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PATTERNS OF POSTZYGOTIC ISOLATION IN LEPIDOPTERA

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Abstract.—I present patterns characterizing the evolution of intrinsic postzygotic isolation in Lepidoptera by analyzing data from the literature on genetic distance, strength of hybrid sterility and inviability, biogeography, and natural hybridization. Using genetic distance as a proxy for time, I investigate the time-course of the evolution of postzygotic isolation and the waiting times to particular hybrid fitness problems. The results show that postzygotic isolation increases gradually as species diverge, but that hybrid sterility evolves faster than hybrid inviability. The overwhelming preponderance of female-specific hybrid problems in Lepidoptera shows that Haldane's rule (the preferential sterility or inviability of the heterogametic sex) is well obeyed. Together the rates and patterns characterizing the accumulation of postzygotic isolation allow several tests of the composite theory of Haldane's rule. Interestingly, comparing these data with those from *Drosophila* reveals that Haldane's rule for sterility evolves as fast (if not faster) in Lepidoptera. Finally, I show that a substantial fraction of sympatric species hybridizes in nature and that the majority of these suffer some level of hybrid sterility or inviability.

Key words.—Haldane's rule, hybrid inviability, hybrid sterility, Lepidoptera, speciation.

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Few topics in evolutionary biology inspire as much controversy as the causes of speciation (Otte and Endler 1989; Coyne and Orr 1998; Howard and Berlocher 1998; Turelli et al. 2001). Hard answers have proven difficult to come by for two reasons. One is that speciation is usually slow and therefore unobservable in real time. The second is that taxa differ in their rates and modes of speciation, both being contingent on details of geography, ecology, mating system, and genetics (Mayr 1942). Drawing broad conclusions about the causes and the importance of any one form of reproductive isolation thus requires a comparative approach using information from many species. Coyne and Orr's (1989a, 1997) classic study of *Drosophila* speciation, for instance, revealed several important patterns that could not have been detected from the study of a single or even a few species pairs, for example, that pre- and postzygotic isolation evolve gradually and at similar rates among allopatric taxa. But can we generalize from these *Drosophila* data? Progress has recently been made on this front through studies of other taxa, at least for one type of reproductive isolation: Patterns characterizing the evolution of intrinsic postzygotic isolation (hybrid sterility and inviability) have recently been reported in frogs (Sasa et al. 1998) and birds (T. Price and M. Bouvier, unpubl. ms.). Here I extend such studies by analyzing patterns of intrinsic postzygotic isolation in Lepidoptera.

Two patterns appear to characterize the evolution of intrinsic postzygotic isolation in flies, frogs, and birds. First, hybrid sterility and inviability evolve gradually, giving rise to a rough ''speciation clock'' (Coyne and Orr 1989a, 1997; Sasa et al. 1998; Orr and Turelli 2001; T. Price and M. Bouvier, unpubl. ms.). The evolution of hybrid fitness problems likely reflects the gradual accumulation of deleterious epistatic interactions between species (Dobzhansky 1937; Muller 1940, 1942). Such hybrid incompatibilities accumulate as a side effect of normal adaptive or neutral divergence (Dobzhansky 1937; Muller 1940, 1942; Orr 1995; Johnson 2000). Although this classical Dobzhansky-Muller model of speciation is generally accepted, special processes might result in instantaneous speciation, for example, microbe-associated incompatibilities (Werren 1998), founder-effect genetic revolutions (Mayr 1954; Carson and Templeton 1984), and polyploid speciation (Stebbins 1950). Such instantaneous speciation would render any speciation clock erratic and, if common enough, would obliterate it.

The second pattern characterizing postzygotic isolation is Haldane's rule, the fact that when one hybrid sex is sterile or inviable, it is usually the heterogametic (XY) sex (Haldane 1922; for simplicity I will speak of X and Y chromosomes, not Z and W). Though many of Haldane's original examples were from Lepidoptera, Haldane's rule is now known to hold across a wide range of animals (Coyne 1992; Wu and Davis 1993; Laurie 1997; Orr 1997; Turelli 1998). Indeed Haldane's rule appears to be an early and nearly obligate phase of speciation, at least in *Drosophila*—almost all cases of hybrid sterility or inviability between young species pairs are limited to males (Coyne and Orr 1989a, 1997). The ubiquity of Haldane's rule suggests that the types of genetic changes underlying it—and thus underlying postzygotic problems generally—may be similar in most or all animals (Coyne 1992; Wu and Davis 1993; Wu and Palopoli 1994; Laurie 1997; Orr 1997; Turelli 1998; Orr and Presgraves 2000).

The possible generalities embodied in Haldane's rule thus largely inspired the renaissance in speciation genetics during the last 15 years (Coyne 1985, 1992; Wu and Davis 1993; Laurie 1997; Orr 1997; Turelli 1998). Most workers now agree that Haldane's rule is a composite phenomenon reflecting the confluence of several evolutionary and genetic factors. First, the dominance theory posits that most hybrid incompatibilities act as partial recessives in hybrids (Muller 1940, 1942; Orr 1993; Turelli and Orr 1995, 2000). Haldane's rule results because XY hybrids suffer the full effects of any recessive X-linked incompatibilities; XX hybrids, despite carrying twice as many X-linked incompatibilities, are heterozygous for the X and therefore protected from most recessive problems (Orr 1993; Turelli and Orr 1995, 2000).

The second factor contributing to Haldane's rule for sterility, at least in male-heterogametic taxa (e.g., *Drosophila*, mammals), is faster-male evolution, which seeks to explain

the more rapid evolution and abundance of hybrid male sterility (Wu and Davis 1993; Wu et al. 1996). The faster-male theory includes two possible explanations. One is that sexual selection, being typically more intense in males, drives the rapid divergence of male fertility factors that later cause hybrid male sterility. The other explanation is that spermatogenesis may be inherently more sensitive to the genetic perturbations experienced by hybrids. In either case, faster-male evolution can explain patterns in comparative and genetic data. Comparative studies, for instance, find far more cases of hybrid male sterility than either female sterility or inviability in male-heterogametic taxa (Wu and Davis 1993; Wu et al. 1996). Although dominance theoretically can account for many or most of these cases, heterogametic male sterility also occurs in species hybrids where dominance cannot contribute, that is, among species lacking a degenerate Y chromosome (Presgraves and Orr 1998). Genetic mapping experiments also find far more hybrid male than female sterility factors between particular species pairs (Hollocher and Wu 1996; True et al. 1996; Sawamura et al. 2000). The composite theory, then, is that dominance contributes to Haldane's rule for sterility and inviability in taxa possessing degenerate Y chromosomes, while faster-male evolution helps cause hybrid male sterility in male-heterogametic taxa.

In this paper, I bring together information from the literature on intrinsic postzygotic isolation and genetic distance among Lepidoptera to describe the time course of speciation, disentangle the forces driving the evolution of postzygotic isolation and Haldane's rule, and compare the rate at which postzygotic isolation evolves in Lepidoptera with that in *Drosophila*. Although much energy has been devoted to the evolution and genetics of intrinsic postzygotic isolation, some question its importance in speciation. Postzygotic barriers are, after all, only realized when prezygotic ones are incomplete or absent. I therefore also gathered data on biogeography and natural hybridization among species to estimate how many species form natural hybrids that experience intrinsic postzygotic isolation.

For a number of reasons, Lepidoptera are ideal for such a study. First, they differ genetically from *Drosophila* in several important ways. In Lepidoptera, females, not males, are the heterogametic sex. Moreover, only about 3–5% of the Lepidopteran genome is X-linked, not 20–40% as in *Drosophila*. Together these differences allow several tests of the composite theory of Haldane's rule. Another difference is that, despite the small size of Lepidopteran X chromosomes, a striking large-X effect exists for phenotypic species differences—more than half of all morphological, behavioral, and physiological species differences show X-linked effects (Sperling 1994; Prowell 1998). This intriguing pattern motivates a test for a large-X effect on intrinsic postzygotic isolation. Next, Lepidoptera have been favorites of naturalists for hundreds of years. There are thus reasonably good data on their geographic distribution and natural hybridization. Finally, Lepidoptera, with roughly 112,000–140,000 species, are the second most species-rich group of animals after Coleoptera (Mitter et al. 1988; Prowell 1998). It seems worth asking if intrinsic postzygotic isolation has played any interesting role in their origin and maintenance.

Materials and Methods

I compiled data from the literature on experimental hybridization, genetic distance, phylogeny, biogeography, and natural hybridization for more than 200 species pairs of Lepidoptera. An extensive downloadable list of references is available online at http://www.rochester.edu/College/BIO/labs/ORRLAB/ORRHOME.HTML. Below I outline criteria for including studies in the dataset. Although I tried to collect as much information as possible, some reports from this expansive literature (particularly those not in English) have no doubt been overlooked. Unfortunately, not all forms of data were available for all species pairs. These data are therefore less complete than those for *Drosophila* (see Coyne and Orr 1989a, 1997). As I argue below, these problems should not systematically bias the results.

Intrinsic Postzygotic Isolation

Assaying hybrid viability requires the production of F_1 hybrids. Assaying hybrid fertility, however, requires at least two generations of hybrids. Because many Lepidoptera have long generation times (e.g., some are univoltine), many hybridization experiments were taken only to the F_1 generation and hybrid fertility was not scored. I therefore provide two measures of postzygotic isolation: a hybrid inviability index and, for those cases in which both hybrid inviability and sterility were measured, a total postzygotic isolation index. The number of observations is obviously smaller for the second index. I collected hybridization data even between species pairs for which genetic distance data were not available.

