Genetic Mapping of Sexual Isolation Between E and Z Pheromone Strains of the European Corn Borer (*Ostrinia nubilalis*)

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ABSTRACT

The E and Z pheromone strains of the European corn borer (ECB) provide an exceptional model system for examining the genetic basis of sexual isolation. Differences at two major genes account for variation in female pheromone production and male behavioral response, components of the pheromone communication system known to be important for mate recognition and mate choice. Strains of ECB are morphologically indistinguishable, and surveys of allozyme and DNA sequence variation have revealed significant allele frequency differences at only a single sex-linked locus, *Tpi*. Here we present a detailed genetic linkage map of ECB using AFLP and microsatellite markers and map the factors responsible for pheromone production (*Pher*) and male response (*Resp*). Our map covers 1697 cM and identifies all 31 linkage groups in ECB. Both *Resp* and *Tpi* map to the Z (sex) chromosome, but the distance between these markers (>20 cM) argues against the hypothesis that patterns of variation at *Tpi* are explained by tight linkage to this "speciation gene." However, we show, through analysis of marker density, that *Tpi* is located in a region of low recombination and suggest that a second Z-linked reproductive barrier could be responsible for the origin and/or persistence of differentiation at *Tpi*.

ALTHOUGH evolutionary biologists and systematists often do not agree on the relative merits of competing species concepts, most students of the speciation *process* adopt the biological species concept (BSC), in which the origin of new species involves the evolution of intrinsic barriers to gene exchange between diverging populations (Coyne and Orr 1998; Harrison 1998). As a consequence, research on the genetic basis for speciation has focused on identification and characterization of the genetic elements that contribute to reproductive barriers. The proximate goal is to define the genetic architecture of the relevant phenotypic traits, that is, to define the number, chromosomal location, and phenotypic effects of genes involved in reproductive isolation (Via and Hawthorne 1998; Orr 2001).

Until very recently, this research agenda was pursued successfully in only a few model organisms and, indeed, most of our knowledge about the genetics of speciation came from detailed studies of hybrid sterility and inviability in Drosophila (Coyne and Orr 1998). A recent review of the evolutionary genetics of speciation (Coyne and Orr 1998) summarized case studies that provided "rigorous" genetic analysis of traits known to cause reproductive isolation. Approximately 75% of the studies used Drosophila as a model system, and 60–70% of those studies focused on postzygotic isolation. Although the genetics of postmating isolation in Drosophila continues to be a major focus for speciation research (*e.g.*, Presgraves *et al.* 2003), premating barriers (*i.e.*, those that derive from behavioral, temporal, or ecological differences) may be the most important components of reproductive isolation in many taxa (Coyne and Orr 1989). Genetic analysis of premating barriers brings added complexity because they are often composed of many context-dependent traits for which individual effects on reproductive isolation are difficult to quantify (Butlin and Ritchie 2001). As a result, studies of the genetics of premating barriers have lagged behind those of postmating barriers. However, the availability of molecular tools facilitates linkage mapping studies in animals and plants of particular interest to speciation researchers and allows identification of genetic factors for ecological, behavioral, or morphological differences associated with (perhaps responsible for) reproductive isolation between closely related species or races (Bradshaw *et al.* 1995; Rieseberg *et al.* 1999; Hawthorne and Via 2001; Peichel *et al.* 2001; Parsons and Shaw 2002).

One of the non-Drosophila model systems mentioned by Coyne and Orr (1998) is the European corn borer (ECB) moth, *Ostrinia nubilalis* (Crambidae). Like many moths, ECB uses a chemical communication system for long-distance mate attraction. Stationary female moths emit a particular pheromone blend that is used by males with the "appropriate" behavioral response for orientation and navigation toward the signaling female. Although in most moths this pheromone system provides species specificity and is invariant within species (Löf-

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stedt 1993), in ECB two pheromone strains exist: one in which females produce and males respond to a 3:97 ratio of E/Z-11-tetradecenyl acetate (Δ11-14:Oac; Z strain) and another in which females produce and males respond to a 99:1 E/Z blend (E strain; Klun *et al.* 1973; Kochansky *et al.* 1975). Both Z and E borers are found in Europe and were introduced into eastern North America early in the 20th century, presumably on shipments of broomcorn (Caffrey and Worthley 1927; Klun *et al.* 1975).

