, the effect was mediated via direct larval contact with plant compounds (20). But, because *D. alloenum* has no direct contact with surface fruit volatiles at any stage of its life before adult eclosion, larval conditioning is unlikely.

Diapause life-history differences represent a second critical ecological barrier to gene flow among *Rhagoletis* flies (21). Sympatric blueberry, apple, and hawthorn flies all eclose as adults at different times in the spring and/or summer, matching the timing of fruit ripeness on their respective



**Fig. 1.** Genetic distance networks depicting relationships of *D. alloenum* wasp populations attacking blueberry (blue), apple (green), hawthorn (red), and snowberry flies (black). (A) Fitch network created on the basis of Nei genetic distances of mtDNA haplotype frequencies in wasp populations. (B) Neighbor-joining Nei genetic distance network of the nine microsatellite loci showing consistent site-to-site host-related frequency differences. Black ovals give bootstrap confidence values (10,000 replicates). Site abbreviations are MI<sub>GR</sub> = Grant, MI; MI<sub>HL</sub> = Holland, MI; MI<sub>EL</sub> = East Lansing, MI; MI<sub>FN</sub> = Fennville, MI; MI<sub>DW</sub> = Dowagiac, MI; IL<sub>UR</sub> = Urbana, IL; PA<sub>SC</sub> = State College, PA; NJ<sub>CW</sub> = Chatsworth, NJ. See fig. S1 and table S1 for a map and descriptions, respectively, of study sites.

host plants (Fig. 3). Because *Rhagoletis* flies have only one generation per year and live for about a month in the field, the differences in the timing of eclosion can allochronically isolate the flies. Laboratory rearing experiments and field studies of *D. alloenum* indicated that similar eclosion time differences exist among blueberry, apple, and hawthorn wasps (Fig. 3 and table S4). The lone exception was a late fruiting apple from the Dowagiac, MI site where apple wasps had a relatively late mean eclosion time of  $112.9 \pm 1.01$  days (SE) ( $n = 102$ ) that overlapped with that of hawthorn wasps ( $111.1 \pm 1.13$  days,  $n = 44$ ). Mean longevity of adult *D. alloenum* was determined to be less than 2 weeks in the laboratory ( $12.9 \pm 0.4$  days SE,  $n = 286$ ). Assuming wasps have a similar lifespan in nature (a likely overestimate), eclosion time differences may decrease

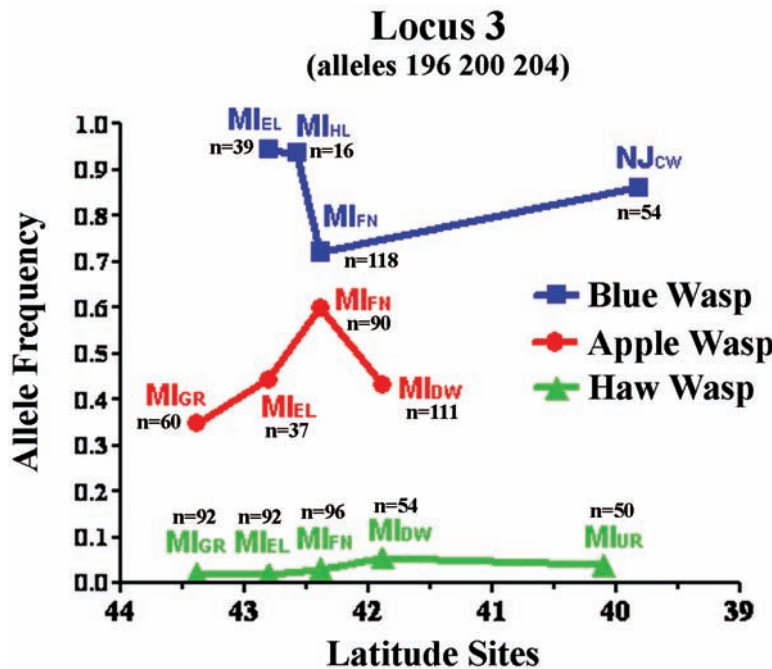
seasonal overlap and mating opportunities between blueberry and apple wasps at the sympatric Fennville, MI site by 28.9%, between apple and hawthorn wasps by 29.8%, and between blueberry and hawthorn wasps by 74.5%.