Hybrid inviability

I followed the criteria used by Coyne and Orr (1989a, 1997 and Sasa et al. (1998) in assigning an inviability index by scoring the number of hybrid sexes shown to be inviable. For each cross I assigned a value between zero (no hybrid sexes inviable) and two (both hybrid sexes inviable). I then divided this index by two. If data were available for both directions of the cross, I assigned a value ranging from zero (no hybrid sexes inviable in either direction) to four (both hybrid sexes inviable in both directions), then divided by four. Inviability indexes thus range from zero (no inviability) to one (complete inviability). A hybrid sex was considered viable if even a single adult was produced. Evidence of fertilization (e.g., dead embryos) was required to assign an index of one (all hybrid progeny inviable). I was therefore able to distinguish cases of complete inviability from cases of postmating, prezygotic incompatibilities in which, for example, sperm may have been incapacitated prior to fertilization.

Total postzygotic isolation

I constructed a similar index for total postzygotic isolation. Only crosses in which both sexes were assayed for both inviability and sterility were included. For this index, I do not distinguish between sterility and inviability because both prevent gene flow between species. As above, I assigned values ranging from zero (no isolation) to two (complete isolation), then divided by two; if reciprocal cross data existed, I assigned values ranging from zero (no isolation) to four (com-

plete isolation in both directions of cross), then divided by four. Total postzygotic isolation indexes thus also range from zero to one. Inviability was scored exactly as described above. Inferring hybrid sterility required evidence of copulation with an individual known to be fertile (e.g., F_1 female $\times$ parental male). Crosses between F_1 males and females were insufficient to infer sterility because failure of this cross to produce progeny could reflect sterility of either or both hybrid sexes. If, however, an $F_1 \times F_1$ cross produced progeny, both hybrid sexes were deemed fertile. A hybrid sex was considered fertile if it produced even a single adult offspring. As in previous studies (e.g., Throckmorton 1982; Coyne and Orr 1989a; Sasa et al. 1998), the measures of postzygotic isolation used here are conservative, representing a minimum estimate of true isolation.

Haldane's rule

I compiled the number of crosses conforming to or violating Haldane's rule for sterility and inviability. Following previous workers (Coyne and Orr 1989b; Coyne 1992; Wu and Davis 1993; Laurie 1997; Orr 1997), I tally reciprocal crosses separately. For Haldane's rule for inviability, I scored complete cases and quantitative cases. Complete cases are those in which only one hybrid sex survived to adulthood, the other sex being completely absent. If one sex was absent, at least eight surviving adults of the other sex had to survive before being counted as obeying or excepting the rule (i.e., a significant excess of the surviving sex as determined by chi-square test corrected for continuity, with $P < 0.05$; see Sokal and Rohlf 1995). Quantitative cases are those in which both hybrid sexes appear, but one sex is significantly rarer than the other (again using a chi-square test corrected for continuity).

Genetic Distance Data

I also collected genetic distance data from the literature. Whenever possible, I used Nei's allozyme genetic distance, *D* (Nei 1972, 1987), for two reasons. First, this was the measure of divergence most commonly reported for species in the hybridization dataset. Second, this was the measure of divergence used in studies of the time-course of speciation in *Drosophila* (Coyne and Orr 1989a, 1997; Turelli and Begun 1997). All Nei's *D* values were estimated from at least thirteen loci (range: 13–52). Using Nei's *D* thus facilitated comparisons between rates of evolution of postzygotic isolation in Lepidoptera vs. *Drosophila*. Nei's *D* estimates the number of codon differences per gene between pairs of taxa (Nei 1972, 1987). Before saturation, and assuming a molecular clock, *D* should rise nearly linearly with time (Nei 1972, 1987).

Unfortunately, some species pairs with hybridization data lack estimates of Nei's *D*. To maximize the number of crosses included in the analysis, I therefore estimated Nei's *D* values using divergence between DNA sequences taken from Genbank. In Appendix 1, I describe the methods for estimating Nei's *D* by taking advantage of the strong positive correlation between Nei's *D* and DNA sequence divergence. Although not ideal, such an approach seemed better than not including information from many hybridizations (particularly given the strong correlations between the two measures of divergence).

Phylogenetic Correction

To ensure that the observations are phylogenetically and statistically independent, I used Coyne and Orr's (1989a, 1997) modification of Felsenstein's (1985) phylogenetically independent contrasts method. Briefly, if species A and B are sister taxa, with C as an outgroup, then data from the A $\times$ C and B $\times$ C hybridizations are not independent; any isolation common to the two hybridizations may have evolved once in the ancestor of A and B. To correct for this, the genetic distances and hybridization results of nonindependent crosses were averaged to give a single comparison. I used published phylogenies estimated from genetic or molecular data. Phylogenies based on morphology or reproductive isolation were not used. The phylogenetic correction procedure reduced the number of observations for the inviability index and total postzygotic isolation index by about 21% each.

Biogeography and Natural Hybridization

I gathered published information on the geographic distribution of nearly all species in the hybridization dataset and categorized each pair as either sympatric or allopatric. Species pairs whose ranges do not overlap or abut at any point were considered allopatric; all others were considered sympatric. Parapatric species are thus included in the sympatric category.

I also recorded any reports of interspecific hybridization in nature. (I did not, however, try to estimate the frequency of hybridization between individuals of particular species pairs.) These data should be treated with caution because there are several sources of bias and error. For instance, there may be false positive reports—cases in which suspected hybrids were simply aberrant parental forms. This would lead to overestimation of the number of species pairs that hybridize in nature. Three classes of species pair may, however, cause true hybrids to go undetected: (1) species that have received little study in the wild, particularly those from remote locations; (2) species pairs with few or no morphological differences, making morphological detection of hybrids difficult; or (3) species pairs that hybridize infrequently. These factors would lead to underestimation of the number of species pairs that hybridize in nature.

Statistical Analyses

The genetic distance and isolation data are not normally distributed. To test for associations, I therefore used nonparametric Spearman rank correlation. To test for differences between means, I used unpaired *t*-tests with null distributions generated by randomization of the observations. Probabilities were calculated from at least 1000 randomizations of the data. All tests of significance are two-tailed.

RESULTS

I obtained hybridization data for 212 crosses involving 182 species, corresponding genetic distances for 69 species pairs, and geographic data for 205 species pairs (Appendix 2). First,

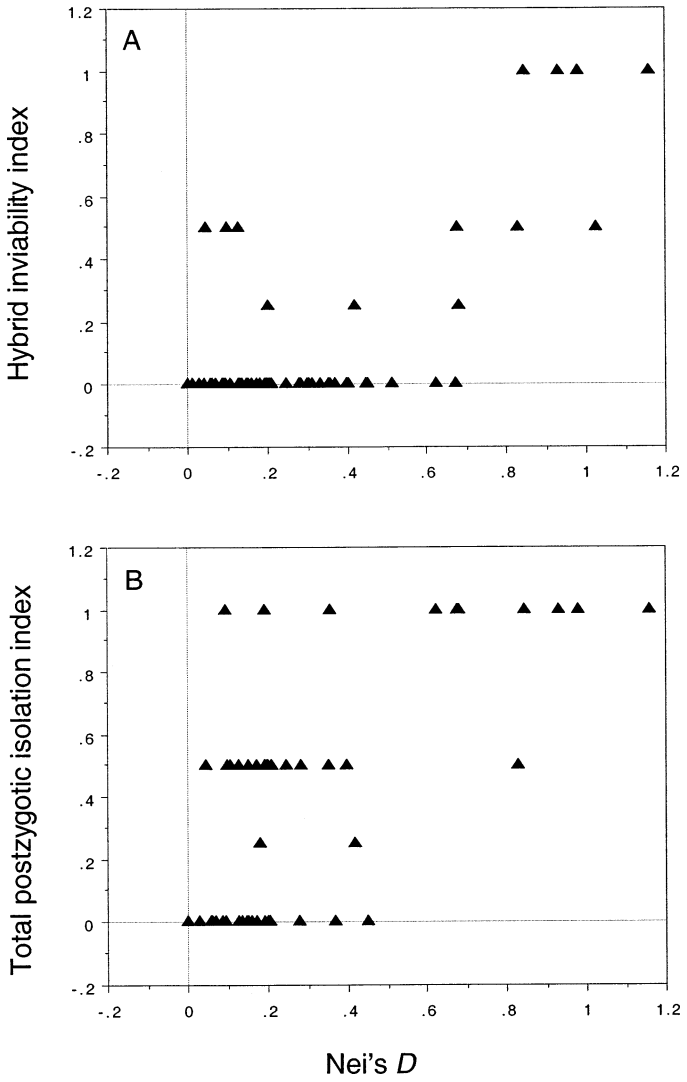

FIG. 1. Strength of (A) hybrid inviability and (B) total intrinsic postzygotic isolation versus genetic distance, Nei's *D* (uncorrected data).

I study the time-course of speciation by intrinsic postzygotic isolation: Does postzygotic isolation evolve gradually in Lepidoptera? I next study Haldane's rule in Lepidoptera in light of the composite theory: How strong is the pattern? What is the waiting time to Haldane's rule for sterility vs. inviability? I then ask if the well-known large-X effect in Lepidoptera extends to intrinsic postzygotic isolation. Last, I estimate the incidence of natural hybridization in Lepidoptera and the frequency with which intrinsic postzygotic problems afflict natural hybrids.