Laboratory analyses of E and Z borers suggest that sexual isolation stems from stereotypic differences in male response to pheromone blend composition (Klun *et al.* 1975; Liebherr and Roelofs 1975; Cardé *et al.* 1978; Glover *et al.* 1991; Linn *et al.* 1997; E. B. Dopman, unpublished results). Z-strain males fly to the 3:97 E/Z pheromone, but not to any other blends (Roelofs *et al.* 1987; Glover *et al.* 1990). In contrast, although most E males respond to the 99:1 E/Z pheromone produced by E females, a substantial proportion fly to intermediate blends and an occasional male is attracted to the Z blend. Thus, in spite of the use of alternative sexual communication systems, cross attraction between moths occurs at a low frequency. Field and laboratory data indicate that hybridization occurs when E males court and mate with Z females (Liebherr and Roelofs 1975; Glover *et al.* 1991). Of 150 females that were analyzed for pheromone production at localities where Z and E populations were sympatric, 13 (8.7%) produced the characteristic 65:35 E/Z hybrid blend (Glover *et al.* 1991). When hybrid males are produced they respond over a broad range of pheromone blends, but rarely to the E blend (Roelofs *et al.* 1987; Glover *et al.* 1990). Hybrid males and females exhibit no obvious reduction in fitness in the laboratory (Liebherr and Roelofs 1975).

Segregation patterns assessed from F_1, F_2, and backcross progeny produced using Z- and E-strain parents indicate that pheromone production and male behavioral response are each determined by single major genes. Pheromone production exhibits autosomal inheritance, whereas male behavioral response is sex linked (Klun and Maini 1979; Roelofs *et al.* 1987; Glover *et al.* 1990). In Lepidoptera, females are the heterogametic sex and are ZW, whereas males are ZZ.

Although differentiated with respect to pheromone communication, the Z and E strains of ECB are otherwise difficult to distinguish (Liebherr 1974), and the taxonomic status of these strains has remained uncertain (Cardé *et al.* 1978). Surveys of allozymes (Harrison and Vawter 1977; Cianchi *et al.* 1980), mitochondrial DNA (Marcon *et al.* 1999), randomly amplified polymorphic DNA (Pornkulwat *et al.* 1998), and a nuclear gene (Willett and Harrison 1999) have revealed significant allele frequency differences at only one locus, the gene encoding the enzyme triose phosphate isomerase (TPI). In upstate New York, E-strain populations are fixed for the *Tpi-1* allele, whereas Z-strain populations are segregating for both *Tpi-1* and *Tpi-2* at intermediate frequencies (Glover *et al.* 1991). Even where they occur together in the same fields, E and Z borers remain differentiated for *Tpi*, suggesting that gene flow is limited or absent for this locus. Because *Tpi* and male pheromone response are both Z linked (Glover *et al.* 1990), the two loci may map close to each other on the Z chromosome. Close physical linkage and/or low recombination between loci could explain the apparent reduction of introgression in spite of ongoing hybridization. Furthermore, a recent selective sweep at the locus for male response could have initially driven differentiation at the *Tpi* locus via genetic hitchhiking.