For *R. pomonella*, the allozymes displaying host-related frequency differences correlate with eclosion time (9), tying together the genetics of host race formation with a trait that allochronically isolates the flies. We tested for similar relationships for the microsatellite loci separately in male and female wasps because of sex-related differences in eclosion time (male wasps eclose several days earlier than females). For *D. alloenum*, the microsatellite loci were significantly correlated with variation in eclosion time for hawthorn ( $r^2$  stepwise multiple regression for females = 0.504,  $P < 0.0001$ , 52 df;  $r^2$  males = 0.525,  $P <$

0.0001, 46 df), apple ( $r^2$  females = 0.163,  $P = 0.0097$ , 54 df;  $r^2$  males = 0.657,  $P < 0.0001$ , 30 df), and blueberry ( $r^2$  females = 0.686,  $P < 0.0001$ , 29 df;  $r^2$  males = 0.511,  $P < 0.0001$ , 53 df; table S5) wasps. In particular, the locus DA003 was significantly correlated with eclosion time variation in blueberry wasps ( $r^2$  females = 0.378,  $P = 0.0003$ , 29 df;  $r^2$  males = 0.511,  $P < 0.0001$ , 53 df) but only moderately in female apple wasps ( $r^2$  females = 0.101,  $P = 0.0182$ , 54 df) and was not a significant predictor for hawthorn wasps. The alleles 196+200+204 at locus DA003 were associated with earlier eclosion times in both female and male blueberry and apple wasps (fig. S5). These three alleles were present in highest frequency for blueberry wasps (0.790,  $n = 205$  total alleles scored), intermediate for apple (0.485,  $n = 260$ ), and lowest for hawthorn wasps (0.029,  $n = 385$ ), corresponding to the order of eclosion from earliest to latest for these populations (Fig. 3). Locus DA003 therefore may represent a naturally segregating, major effect quantitative trait locus for diapause that is associated with ecological reproductive isolation among *D. alloenum* populations.

Our results imply that sympatric host shifts of *R. pomonella* onto new plants may have triggered a reciprocal and rapid starburst of adaptive radiation for its parasitoid, *D. alloenum*. We found that host-related ecological effects initiating speciation for *Rhagoletis* rippled through the community and may have amplified diversity for the wasp. Although we were unable to unambiguously resolve the source of the recently formed apple wasp race in this study, the absence of the 196 allele at microsatellite locus DA003 in the hawthorn wasp race suggests that the hawthorn wasp race is not the sole progenitor of the apple wasps. Together with the mtDNA data, allele 196 and its association with early eclosion instead suggest that blueberry wasps may have given rise to the apple wasp population. It is possible, however, that apple wasps are hybrids resulting from crosses between hawthorn and blueberry wasps on the basis of both their intermediate position in the microsatellite distance network and their intermediate overlapping eclosion time. Regardless, our study demonstrates that the origin of the apple wasp was not a result of strict 1:1 cladogenesis with apple-infesting *Rhagoletis* flies. One intriguing possibility is that the wasps themselves may be promoting *Rhagoletis* host shifts. Lower parasitism rates for *R. pomonella* in derived hosts like apple [i.e., enemy-free space (22)] could favor race formation for the fly, creating new niches promoting wasp divergence. We must also determine whether non-host-related premating and intrinsic postzygotic isolation exist in the genus *Diachasma*. The sequential radiation hypothesis predicts that if nonecological barriers exist they should arise after host-related barriers evolve.

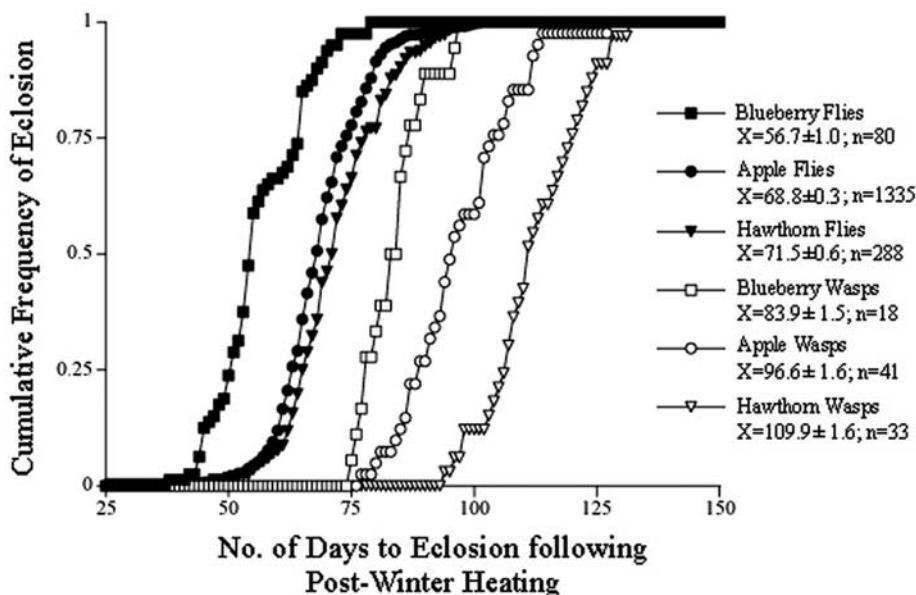
We have presented one case study supporting sequential speciation. Other studies have identified potential examples (7), and, because *D. alloenum* is just one member of a guild of parasitic braconids attacking *R. pomonella*, it is possible that sympatric host races of *Diachasmimorpha mellea*