The Time-Course of Postzygotic Isolation

Seventy-seven percent of species pairs in the present dataset show some postzygotic isolation. Figure 1 shows the positive relationships of genetic distance with hybrid inviability (Fig. 1A; phylogenetically corrected $r_s = 0.584$, $N = 53$, $P < 0.0001$) and total postzygotic isolation (Fig. 1B; corrected $r_s = 0.573$, $N = 40$, $P = 0.0003$). A rough speciation clock is evident for both indexes: Isolation accumulates gradually as species diverge. I estimated the mean age of taxa showing complete postzygotic isolation in two ways. First, I simply calculated the mean age of completely isolated taxa: $D = 0.705$ ($N = 9$). Second, I used linear regression to model the increase in postzygotic isolation with genetic distance; then, using the model, I calculated the Nei's *D*-value at which the isolation index = one. The linear model was highly significant (corrected $r^2 = 0.716$, $N = 40$, $P < 0.0001$) and fit the data better than more complex ones. By this method, the age of taxa separated by complete isolation is $D = 0.907$.

TABLE 1. Haldane's rule in Lepidoptera.

	Haldane's rule (females afflicted)	Exceptions (males afflicted)
Sterility	29	1
Inviability		
Quantitative cases	25	2
Complete cases	56	1
Subtotal	81	3

Haldane's Rule in Lepidoptera

Table 1 summarizes the outcome of all hybridizations yielding sex-specific inviability or sterility, including those for which genetic distance data were unavailable. Haldane's rule is very common in Lepidoptera and is obeyed equally well for sterility and inviability ($\chi^2 = 0.218$, $P = 0.640$). Ninety percent of species pairs with intermediate inviability indexes (0.25–0.75) show Haldane's rule for inviability, and 74% with intermediate total postzygotic isolation indexes (0.25–0.75) show Haldane's rule for either inviability or sterility.

To test if species pairs evolve particular forms of isolation at different rates, I estimated waiting times by comparing the age of species pairs falling into the following four groups for both sterility and inviability: (1) neither sex affected; (2) Haldane's rule; (3) exceptions to Haldane's rule; and (4) both sexes affected (note that this classification scheme does not allow phylogenetic correction; see note in Table 2). The mean ages of species pairs showing neither sterility nor inviability were not different from each other (Table 2). In general, species pairs obeying Haldane's rule for sterility and inviability are of young and intermediate age, respectively (Table 2). This finding, along with the ubiquity of female-specific hybrid problems among taxa separated by intermediate levels of isolation, suggests that Haldane's rule may be an early, nearly obligate step in the evolution of complete postzygotic isolation in Lepidoptera (as in *Drosophila*; Coyne and Orr 1989a, 1997).

The evolution of hybrid sterility and inviability appear, however, to follow different time-courses. Taxa suffering Haldane's rule for sterility do not differ in age from those in which both hybrid sexes remain fertile, suggesting that sterility evolves quickly. Taxa suffering Haldane's rule for inviability, however, are significantly older that those in which both hybrid sexes remain viable, suggesting that inviability evolves more slowly than does sterility. This pattern

TABLE 2. Mean genetic distance for stages of postzygotic isolation.*

	Hybrid sterility	Hybrid inviability
Neither sex affected	0.175^{a} (N = 38, SE = 0.018)	0.198^{a} (76, 0.017)
One sex affected		
Haldane's rule	$0.231^{a,b}$ (10, 0.023)	0.629^{b} (5, 0. 145)
Quantitative	—	0.321^{b} (5, 0.103)
Exceptions	0.355	—
Quantitative	—	0.202
Both sexes affected	0.324^{b} (5 0.089)	0.608^{b} (8, 0.159)

* Within each class (hybrid sterility and inviability), D-values with different superscripts are significantly different by two-tailed randomization t-tests. The least significant differences are $P = 0.0378$ and $P = 0.0078$ for hybrid sterility and inviability, respectively. Contrasts involving exceptions to Haldane's rule were not made because these were represented by single observations in each class. This classification scheme is not amenable to phylogenetic correction. To phylogenetically correct continuous variables (e.g., isolation indexes), an average value is taken; but this is not possible for the categorical variables used here (e.g., Haldane's rule, both sexes affected).

is borne out by another, more direct comparison: Haldane's rule for sterility evolves significantly faster than Haldane's rule for inviability (randomization test: uncorrected $P = 0.0012$; corrected $P = 0.0036$).

Three findings appear to weigh against the importance of faster-male evolution in Lepidoptera. First, faster-male evolution should cause hybrid male, not female, sterility to evolve quickly, giving rise to exceptions to Haldane's rule for sterility in Lepidoptera. I find however only a single such exception (Table 1). Indeed, Haldane's rule for sterility is obeyed as well in Lepidoptera, in which faster-male evolution opposes the rule, as it is in *Drosophila*, in which faster-male evolution is purported to be a major cause of the rule (in *Drosophila*: 108 cases, one exception; $\chi^2 = 0.968$, $P = 0.325$).

Second, of 29 crosses showing hybrid female inviability, 18 are cases in which the surviving hybrid males are sterile ($D = 0.677$, $N = 1$), but 11 are cases in which hybrid males remain fertile (mean $D = 0.482 \pm 0.183$, $N = 3$). Thus, in 11 of 29 observations (38%), complete hybrid female inviability evolves before hybrid male sterility. This is difficult to explain if faster-male evolution is a strong force.

Last, the preponderance of cases of Haldane's rule for sterility versus inviability in flies (108 vs. 19) has sometimes been taken as evidence for faster-male evolution (e.g., Wu and Davis 1993; Turelli 1998). At first sight, the opposite appears true in Lepidoptera—there are more cases of inviability (56) than sterility (29)—perhaps suggesting that faster-male evolution opposes the rule for sterility in female-heterogametic taxa (Wu and Davis 1993; Turelli 1998). But this ratio of 56/29 cannot be taken at face value because a strong observational bias exists: The long generation times of some Lepidoptera make it easier to score inviability (requiring one generation) than sterility (requiring two). Indeed, tallying the relative frequencies of the two rules while controlling for the bias yields a different answer. In the present data, hybrid inviability was assayed in 281 crosses, 56 of which obey Haldane's rule (20%). Hybrid sterility was assayed in 111 crosses, 29 of which obey Haldane's rule (26%). Thus Haldane's rule for sterility is, if anything, slightly more common than that for inviability. Although the fraction of cases obeying the rule for sterility in Lepidoptera remains less than that in *Drosophila*, this might simply reflect the fact that Haldane's rule for inviability is weakly obeyed in flies.

These findings—in particular, the accelerated evolution of hybrid female sterility, the lack of exceptions to Haldane's rule for sterility, and the fact that complete hybrid female inviability often evolves before hybrid male sterility—suggest that faster-male evolution is not a predominant force shaping patterns of postzygotic isolation in Lepidoptera.

A Large-X Effect in Lepidoptera?

There is a well-known disproportionately large effect of the Lepidopteran X on morphological and behavioral traits distinguishing closely related species (e.g., color, pheromones; Sperling 1994; Prowell 1998). Before testing whether this apparent large X effect extends to intrinsic postzygotic isolation, I confirmed that Lepidopteran X chromosomes are not atypical in size compared to the autosomes, either physically or genetically. Cytological work shows that Lepidoptera possess, on average, 31 similarly sized chromosomes (Robinson 1971; Traut and Marec 1996). Consistent with this observation, the *Bombyx* linkage map shows that DNA marker densities are homogeneously distributed among chromosomes and not concentrated on the X (Yasukochi 1998; Tan et al. 2001). This suggests that the Lepidopteran X is not atypical in size and represents, conservatively for our purposes, about 3–5% of the genome.

To test for the presence of a large X effect on postzygotic isolation in Lepidoptera, I used a prediction of the dominance theory as a benchmark (Turelli and Orr 1995). Turelli and Begun (1997) showed that *Drosophila* species with large X chromosomes (those in which 40% of the genome is X-linked) evolve Haldane's rule for sterility faster than do *Drosophila* species with small X Chromosomes (20%). The apparent reason is that large-X species suffer roughly twice as many recessive X-linked incompatibilities as small-X species. The dominance theory therefore predicts that, because the Lepidopteran X is much smaller than the *Drosophila* X chromosomes, the waiting time to Haldane's rule for sterility in Lepidoptera, all else being equal, should be much longer than the waiting time in *Drosophila*. (It is important to note that waiting time is measured in units of genetic distance and not absolute time. Although Nei's D correlates with absolute time, there is no guarantee that D increases at identical rates in Lepidoptera and *Drosophila*. The comparisons that follow are nonetheless informative about the average amount of genetic divergence underlying Haldane's rule.) Surprisingly, the mean waiting time to Haldane's rule for sterility in Lepidoptera is similar to that seen in large-X *Drosophila* (randomization test: uncorrected $P = 0.712$; corrected $P = 0.284$) and nearly half that of small-X *Drosophila* (randomization test: uncorrected $P = 0.025$; corrected $P = 0.109$; Fig. 2). Thus, despite having tiny X chromosomes, Lepidoptera show a relatively short waiting time to Haldane's rule for sterility, consistent with a Lepidopteran large-X effect on hybrid fitness problems. Unfortunately, similar comparisons for Hal-

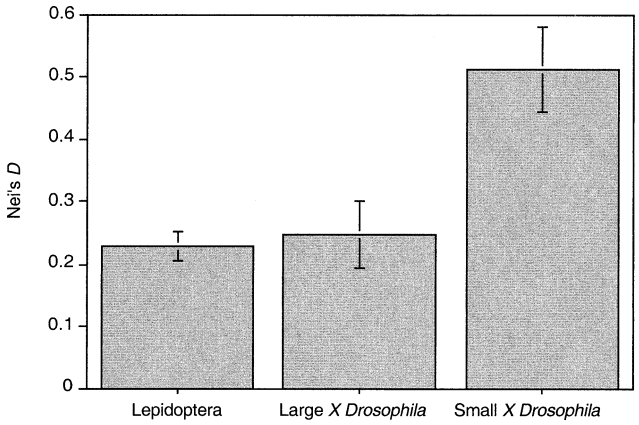

FIG. 2. Comparison of average genetic distance between species pairs whose hybrids suffer Haldane's rule for sterility in Lepidoptera, *Drosophila* with large X chromosomes and *Drosophila* with small X chromosomes.

dane's rule for inviability could not be made because too few cases exist in *Drosophila* that have Nei's *D* estimates.