Here we describe the first genetic linkage map for ECB based on a combination of dominant amplified fragment length polymorphism (AFLP) and codominant microsatellite molecular markers. Because crossing over during oogenesis does not occur in female Lepidoptera, families can be generated in which all markers on the same chromosome cosegregate as a single unit. Conversely, crossing over during spermatogenesis in male Lepidoptera allows families to be generated in which recombinational distance affects the cosegregation of markers on the same chromosome. This "biphasic" nature of crossing over facilitates mapping of major loci underlying phenotypes by using a sequential analysis of female- and male-informative crosses (Heckel *et al.* 1999). We used a female-informative backcross to establish linkage groups for segregating markers and traits (BC1F) and a male-informative backcross to order markers and traits within linkage groups (BC1M). Within the context of this linkage map, we locate the *Tpi* gene and the factors responsible for pheromone blend production and male behavioral response. In addition, marker densities across the Z chromosome are used to estimate changes in local recombination rates.

MATERIALS AND METHODS

Insect populations: Two cultures of ECB, maintained by Wendell Roelofs and colleagues at the New York State Agricultural Experiment Station (NYSAES) in Geneva, New York, were used as sources for initiating mapping families. The first culture consisted of Z-strain insects that were isolated from field-collected larvae, pupae, and adults in corn stubble from Bouckville, New York in April 1994. The second culture consisted of E-strain insects isolated from corn stubble near Geneva, New York in May 1996. We set up 12 F_1 crosses between E females and Z males and chose 1 cross that was segregating at the *Tpi* locus to begin the backcross generation. Backcross families were started using an F_1 female crossed to an E male for BC1F and an F_1 male crossed to an E female for BC1M (Figure 1). All insects were reared under a 16:8 L:D photoperiod at 25°–30° and 50–60% relative humidity.

Marker development: Genomic DNA was extracted from adult moths using a DNeasy tissue kit (QIAGEN, Valencia, CA). AFLP markers (Vos *et al.* 1995) were developed with the restriction enzymes *Eco*RI and *Mse*I (New England Biolabs, Beverly, MA) using the AFLP plant-mapping kit for small ge-

nomes (50–500 Mb) according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). We developed markers for ECB using 12 primer pairs for BC1F, 9 of which were used for BC1M plus 1 additional primer pair not used in BC1F. For the selective amplification, the *Eco*RI primer was fluorescently labeled, allowing detection of fragments on an ABI 377 automated sequencer (Applied Biosystems). Primers generated between 50 and 100 AFLP bands per primer pair, and detected fragments ranged in size from ~50 to ~450 bp. AFLP fragments were sized in GENESCAN (version 3.1; ABI) and segregating markers were visualized with GENOTYPER (version 2.1; ABI).

Sequences containing microsatellites were isolated as described by Hamilton *et al.* (1999), with some modifications. Genomic DNA digestion and linker ligation occurred simultaneously, and these fragments were enriched for microsatellites by hybridization to biotinylated dimeric and tetrameric oligonucleotides. After magnetic capture, double-stranded DNA was recovered by PCR and ligated to pUC 19. Aliquots of this ligation were used to transform *Escherichia coli* (DH5-α) cells. Colonies were plated on Luria agar supplemented with ampicillin, replicated onto nylon membranes, and probed with the same oligonucleotides (now radiolabeled) that were used in the enrichment. Sequences from positive colonies were obtained with universal M13 primers that flank the cloning site. Primers were designed using PRIMERSELECT (DNASTAR, Madison, WI), and unlabeled and 5′ fluorescently labeled primers (HEX, 6-FAM) were ordered from Integrated DNA Technologies (Coralville, IA).

PCR reactions for genotyping individuals contained 1 μl (~10 ng) genomic DNA in a 10-μl total volume that included 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 2 mm $MgCl_2$, 0.2 mm each dNTP, 1.5 pmol each primer, and 0.5 units Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Thermal cycling consisted of a "touchdown" procedure that began at 95° for 50 sec, 67° for 1 min, and 72° for 1 min. The annealing temperature was dropped 2° per cycle for the first 7 cycles (range of temperatures 67°–55°), followed by 28 cycles with an annealing temperature of 52°. Up to six loci were multiplexed per lane on an ABI 377 automated sequencer according to the manufacturer's protocols. Fragments were analyzed identically to AFLPs.