**Fig. 2.** Combined 196+200+204 allele frequencies for locus DA003 for *D. alloenum* wasp populations attacking blueberry (blue sites), apple (green sites), and hawthorn (red sites) arrayed by latitude in the United States.  $n$  is number of alleles scored for site. See Fig. 1 legend for site abbreviations.

**Table 1.** Percentages of increase (+ indicates preference) or decrease (–, antagonism or avoidance) in the behavioral orientation of populations of *D. alloenum* wasps to the arm of a y-tube olfactometer containing the indicated host fruit volatiles compared with blank control experiments. Control values (bottom row) denote the percentage of wasps orienting to either the right or the left arm of the y-tube in experiments when no odor was present in both arms. There was no directional preference for either arm of the y-tube in the blank control runs, and the remaining ~23% of wasps that did not orient right or left failed to move up the y-tube from their release point. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and †not significant.

Host fruit volatiles	Apple wasps $n = 201$	Blueberry wasps $n = 110$	Hawthorn wasps $n = 330$	Snowberry wasps $n = 9$
Apple	+54%***	–29%**	–18%**	–14%†
Blueberry	–23%***	+42%***	–28%***	–14%†
Hawthorn	–33%***	–34%***	+44%***	–14%†
Snowberry	–46%***	–11%†	–77%***	0%†
Dogwood	–36%***	–53%***	–16%***	–14%†
Control	38%	39%	38%	39%



**Fig. 3.** Cumulative eclosion curves for blueberry-, apple-, and hawthorn-infesting *Rhagoletis* flies (solid shapes) and their associated *D. alloem* wasp parasites (open shapes) at a sympatric field site near Fennville, MI. Mean times to adult emergence after overwintering (in days) and sample sizes (*n*) are given in the figure key.

and *Uteles canaliculatus* wasps are also associated with *R. pomonella*. Given that over half of all animals may be parasites in a broad sense (23), that there are more phytophagous insects than any other life form (23), and that 20% of all insects may be parasitic wasps (24), there is a world of opportunity for sequential speciation in nature.

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**Supporting Online Material**

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 Materials and Methods  
 SOM Text  
 Figs. S1 to S5  
 Tables S1 to S5  
 SOM Appendix

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# Evolution of the *Drosophila* Nuclear Pore Complex Results in Multiple Hybrid Incompatibilities

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Speciation often involves the evolution of incompatible gene interactions that cause sterility or lethality in hybrids between populations. These so-called hybrid incompatibilities occur between two or more functionally divergent loci. We show that the *nucleoporin 160kDa* (*Nup160*) gene of the fruitfly *Drosophila simulans* is incompatible with one or more factors on the *D. melanogaster* X chromosome, causing hybrid lethality. *Nup160* encodes a nuclear pore complex protein and shows evidence of adaptive evolution. Furthermore, the protein encoded by *Nup160* directly interacts with that of another hybrid lethality gene, *Nup96*, indicating that at least two lethal hybrid incompatibility genes have evolved as byproducts of divergent coevolution among interacting components of the *Drosophila* nuclear pore complex.

As species diverge from one another, they accumulate genetic substitutions that function within their own genomic back-

ground but, when brought together in hybrids, can disrupt gametogenesis (causing hybrid sterility) or development (causing hybrid lethality) (1, 2).

The evolution and genetics of these hybrid incompatibilities have been shown to follow specific rules. For instance, hybrid incompatibilities tend to accumulate gradually as species diverge (3); behave as partial recessives in hybrids (4); follow Haldane’s rule [that is, the preferential sterility or inviability of hybrids of the heterogametic (XY or ZW) sex (5)]; and accumulate disproportionately on the X chromosome (that is, the large X-effect) (6, 7). The molecular biology of hybrid incompatibilities has revealed that five of six hybrid incompatibility genes identified so far show signatures of recurrent adaptive evolution (8–13), which suggests that hybrid sterility and inviability generally evolve as incidental byproducts of positive natural selection.

To test whether this emerging molecular rule of speciation holds for additional hybrid incompatibility loci, we performed a genetic screen for lethal hybrid incompatibilities between *Drosophila melanogaster* and *D. simulans*, two species that diverged ~3 million years ago. Because all hybrids between *D. melanogaster* and