Natural Hybridization and the Geography of Postzygotic Isolation

The question of the relevance of intrinsic postzygotic isolation to speciation remains. There have been recent suggestions that hybrid sterility and inviability play little or no role in speciation. To begin to assess the importance of postzygotic isolation as a barrier to gene flow, I gathered information on biogeography and the occurrence of natural hybridization among species pairs used in the analyses above.

Hybridization is common among Lepidoptera: 19% of sympatric (15% of all) species in the present dataset hybridize to some degree in nature. A surprisingly large fraction of these naturally hybridizing species (67%) show some intrinsic hybrid fitness problems (i.e., an isolation index > 0). Intrinsic postzygotic isolation does not, however, appear to be a prerequisite for stable coexistence in sympatry because 25% of sympatric species show no intrinsic hybrid fitness problems, at least by the strict criteria used here. It is important to note, however, that these figures are underestimates, possibly serious ones, of the incidence of total postzygotic isolation in nature because the isolation indexes do not include certain classes of postzygotic problems. They do not, for example, include more subtle intrinsic postzygotic problems, such as the behavioral sterility suffered by naturally occurring *Anartia* hybrids (Davies et al. 1997). Nor do they include cases in which hybrids suffer extrinsic postzygotic problems, such as the increased predation probably suffered by naturally occurring hybrids among *Heliconius* warning color races (Mallet and Barton 1989).

Certain biases in the above estimates can be ruled out. There is, for instance, no systematic difference between the mean age of allopatric ($D = 0.301 \pm 0.079$, $N = 16$) and sympatric ($D = 0.301 \pm 0.035$, $N = 52$) species pairs (randomization test: uncorrected $P = 0.591$). Similarly, there is no systematic difference between the mean age of sympatric taxa that produce natural hybrids ($D = 0.249 \pm 0.032$, $N = 19$) and those not known to ($D = 0.331 \pm 0.051$, $N = 33$; randomization test: uncorrected $P = 0.289$). Nor is there a difference in the incidence of postzygotic isolation among allopatric taxa, sympatric taxa that form hybrids, and sympatric taxa with no known hybrids (Table 3, $\chi^2 = 3.455$, $P = 0.178$), as might be expected for similarly aged taxa.

TABLE 3. Natural hybridization and postzygotic isolation.

Species distribution	Natural hybridization	Intrinsic postzygotic isolation		
		−	+	Percent
Sympatric	no hybrids known	19	80	80.8
	hybrids reported	8	16	66.7
Allopatric	no hybrids	11	24	68.6

These findings suggest that natural hybridization is common and that intrinsic postzygotic isolation contributes to reproductive isolation between sympatric Lepidoptera.

DISCUSSION

Five patterns emerge from the present survey: (1) intrinsic postzygotic isolation evolves gradually; (2) Haldane's rule is well obeyed in Lepidoptera; (3) the evolution of sterile, then inviable, hybrid females (i.e., Haldane's rule) appears to be the common pathway toward complete postzygotic isolation; (4) the large X effect on phenotypic differences between Lepidopteran species also appears to extend to intrinsic hybrid fitness problems; and (5) natural hybridization is common among sympatric species and most of these suffer intrinsic postzygotic problems. I discuss each of these conclusions in turn.

Postzygotic Isolation Evolves Gradually

The increase of intrinsic postzygotic isolation with time is consistent with most theories of speciation in which isolation is the by-product of gradual genetic divergence. The only theories not supported are those that predict instantaneous speciation (e.g., genetic revolutions, frequent microbe-induced isolation, polyploid speciation), for two reasons. First, instantaneous speciation should regularly produce very young pairs of taxa showing strong reproductive isolation. There are, however, few such cases. Second, intermediate phases of isolation in Lepidoptera are usually cases of female-specific sterility and inviability, which is not typical of polyploid incompatibilities (which affect both sexes) or cytoplasmic microbe-induced incompatibilities (which preferentially affect males). Instead, the pattern of gradual evolution of postzygotic isolation likely reflects the accumulation of epistatic hybrid incompatibilities.

The most common evolutionary pathway to complete postzygotic isolation in Lepidoptera begins with the early evolution of hybrid female sterility, usually (but not always) followed by hybrid male sterility, then by hybrid female inviability, and finally by the inviability of both sexes. The ubiquity of hybrid female fitness problems shows that Haldane's rule is a common outcome of hybridization between closely-related species of Lepidoptera. Because the evolutionary and genetic causes of Haldane's rule probably differ for inviability and sterility, I discuss them separately.

Haldane's Rule for Inviability

Haldane's rule for inviability is obeyed extraordinarily well in Lepidoptera. Indeed, the rule for inviability is obeyed significantly better in Lepidoptera than in *Drosophila* (Lepidoptera, Table 1; *Drosophila*: 19 cases, four exceptions; $\chi^2 = 6.839$, $P < 0.009$). Here I consider four possible explanations.

First, we can safely rule out the disruption of dosage compensation as a cause of XY hybrid fitness problems because such compensation appears absent in Lepidoptera (Johnson and Turner 1979; Suzuki et al. 1998).

Second, as Sturtevant (1920) pointed out, the absence of one hybrid sex from a species cross could result from either sex-specific inviability or sex transformation. Species crosses that regularly produce sex-transformed hybrids are rare (Dobzhansky 1937). By far the best-known case is Goldschmidt's (1931) classic work with the gypsy moth, *Lymantria dispar*. Crosses between some geographic races produce only hybrid males, half of which, according to Goldschmidt, are sex-transformed females. But this work has more recently been questioned (Clarke and Ford 1980, 1982, 1983; Clarke 1984). Clarke and Ford (1982) repeated Goldschmidt's *L. dispar* crosses and showed that the absence of hybrid females was caused by inviability, not sex-transformation. Thus, the best-known case of hybrid sex transformation appears to be false.

The third explanation therefore seems more likely: Haldane's rule in Lepidoptera, as in other taxa, probably results from lethal hybrid incompatibilities between X-linked and autosomal loci. When hybrid lethals act, on average, as partial recessives, such X-autosome incompatibilities give rise to Haldane's rule (Orr 1993; Turelli and Orr 1995).

Finally, the ubiquity of Haldane's rule for inviability in Lepidoptera may be explained by an additional class of incompatibilities—one that male-heterogametic taxa never experience (Turelli and Orr 2000). Lepidopteran hybrid females inherit their only X chromosome from one species (paternally) and their cytoplasm from the other (maternally). They consequently suffer from any recessive paternal X–maternal incompatibilities. Lepidopteran hybrid males, however, being heterozygous for the X, are protected from such incompatibilities. In contrast, hybrids between male-heterogametic taxa never experience recessive paternal X–cytoplasm incompatibilities: XY males inherit their only X and their cytoplasm from a single species (maternally), whereas XX females suffer no recessive incompatibilities. Recessive paternal X–maternal cytoplasm incompatibilities can therefore contribute to Haldane's rule for inviability only in female-heterogametic taxa.

Haldane's Rule for Sterility

The different causes of Haldane's rule for sterility versus inviability reflect two facts: fertility-essential genes, unlike viability-essential ones, are usually sex specific (Lindsley and Tokuyasu 1980); and sex-related genes often evolve rapidly (Civetta and Singh 1995, 1998; Swanson et al. 2001a,b). Haldane's rule for sterility is thus thought to be a composite phenomenon caused both by the recessivity of incompatibilities (i.e., the dominance theory) and, in male-heterogametic taxa, by the rapid divergence of male-specific fertility factors (i.e., faster-male evolution). Although dominance can explain why heterogametic hybrids, regardless of sex, preferentially suffer sterility, faster-male evolution should work against Haldane's rule in female-heterogametic taxa, such as Lepidoptera. Orr and Turelli (1996) showed, however, that Haldane's rule in female-heterogametic taxa can still result even when hybrid male-steriles outnumber hybrid female-steriles as long as the incompatibilities are sufficiently recessive. The strength of Haldane's rule for sterility in Lepidoptera is therefore consistent with the composite theory.

But several findings suggest that faster-male evolution makes little or no contribution to patterns of postzygotic isolation in Lepidoptera. First, the faster-male theory predicts exceptions to Haldane's rule in female-heterogametic taxa. But there is only a single exception. Second, the faster-male theory predicts that hybrid male sterility, not female, should evolve rapidly. The present findings, however, show that hybrid female sterility evolves very rapidly. Finally, the faster-male theory predicts that, even when hybrid female sterility evolves first, hybrid male sterility should follow soon after. Instead, hybrid females often evolve complete inviability before hybrid males evolve sterility. These findings—and the fact that hybrid female sterility evolves before hybrid female inviability—strongly suggest that the sex-related genes of both sexes diverge faster than viability-essential ones. There is in fact some evidence for this. Protein and DNA sequence data show elevated divergence rates for male- and female-specific genes, with some suggesting only a moderately faster rate for males (Civetta and Singh 1995; Swanson et al. 2001b). One of two conclusions thus follows from these Lepidoptera results: Either there is no faster-male evolution (relative to female), or faster-male evolution has fixed more hybrid male (than female) sterility factors between species, but because these factors are very recessive, hybrid male sterility is rarely expressed in F_1 hybrids (see Orr and Turelli 1996). Ultimately, only high-resolution genetic analyses measuring the density and dominance of male versus female sterility factors in Lepidoptera will resolve the issue.