Genotyping moths for *Tpi*: Moths were genotyped for *Tpi* either by characterizing protein mobility on cellulose acetate plates or by use of a diagnostic restriction site difference. For protein electrophoresis, three legs from adult ECB in BC1M were removed prior to genomic DNA extraction, and homogenized leg tissue was applied to cellulose acetate plates for allozyme analysis. Samples were electrophoresed for 13 min at 200 V in Tris/glycine buffer. Agar-overlay stain components (Sigma, St. Louis) for single 76- × 76-mm acetate plates included 3.5 mg dihydroxyacetone phosphate as a substrate together with 2 ml NAD (2 mg/ml), 10 mg $Na_2HA_sO_4$, 130 μl MTT (10 mg/ml), 130 μl PMS (2 mg/ml), 20 μl G3PDH, and 3 ml agar (16 mg/ml). From *Tpi* DNA sequence data (E. B. Dopman, L. Perez, S. M. Bogdanowicz and R. G. Harrison, unpublished results) we identified a diagnostic *Bsr*I restriction site between the *Tpi-1* and *Tpi-2* alleles. Following amplification of genomic DNA using primers that flank the recognition site, the PCR product was digested with *Bsr*I (New England Biolabs) following the manufacturer's protocol.

Female pheromone blend and male behavioral response assays: Adult 1- to 2-day-old females in the BC1F and BC1M mapping populations were prepared for the pheromone blend assay by being placed in 10-ml plastic cups covered with aluminum foil. Following an 18- to 24-hr period in the dark, abdominal tips were exposed and clipped into one drop of HPLC-grade hexane (Sigma). Tips were removed after 1 hr and solutions were stored at −80°. Pheromone samples were concentrated to 1 μl under an N_2 stream before being injected into a HP 5890 GC (for BC1F) or a Shimadzu GC-17A (for BC1M) gas chromatograph, both of which were equipped with 30-m polar EC-Wax columns (Alltech, Deerfield, IL). The running program for the HP 5890 began at 80° for 2 min followed by a ramping rate of 15°/min to 190°, 4°/min to 220°, and ending at 200° for 4 min, whereas for the Shimadzu, running conditions were 40° for 2 min, increased at 15°/min to 250°, and held for 10 min. Under these conditions, females that are E/Z at the production locus exhibit a 65:35 ratio of E/Z Δ11-14:OAc and females that are E/E at this locus exhibit a 99:1 ratio of E/Z Δ11-14:OAc (Roelofs *et al.* 1987).

Male behavioral response for BC1M males was characterized by flying 1- to 2-day-old males to synthetic pheromone blends in a sustained-flight tunnel according to the conditions and protocol of Linn *et al.* (1997). Flight tunnel conditions were 19°, 50–60% relative humidity, 0.5 m/sec air speed, and 11-lx incandescent red-light intensity. Males were taken to the flight tunnel room 1 hr before lights off and placed individually in 12- × 5-cm screen cages for acclimatization. Moths were first assayed with a 99:1 E/Z blend followed by 3:97 blend of E/Z. Pheromones (30 μg) were impregnated on rubber septa placed upwind of males and a behavioral sequence was recorded that included activation, taking flight, orientation in the odor plume, upwind flight at least 10 cm from release point, upwind flight to the midpoint in the tunnel, upwind flight at least 10 cm from the pheromone source, and contact with the pheromone source. Positive responses were those for which the male exhibited upwind flight to within 10 cm of the pheromone source. Under these conditions, all responding control males with the E/E genotype for male response flew to the 99:1 E/Z source, but not to 3:97 E/Z. Conversely, all responding control hybrid males with the E/Z genotype for male response flew to only the 3:97 E/Z blend or to both blends. Thus, test males flying to only the 99:1 E/Z source were E/E at the response locus and test males flying to both or to only the 3:97 E/Z source were E/Z at the response locus.