A composite theory comprising dominance and faster-sex evolution (in which sex-related genes of both sexes evolve rapidly, with perhaps a small bias toward faster divergence of male-specific factors) explains two pervasive patterns in animal speciation: Haldane's rule and the faster evolution of hybrid sterility versus inviability in male- and female-heterogametic taxa (as seen, e.g., in flies, frogs, birds, and Lepidoptera).

A Large-X Effect for Postzygotic Problems in Lepidoptera?

One finding remains puzzling. The dominance theory predicts that Lepidoptera, having much smaller X chromosomes, should require a longer waiting time to heterogametic hybrid sterility than *Drosophila* (see Turelli and Orr 1995; Turelli and Begun 1997), all else being equal. (It is also worth noting that the faster-male theory makes the same prediction, that is, rapid evolution of Haldane's rule for sterility in flies but not in Lepidoptera.) Despite this, Lepidoptera evolve Haldane's rule for sterility as fast as *Drosophila* (if not faster). The most likely reason is that all is else is probably not equal among the two groups. Below I consider two taxon-specific

differences that might explain the disparity: faster-X evolution and Y-linked incompatibilities.

Faster-X evolution has been offered as a plausible explanation of the large-X effect on phenotypic differences between closely related Lepidoptera species (Sperling 1994; Prowell 1998). Briefly, Charlesworth et al. (1987) showed that rates of substitution for X-linked loci will outpace those of autosomal ones as long as new favorable mutations are, on average, partially recessive (i.e., $\bar{h} < 0.5$). The disparity in substitution rates between the X chromosome and autosomes is further enhanced for sex-specific alleles (Charlesworth et al. 1987; Coyne and Orr 1989b), which makes this an attractive theory for the rapid evolution of hybrid sterility. But the faster-X explanation suffers three shortcomings. First, the faster-X process alone cannot cause Haldane's rule; regardless of the rate of X-linked evolution, XX hybrids carry twice as many divergent X-linked genes as XY hybrids (Orr 1997) and Haldane's rule emerges only if X-linked incompatibilities act as partial recessives (Orr 1993; Turelli and Orr 1995), as appears to be the case (see Turelli and Orr 2000; Orr and Presgraves 2000). Second, and more seriously, theory shows conditions for faster-X evolution to be more restrictive in taxa lacking dosage compensation (i.e., $\bar{h} < 0.25$ instead of $\bar{h} < 0.5$; Charlesworth et al. 1987). *Drosophila* (which have compensation) should therefore be more prone to faster-X evolution than Lepidoptera (which lack it). The relatively rapid evolution of heterogametic hybrid sterility in Lepidoptera, however, suggests the opposite. Third, a recent study of sequence divergence at more than 250 loci in *Drosophila* found no evidence for faster-X evolution—rates of substitution at X-linked loci do not differ from those at autosomal ones (A. Betancourt and D. Presgraves, unpubl. ms.). Although this test is from *Drosophila*, it is difficult to imagine that the distribution of dominance effects among new favorable mutations systematically differs between flies and Lepidoptera.

A second taxon-specific explanation therefore seems more likely to explain the Lepidopteran large-X effect for postzygotic problems: Y-linked incompatibilities may evolve earlier between Lepidoptera species than *Drosophila* ones owing to a less degenerate Y. Turelli and Orr (2000) report that the *Drosophila* Y causes hybrid male sterility in 10 of 11 species crosses despite harboring only on the order of a dozen genes (Carvalho et al. 2001). If Lepidopteran Y chromosomes harbor more functional loci, and thus provide more substrate for the evolution of incompatibilities, the waiting time to Haldane's rule for sterility will be reduced. Such Y-linked incompatibilities fit well with the composite theory outlined above. For one, diverging X- and Y-linked loci are particularly susceptible to recessive-recessive incompatibilities (Turelli and Orr 2000). For another, the Y is expected to harbor sex-specific genes (Fisher 1931, 1932), especially fertility-essential ones (e.g., Carvalho et al. 2001), that may evolve rapidly. Unfortunately, little is known about the number of fertility-essential loci on the Lepidopteran Y. But genetic analyses suggest that at least two morphological traits map to the Y suggesting that, at least in some Lepidoptera, the Y retains functional loci (Prowell 1994; Scriber et al. 1996).

In summary, the common patterns of postzygotic isolation between Lepidoptera and flies fit well with the composite theory of Haldane's rule, whereas differences between Lepidoptera and flies are probably best explained by taxon-specific factors.

Natural Hybridization and Postzygotic Isolation

Although intrinsic postzygotic isolation can tell us about the forces shaping functional divergence between species, its relevance to the historical process of speciation remains controversial. Most likely, the role of intrinsic postzygotic isolation in speciation varies among taxa.

For Lepidoptera, 25% of species pairs in the present dataset coexist sympatrically without strong intrinsic postzygotic isolation. This suggests that other barriers to gene exchange are important and, not surprisingly, that intrinsic postzygotic isolation is not necessary for the coexistence of species in sympatry. But there are also many sympatric species that hybridize in nature (~19%) and a large fraction of these (conservatively, ~67%) suffer intrinsic hybrid problems. Intrinsic postzygotic isolation is therefore an active barrier to gene flow between these species. It is difficult to escape the conclusion that intrinsic postzygotic isolation contributes (and not rarely) to the maintenance of the integrity of sympatric species in Lepidoptera. This would seem contrary to recent claims (albeit ones based on studies of relatively few taxa) that intrinsic postzygotic isolation is of little relevance to speciation in Lepidoptera (e.g., Mallet et al. 1998).

Postzygotic barriers, both intrinsic and extrinsic, might also fuel the evolution of prezygotic ones because natural selection strengthens mate preferences that prevent the production unfit hybrids (i.e., reinforcement; Dobzhansky 1937; Howard 1993; Noor 1995, 1999). Coyne and Orr's (1989a, 1997) study of speciation in *Drosophila* showed that prezygotic isolation evolves especially fast in sympatry, a pattern consistent with a history of reinforcement. The importance of reinforcement in Lepidoptera remains unclear. Unfortunately, the present data provide no way of testing how many species pairs that once hybridized in nature no longer do so. A broad survey of prezygotic isolation in Lepidoptera might reveal patterns left by reinforcement, but the heterogeneous nature of the data complicates this task. The present findings do, however, suggest that there is at least ample opportunity for reinforcement in Lepidoptera.

Conclusions

The data presented here suffer several shortcomings and should be interpreted with some caution. First, the Lepidoptera data are less complete than those from *Drosophila*. An ideal analysis would use reciprocal cross data for all species pairs as well as systematic data on prezygotic isolation. The most serious problem, however, is that there is no common genetic distance measure among all species. The approach of estimating some Nei's *D*-values from DNA sequence divergence may introduce some error into the estimates of divergence time, but these would seem to be unsystematic and so should not artificially give rise to the sorts of patterns described here. Eventually DNA sequence data will become available for more species, and the questions addressed here could be revisited. These problems notwithstanding, the last

century's work on speciation and systematics in Lepidoptera has allowed us to infer a rough portrait of the evolution of intrinsic postzygotic isolation.

Coyne and Orr (1989a) concluded their study of *Drosophila* speciation with a call for similar comparative surveys of reproductive isolation from other taxa. Over a decade later, patterns of intrinsic postzygotic isolation can now be compared among *Drosophila*, frogs, birds, and Lepidoptera. The broad patterns characterizing intrinsic postzygotic isolation—its gradual evolution, the ubiquity of Haldane's rule, the rapid evolution of sterility versus inviability—suggest that common themes pervade animal speciation. Nevertheless, some details differ among groups. In birds, for instance, intrinsic hybrid problems appear to evolve slowly (T. Price and M. Bouvier, unpubl. ms.). Because many bird species hybridize in nature (~10%; Grant and Grant 1992), other forms of isolation must explain the maintenance of species' integrity, for instance, prezygotic isolation and extrinsic hybrid problems of sexual or ecological unfitness. In *Drosophila*, however, intrinsic hybrid problems appear to evolve fairly rapidly—as fast as prezygotic barriers in allopatry. And despite the fact that hybridization appears rare among extant *Drosophila* species (Kaneshiro 1990), patterns of prezygotic isolation and DNA sequence data strongly suggest a history of hybridization and reinforcement (Coyne and Orr 1989a, 1997; Wang and Hey 1996; Ballard 2000). In Lepidoptera intrinsic postzygotic problems also appear to evolve rapidly. Moreover, the occurrence of intrinsic postzygotic isolation between naturally hybridizing species suggests that postzygotic barriers may play an important role in the maintenance of species boundaries. It is important to emphasize that the present study underestimates the contribution of postzygotic isolation to speciation because the analysis is conservative and restricted to intrinsic hybrid problems. When considered with extrinsic hybrid problems, the role of postzygotic isolation and the opportunity for reinforcement in Lepidoptera seem considerable.

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Appendix 1

Here I present the methods for estimating Nei's *D* from data on DNA sequence divergence. I first compiled as many estimates of Nei's *D* based on allozymes from Lepidoptera as possible ($N = 362$), including species pairs for which we lack information on postzygotic isolation. DNA sequences from corresponding species pairs were then downloaded from Genbank (accession numbers available upon request). In the end, I relied exclusively on the two most commonly used loci: the mitochondrial gene *cytochrome oxidase I* (*COI*) and the nuclear gene *elongation factor 1-alpha (ef1-α)*. I aligned homologous sequences manually

in SeAl version 1.0a1 (Rambaut 2002) and estimated pairwise HKY sequence-based distances (Hasegawa et al. 1985), which correct for multiple and parallel substitutions, using PAUP* 4.0 beta (Swofford 2001).

Nei's *D* and the HKY distances are strongly correlated for both genes (*COI:* $r_s = 0.831$, $N = 52$, $P < 0.0001$; *ef1-α:* $r_s = 0.715$, $N = 22$, $P = 0.0011$). The best-fit lines relating Nei's *D* to the HKY distances of each gene, forced through the origin, show that DNA sequence divergence is a very good predictor of Nei's *D* (Fig. A1); coefficients of determination (r^2) of the relationships of Nei's *D* with *COI* and with *ef1-α* were both very high (0.866 and 0.930, respectively).

I therefore calculated Nei's *D*-values for those species pairs lacking a direct estimate using the equations of the best-fit lines. If HKY distances were available from both *COI* and *ef1-α*, I simply used the mean of the estimates of Nei's *D* from each. None of the results reported here depend on the choice of gene. Although probability values differ slightly, all significant findings remain so regardless of the gene used.

Of the 69 Nei's *D*-values used in the analysis of postzygotic isolation, 43 (62%) were direct allozyme-based estimates obtained from the literature, and 26 (38%) were statistically estimated from sequence data as described here (six using *COI*; 12 using *ef1-α*; and eight using the mean distance of *COI* and *ef1-α*;).

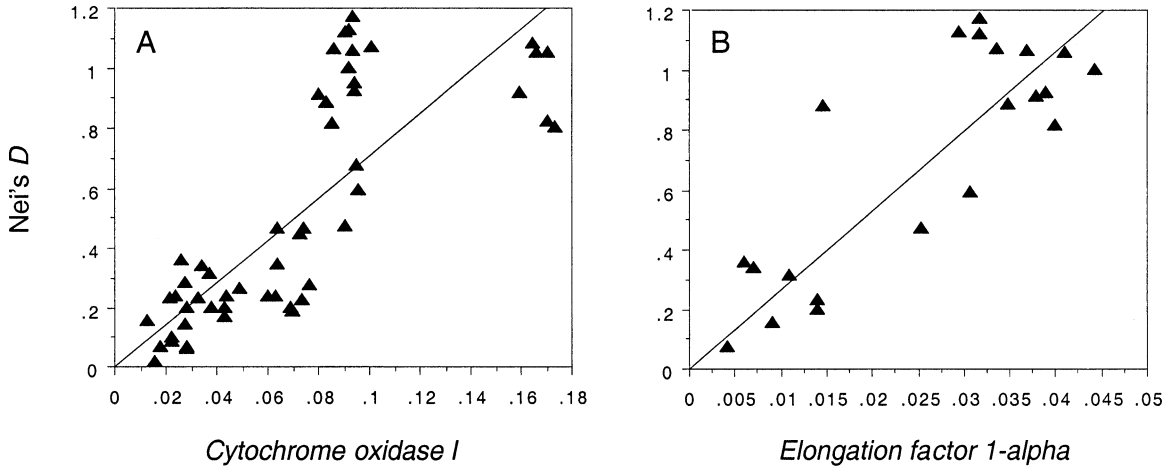

FIG. A1. Nei's allozyme genetic distance, *D*, versus HKY DNA sequence distances from (A) the mitochondrial gene, *COI*, and (B) the nuclear gene, *egf1-α*, among Lepidoptera.

APPENDIX 2.

Summary of literature data on genetic distance, intrinsic postzygotic isolation, biogeography, and natural hybridization.[1]

Family	Species 1	Species 2	Nei's *D*[2]	Inviability index[3]	Total isolation index	Sympatric/ allopatric	Natural hybrids[4]
Geometridae	*Lycia hirtaria*	*Nyssia alpina*		0.500 [2]			
Geometridae	*Lycia hirtaria* (English)	*Nyssia zonaria*		0.250 [2]	0.500 [1]	S	?
Geometridae	*Lycia hirtaria* (English)	*Poecilopsis isabellae*		0.000 [2]	1.000 [1]	S	?
Geometridae	*Lycia hirtaria* (English)	*Poecilopsis pomonaria*		0.000 [2]	0.250 [2]	S	?
Geometridae	*Lycia hirtaria* (English)	*Poecilopsis isabellae*		0.000 [1]		S	?
Geometridae	*Lycia hirtaria* (Scottish)	*Poecilopsis pomonaria*		0.000 [1]	0.500 [1]	S	?
Geometridae	*Lycia hirtaria*	*Poecilopsis lapponaria*		0.000 [2]	1.000 [1]	S	?
Geometridae	*Nyssia alpina*	*Poecilopsis lapponaria*		0.500 [1]			
Geometridae	*Nyssia graecaria*	*Lycia hirtaria*		0.500 [1]	1.000 [1]	S	?
Geometridae	*Nyssia zonaria*	*Poecilopsis isabellae*		0.250 [2]	1.000 [1]	S	?
Geometridae	*Nyssia zonaria*	*Poecilopsis lapponaria*		0.000 [2]		S	?
Geometridae	*Nyssia zonaria*	*Poecilopsis pomonaria*		0.000 [2]	1.000 [1]	S	?
Geometridae	*Nyssia zonaria*	*Poecilopsis rachelae*		0.000 [1]		A	N/A
Geometridae	*Oporabia dilutata*	*Oporabia autumnata*		0.000 [2]	0.250 [2]	S	?
Geometridae	*Oporabia filigrammaria*	*Oporabia dilutata*		1.000 [2]	1.000 [1]	S	?
Geometridae	*Oporabia filigrammaria*	*Oporabia autumnata*		0.000 [2]	0.000 [2]	S	?
Geometridae	*Poecilopsis isabellae*	*Poecilopsis lapponaria*		0.000 [2]	0.250 [2]	S	?
Geometridae	*Poecilopsis isabellae*	*Poecilopsis pomonaria*		0.000 [2]	0.000 [2]	S	?
Geometridae	*Poecilopsis lapponaria*	*Poecilopsis pomonaria*		0.000 [2]	0.250 [2]	S	?
Geometridae	*Selenia bilunaria*	*Selenia tetralunaria*		0.250 [2]		S	?
Geometridae	*Thera obeliscata*	*Thera variata*		0.000 [2]		S	?
Lasiocampidae	*Malacosoma franconica*	*Malacosoma neustria*		0.500 [1]		S	?
Lasiocampidae	*Malacosoma castrensis*	*Malacosoma neustria*		0.000 [1]		S	?
Lasiocampidae	*Phyllodesma tremulifolia*	*Phyllodesma ilicifolia*		0.000 [2]	0.250 [2]	S	?
Lymantriidae	*Orgyia thyellina*	*Orgyia antiqua*		0.000 [1]		S	?
Noctuidae	*Heliothis virescens*	*Heliothis subblexa*	0.194[c]	0.000 [2]	0.000 [2]	S	?
Noctuidae	*Helicoverpa zea*	*Helicoverpa armigera*	0.369[c]	0.000 [2]	0.000 [2]	A	N/A
Noctuidae	*Xanthia fulvago*	*Xanthia ocellaris*		0.000 [1]			
Notodontidae	*Cerura erminea*	*Cerura vinula*		0.000 [1]		S	?
Notodontidae	*Clostera anachoreta*	*Clostera curtula*		0.000 [2]	0.500 [2]	S	?
Notodontidae	*Clostera anachoreta*	*Clostera pigra*		0.000 [1]	0.500 [1]	S	?
Notodontidae	*Clostera apicalis*	*Clostera pigra*		0.000 [2]		A	N/A
Notodontidae	*Clostera curtula*	*Clostera pigra*		0.000 [2]	0.000 [1]	S	?
Notodontidae	*Drepana falcataria*	*Drepana curvatula*		0.250 [2]	0.250 [2]	S	?
Nymphalidae	*Erebia nivalis*	*Erebia cassioides*	0.358[a]	0.000 [1]	1.000 [1]	S	?
Nymphalidae	*Erebia cassioides*	*Erebia calcarius*	0.096[a]	0.000 [1]	1.000 [1]	S	?
Nymphalidae	*Erebia calcarius*	*Erebia tyndarus*	0.147[a]	0.000 [1]	0.000 [1]	A	N/A
Nymphalidae	*Erebia tyndarus*	*Erebia cassioides*	0.147[a]	0.000 [1]	0.000 [1]	S	?
Nymphalidae	*Phyciodes tharos*	*Phyciodes batesii*	0.194[b]	0.000 [2]	0.000 [2]	S	?
Nymphalidae	*Phyciodes tharos*	*Phyciodes campestris*		0.250 [2]	0.250 [2]	S	?
Nymphalidae	*Phyciodes tharos*	*Phyciodes cocyta*	0.095[a]	0.000 [1]	0.000 [1]	S	?
Nymphalidae	*Limenitis archippus*	*Limenitis arthemis*		0.000 [2]	0.500 [1]	S	Y
Nymphalidae	*Limenitis lorquini*	*Limenitis archippus*		0.500 [1]		S	Y
Nymphalidae	*Limenitis weidemeyerii*	*Limenitis archippus*		0.000 [1]		S	Y
Nymphalidae	*Anartia amathea*	*Anartia fatima*	0.200[a]	0.000 [2]	0.000 [2]	S	Y
Nymphalidae	*Heliconius erato*	*Heliconius himera*	0.280[a]	0.000 [2]	0.000 [2]	S	Y
Nymphalidae	*Heliconius cydno*	*Heliconius melponeme*	0.247[b]	0.000 [1]	0.500 [1]	S	Y
Nymphalidae	*Heliconius ismenius*	*Heliconius cydno*	0.396[b]	0.000 [1]	0.500 [1]	S	?
Nymphalidae	*Heliconius pachinus*	*Heliconius cydno*	0.162[b]	0.000 [1]	0.000 [1]	S	Y
Nymphalidae	*Heliconius melpomene* (Panama)	*Heliconius melpomene* (French Guiana)	0.182[b]	0.000 [2]	0.250 [2]	A	N/A
Papilionidae	*Papilio aegeus*	*Papilio macilentus*		0.500 [1]	1.000 [1]	A	N/A

APPENDIX 2. Continued

Family	Species 1	Species 2	Nei's D[2]	Inviability index[3]	Total isolation index	Sympatric/ allopatric	Natural hybrids[4]
Papilionidae	*Papilio alexiares*	Papilio glaucus	0.159[a]	0.000 [2]		S	?
Papilionidae	*Papilio alphenor*	Papilio polytes		0.000 [1]	0.000 [1]	S	?
Papilionidae	*Papilio asterius*	*Papilio machaon*		0.000 [2]		S	?
Papilionidae	*Papilio bairdi brucei*	*Papilio hippocrates*		0.000 [1]		S	?
Papilionidae	*Papilio bairdy brucei*	*Papilio xuthus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio bairdy brucei*	*Papilio polyxenes*		0.250 [2]	0.250 [2]	S	?
Papilionidae	*Papilio bianor*	*Papilio arcturus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio bianor amamiensis*	*Papilio b. dehaanii*		0.000 [1]		A	N/A
Papilionidae	*Papilio bianor dehaanii*	*Papilio b. junia*		0.000 [2]		A	N/A
Papilionidae	*Papilio bianor dehaarti*	*Papilio hippocrates*		0.750 [2]	1.000 [1]	S	?
Papilionidae	*Papilio bianor junia*	*Papilio b. okinawensis*		0.250 [2]		A	N/A
Papilionidae	*Papilio bianor junia*	*Papilio b. takasago*		0.000 [2]		A	N/A
Papilionidae	*Papilio bianor okinawensis*	*Papilio b. amamiensis*		0.000 [1]	0.000 [1]	A	N/A
Papilionidae	*Papilio bianor okinawensis*	*Papilio b. dehaanii*		0.000 [1]	0.500 [1]	A	N/A
Papilionidae	*Papilio bianor takasago*	*Papilio b. dehaanii*		0.000 [1]		A	N/A
Papilionidae	*Papilio bianor takasago*	*Papilio b. okinawensis*		0.500 [1]		A	N/A
Papilionidae	*Papilio bianor tokaraensis*	*Papilio b. dehaanii*		0.000 [1]		A	N/A
Papilionidae	*Papilio bianor dehaanii*	*Papilio maackii*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio brevicauda*	*Papilio machaon*		0.000 [1]	0.500 [1]	A	N/A
Papilionidae	*Papilio brevicauda*	*Papilio polyxenes*		0.000 [1]	0.500 [1]	S	?
Papilionidae	*Papilio canadensis*	*Papilio glaucus*	0.154[a]	0.000 [2]	0.000 [1]	S	Y
Papilionidae	*Papilio constantinus*	*Papilio phorcas*	0.677[d]	0.500 [1]	1.000 [1]	S	Y
Papilionidae	*Papilio demoleus*	*Papilio hippocrates*		1.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio demoleus*	*Papilio maackii*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio demoleus*	*Papilio xuthus*	0.997[d]	1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio eurymedon*	*Papilio glaucus*	0.418[a]	0.250 [2]	0.250 [2]	S	?
Papilionidae	*Papilio eurymedon*	*Papilio rutulus*	0.092[a]	0.000 [2]		S	Y
Papilionidae	*Papilio eurymedon*	*Papilio multicaudatus*	0.403[a]	0.000 [1]		S	?
Papilionidae	*Papilio fuscus*	*Papilio macilentus*		0.500 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio glaucus*	*Papilio cleotus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio glaucus*	*Papilio multicaudatus*	0.334[a]	0.000 [1]		S	?
Papilionidae	*Papilio glaucus*	*Papilio rutulus*	0.355[a]	0.000 [2]		S	Y
Papilionidae	*Papilio glaucus*	*Papilio scamander*	0.511[b]	0.000 [1]		S	?
Papilionidae	*Papilio glaucus*	*Papilio xuthus*	1.161[d]	1.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio gothica*	*Papilio polyxenes*		0.250 [2]		S	?
Papilionidae	*Papilio helenus*	*Papilio aegeus*		0.500 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio helenus*	*Papilio hystaspes*		0.000 [1]	0.500 [1]	S	?
Papilionidae	*Papilio helenus*	*Papilio memnon*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio helenus*	*Papilio nepheles*		0.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio helenus*	*Papilio protenor*		0.500 [1]	1.000 [1]	S	Y
Papilionidae	*Papilio hippocrates*	*Papilio aegeus*		1.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio hippocrates*	*Papilio bianor*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio britannicus*		0.000 [1]		S	?
Papilionidae	*Papilio hippocrates*	*Papilio m. gorganus*		0.000 [1]	0.500 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio maackii*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio macilentus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio polytes*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio polyxenes*		0.000 [1]	0.000 [1]	A	N/A
Papilionidae	*Papilio hippocrates*	*Papilio protenor*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio xuthus*		0.500 [2]	0.750 [2]	S	?
Papilionidae	*Papilio hippocrates*	*Papilio zelicaon*		0.000 [1]	0.500 [1]	A	N/A

APPENDIX 2. Continued.

Family	Species 1	Species 2	Nei's D[2]	Inviability index[3]	Total isolation index	Sympatric/ allopatric	Natural hybrids[4]
Papilionidae	*Papilio maackii*	*Papilio aegeus*		1.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio machaon*	*Papilio asterius*		0.000 [2]		S	?
Papilionidae	*Papilio machaon*	*Papilio polyxenes*	0.353[d]	0.000 [2]	0.500 [2]	S	Y
Papilionidae	*Papilio machaon*	*Papilio zelicaon*	0.193[d]	0.000 [1]	1.000 [1]	S	Y
Papilionidae	*Papilio machaon*	*Papilio hospiton*	0.159[c]	0.000 [1]	0.000 [1]	S	Y
Papilionidae	*Papilio memnon*	*Papilio ascalaphus*		0.500 [1]	0.500 [1]	S	?
Papilionidae	*Papilio memnon*	*Papilio macilentus*		0.500 [1]	1.000 [1]	S	Y
Papilionidae	*Papilio memnon*	*Papilio polymnestor*		0.000 [1]	0.000 [1]	S	Y
Papilionidae	*Papilio memnon*	*Papilio rumanzovia*		0.500 [1]	0.500 [1]	A	N/A
Papilionidae	*Papilio palamedes*	*Papilio scamander*	0.931[d]	1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio paris*	*Papilio bianor*		0.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polyctor*	*Papilio bianor*		0.000 [1]		S	?
Papilionidae	*Papilio polyctor*	*Papilio maackii*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polymnestor*	*Papilio rumanzovia*		0.500 [1]	0.500 [1]	A	N/A
Papilionidae	*Papilio polytes*	*Papilio aegeus*		0.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio polytes*	*Papilio fuscus*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio helenus*		0.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio hipponous*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio ledebouria*		0.000 [1]	0.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio memnon*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio nepheles*		0.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polytes*	*Papilio protenor*		0.500 [1]	1.000 [1]	S	?
Papilionidae	*Papilio polyxenes*	*Papilio m. gorganus*	0.451[c]	0.000 [1]	0.000 [1]	A	N/A
Papilionidae	*Papilio polyxenes*	*Papilio xuthus*	0.826[d]	0.500 [1]	0.500 [1]	A	N/A
Papilionidae	*Papilio polyxenes*	*Papilio zelicaon*	0.202[d]	0.250 [2]	0.500 [1]	S	Y
Papilionidae	*Papilio rutulus*	*Papilio multicaudatus*	0.311[a]	0.000 [1]		S	Y
Papilionidae	*Papilio xuthus*	*Papilio aegeus*		1.000 [1]	1.000 [1]	A	N/A
Papilionidae	*Papilio xuthus*	*Papilio bianor*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio xuthus*	*Papilio helenus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio xuthus*	*Papilio maackii*		0.500 [1]		S	?
Papilionidae	*Papilio xuthus*	*Papilio macilentus*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio xuthus*	*Papilio memnon*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio xuthus*	*Papilio paris*		1.000 [1]	1.000 [1]	S	?
Papilionidae	*Papilio xuthus*	*Papilio protenor*		1.000 [1]	1.000 [1]	S	?
Pieridae	*Anthocharis euphenoides*	*Anthocharis cardamines*	0.301[a]	0.000 [1]		S	?
Pieridae	*Anthocharis cardamines*	*Euchloe ausonia*		0.000 [1]	1.000 [1]	S	?
Pieridae	*Anthocharis cardamines*	*Euchloe simplonia*	1.022[a]	0.500 [1]		S	?
Pieridae	*Colias australis*	*Colias hyale*	0.198[a]	0.000 [1]	0.500 [1]	S	?
Pieridae	*Colias crocea*	*Colias hyale*	0.446[a]	0.000 [1]		S	Y
Pieridae	*Colias erate*	*Colias eurytheme*	0.621[b]	0.000 [1]	1.000 [1]	A	N/A
Pieridae	*Colias eurytheme*	*Colias alexandra*	0.305[a]	0.000 [1]		S	?
Pieridae	*Colias eurytheme*	*Colias philodice*	0.207[a]	0.000 [2]	0.000 [2]	S	Y
Pieridae	*Colias eurytheme*	*Colias interior*		0.000 [1]		S	Y
Pieridae	*Pieris adalwinda*	*Pieris bryoniae*	0.030[a]	0.000 [2]	0.000 [1]	S	?
Pieridae	*Pieris adalwinda*	*Pieris napi*	0.030[a]	0.000 [1]	0.000 [1]	S	?
Pieridae	*Pieris bryoniae*	*Pieris dubiosa*		0.000 [1]		A	N/A
Pieridae	*Pieris bryoniae*	*Pieris napi*	0.000[a]	0.000 [2]	0.000 [1]	S	?
Pieridae	*Pieris callidice*	*Pieris nelsoni*		1.000 [1]	1.000 [1]	A	N/A
Pieridae	*Pieris ergane*	*Pieris napi*	0.371[a]	0.000 [1]	0.000 [1]	S	?
Pieridae	*Pieris flavenscens*	*Pieris bryoniae*		0.000 [1]	0.000 [1]	S	?
Pieridae	*Pieris manni*	*Pieris napi*	0.511[a]	0.000 [1]		S	?

APPENDIX 2. Continued.

Family	Species 1	Species 2	Nei's D[2]	Inviability index[3]	Total isolation index	Sympatric/ allopatric	Natural hybrids[4]
Pieridae	*Pieris manni*	*Pieris rapae*	0.105[a]	0.000 [2]	0.500 [1]	S	?
Pieridae	*Pieris melete*	*Pieris napi*	0.211[a]	0.000 [1]	0.500 [1]	A	N/A
Pieridae	*Pieris napi*	*Pieris dulcinea pseudonapi*		0.000 [1]	0.000 [1]	A	N/A
Pieridae	*Pieris neobryoniae*	*Pieris adalwinda*		0.000 [1]	0.500 [1]		
Pieridae	*Pieris nesis*	*Pieris napi*	0.151[a]	0.000 [1]	0.500 [1]	A	N/A
Pieridae	*Pieris protodice*	*Pontia daplidice*		0.000 [1]		A	N/A
Pieridae	*Pieris rapae*	*Pieris napi*	0.673[a]	0.000 [1]		S	?
Pieridae	*Pontia daplidice*	*Pieris rapae*	0.844[a]	1.000 [1]	1.000 [1]	S	?
Pieridae	*Pontia edusa*	*Pontia daplidice*	0.174[a]	0.000 [1]		A	N/A
Psychidae	*Fumea affinis*	*Fumea nitidella*		0.500 [2]			
Psychidae	*Solenobia thomanni*	*Solenobia generosensis*		0.000 [2]	0.750 [2]		
Rosidae	*Ectropis bistortata*	*Ectropis crepuscularia*		0.000 [2]	0.000 [2]	A	N/A
Saturniidae	*Callosamia angulifera*	*Callosamia securifera*	0.085[c]	0.000 [2]	0.000 [2]	S	?
Saturniidae	*Callosamia angulifera*	*Hyalophora cecropia*	0.680[c]	0.250 [2]	1.000 [1]	S	?
Saturniidae	*Callosamia promethea*	*Callosamia angulifera*	0.042[c]	0.000 [2]		S	?
Saturniidae	*Callosamia securifera*	*Callosamia promethea*	0.130[c]	0.000 [1]		S	?
Saturniidae	*Hyalophora cecropia*	*Hyalophora gloveri*	0.281[c]	0.000 [2]	0.500 [2]	S	Y
Saturniidae	*Hyalophora cercropia*	*Hyalophora nokomis*		0.000 [1]	0.500 [1]	S	Y
Saturniidae	*Hyalophora euryalis*	*Hyalophora cecropia*	0.194[c]	0.000 [1]	0.500 [1]	S	Y
Saturniidae	*Hyalophora euryalis*	*Hyalophora gloveri*	0.173[c]	0.000 [1]	0.500 [1]	S	Y
Saturniidae	*Philosamia ricini*	*Philosamia cynthia*	0.070[a]	0.000 [1]	0.000 [1]		
Saturniidae	*Saturnia mendocino*	*Saturnia walterorum*		0.000 [2]	0.000 [2]	S	Y
Saturniidae	*Saturnia pavonia*	*Saturnia spini*		0.000 [2]	0.500 [2]	S	?
Saturniidae	*Saturnia pavonia*	*Saturnia pyri*		0.000 [1]	0.500 [1]	S	?
Sphingidae	*Amorpha austauti*	*Smerinthus atlanticus*		0.000 [2]		S	?
Sphingidae	*Amorpha austauti*	*Smerinthus ocellata*		0.000 [1]		A	N/A
Sphingidae	*Amorpha populi*	*Smerinthus atlanticus*		0.500 [2]		S	?
Sphingidae	*Amorpha populi*	*Smerinthus ocellata*		0.500 [1]		S	Y
Sphingidae	*Celerio dalhi*	*Celerio galii*		0.500 [1]		S	?
Sphingidae	*Celerio elpenor*	*Celerio livornica*		0.250 [2]		S	?
Sphingidae	*Celerio euphorbiae*	*Celerio gallii*		0.000 [2]	0.000 [1]	S	?
Sphingidae	*Celerio euphorbiae*	*Celerio hippophaes*		0.000 [2]	0.000 [2]	S	?
Sphingidae	*Celerio euphorbiae*	*Celerio lineata*		0.000 [2]	0.000 [1]	A	N/A
Sphingidae	*Celerio euphorbiae*	*Celerio vespertilio*		0.000 [2]	0.000 [2]	S	?
Sphingidae	*Celerio euphorbiae*	*Deilephila elpenor*		0.500 [2]	1.000 [1]	S	?
Sphingidae	*Celerio euphorbiae*	*Deilephila porcellus*		0.250 [2]		S	?
Sphingidae	*Celerio galii*	*Celerio lineata*		0.000 [2]		S	?
Sphingidae	*Celerio galii*	*Celerio livornica*		0.250 [2]		S	?
Sphingidae	*Celerio galii*	*Celerio vespertillo*		0.000 [2]		S	?
Sphingidae	*Celerio galii*	*Deilephila elpenor*		0.250 [2]	1.000 [1]	S	?
Sphingidae	*Celerio hippophaes*	*Celerio galii*		0.000 [1]		S	?
Sphingidae	*Celerio hippophaes*	*Celerio lineata*		0.000 [1]		A	N/A
Sphingidae	*Celerio hippophaes*	*Celerio vespertillo*		0.000 [2]		S	?
Sphingidae	*Celerio hippophaes*	*Deilephila elpenor*		0.500 [2]		S	?
Sphingidae	*Celerio vespertilio*	*Deilephila elpenor*		0.500 [2]		S	?
Sphingidae	*Deilephelia elpenor*	*Celerio lineata*		0.500 [1]		A	N/A
Sphingidae	*Deilephelia elpenor*	*Deilephila porcellus*		0.250 [2]	0.250 [2]	S	Y
Sphingidae	*Deilephila porcellus*	*Celerio galii*		0.000 [1]		S	?
Sphingidae	*Smerinthus ocellata*	*Mimas tiliae*		0.500 [1]		S	?
Tortricidae	*Choristoneura biennis*	*Choristoneura fumiferana*	0.137[a]	0.000 [2]	0.000 [2]	A	N/A
Tortricidae	*Choristoneura fumiferana*	*Choristoneura pinus*	0.057[a]	0.000 [2]	0.000 [2]	A	N/A

APPENDIX 2. Continued.

Family	Species 1	Species 2	Nei's D[2]	Inviability index[3]	Total isolation index	Sympatric/ allopatric	Natural hybrids[4]
Tortricidae	*Choristoneura occidentalis*	*Choristoneura fumiferana*	0.063[a]	0.000 [2]	0.000 [2]	A	N/A
Tortricidae	*Choristoneura occidentalis*	*Choristoneura pinus*	0.013[a]	0.000 [2]		A	N/A
Tortricidae	*Choristoneura occidentalis*	*Choristoneura retiniana*	0.129[a]	0.000 [2]	0.000 [1]	A	N/A
Tortricidae	*Choristoneura retiniana*	*Choristoneura fumiferana*	0.173[a]	0.000 [2]	0.000 [2]	S	?
Tortricidae	*Choristoneura retiniana*	*Choristoneura pinus*	0.128[a]	0.000 [2]		A	N/A
Yponomeutidae	*Yponomeuta malinellus*	*Yponomeuta cagnagellus*	0.126[a]	0.500 [2]	0.500 [2]	S	?
Yponomeutidae	*Yponomeuta cagnagellus*	*Yponomeuta padellus*	0.099[a]	0.500 [2]	0.500 [2]	S	?
Yponomeutidae	*Yponomeuta malinellus*	*Yponomeuta padellus*	0.021[a]	0.500 [2]	0.500 [2]	S	Y

[1] Downloadable list of references is available at http://www.rochester.edu/College/BIO/labs/ORRLAB/ORRHOME.HTML.
[2] Source of Nei's D estimate: a, allozymes; b, mtDNA (*COI*); c, nDNA (*ef1-α*); d, mean of both mtDNA (*COI*) and nDNA (*ef1-α*). See Appendix 1 for further details.
[3] [1], cross in one direction; [2], cross performed in both directions.
[4] N/A, allopatric taxa with no opportunity for natural hybridization; Y, sympatric taxa with reported natural hybrids; ?, sympatric taxa with no known natural hybrids.