BC1F—linkage group construction and test of effect on pheromone production: In BC1F, informative markers were those present in the F_1 mother and absent in the recurrent E male parent (Figure 1). Using the labeling scheme developed for use in MAPMAKER/EXP 3.0 (Lander *et al.* 1987), the presence of alleles inherited from the paternal Z grandparent was scored as "H" and absence as "A", whereas the presence and absence of alleles were scored as "A" and "H," respectively, for alleles inherited from the maternal E grandparent. Since pheromone production and male response exhibit simple Mendelian inheritance, traits were similarly coded in BC1F and BC1M. Markers were sorted into linkage groups with a LOD score of 3.0 and a maximum recombinational distance of 40 cM. Because of the absence of crossing over in the F_1 mother, 31 groups of cosegregating markers were expected, each group segregating in a 1:1 ratio. These groups represent different chromosomes inherited from the Z and E grandparents through the F_1 mother, and the contribution of each chromosome to pheromone production was tested using the interaction chi-square statistic at $\alpha = 0.05/31$ where the counts of BC1F females' pheromone blend (99:1 E/Z or 65:35 E/Z) were compared to chromosomal origin (Z or E grandparent).

BC1M—mapping markers and traits within linkage groups: In BC1M, informative markers were those present in the F_1 father and absent in the recurrent E female parent (Figure 1). Segregation ratios (1:1) were tested using a chi-square test and homologous markers from BC1F were noted to identify groups of markers originating from the same chromosome. Linkage was established under an initial score of LOD ≥

3.0 with a maximum distance of 40 cM. For linkage groups possessing <9 markers, the "compare" command was used to order markers and traits within linkage groups and distances were estimated using the Kosambi mapping function. The "suggest subset" command was used to identify equally spaced markers for linkage groups with 10 or more markers and the "build" or "try" commands were used to place additional markers at a threshold of 2.0. Finally, marker order was tested using the "ripple" command. Markers that were not automatically placed because of a low number of recombinants were added to the map under their most likely positions as long as their addition did not result in substantial map extension.

Estimation of recombination rate: For the majority of species in which a physical map is unknown, information about genome-wide recombination patterns can be obtained indirectly. Lyon (1976) suggested that genomic variation in chiasmata would create a nonrandom distribution of marker densities on a genetic map, provided that markers exhibit a random physical distribution. Areas of high recombination would exhibit low marker clustering, whereas in areas of low recombination markers would cluster. Thus, the inverse of marker density on the genetic map provides a proportional estimate of local recombination rate. Using a microsatellite-based genetic map, Lyon's indirect approach correctly identified regions of low recombination in *Drosophila melanogaster* (Nachman and Churchill 1996), and this method has been applied to *Mus musculus* and sea beet (*Beta vulgaris* subsp. *maritima*) using microsatellites and restriction fragment length polymorphism markers, respectively (Nachman and Churchill 1996; Kraft *et al.* 1998).

We compared the number of markers per linkage group with those expected under a Poisson distribution to test for a random physical distribution of AFLP and microsatellite markers among ECB chromosomes. Within each linkage group, the number of markers in a 10-cM bandwidth on the genetic map was compared to that expected under a Poisson distribution to detect significant marker density variation. Density estimates could be inflated due to codominant AFLP markers mapping to the same position; however, codominance would be possible only if markers exhibited complementary segregation and if markers mapping to the same position were derived from the same primers. We checked for these allelic effects and markers that exhibited this pattern were removed from the analysis. Local recombination rates were estimated for the Z chromosome by taking the inverse of the cosine-kernel density function with an 8-cM bandwidth using the R environment (version 1.7-18; ISBN 3-901167-51-X). A reflecting boundary was employed to minimize biased estimates near edges (see Nachman and Churchill 1996), and rates were scaled to those expected with equal-sized chromosomes and a genome size of 500 Mb (the size estimated for the pyralid moth *Galleria melonella*, a close relative of ECB; Gregory 2001).

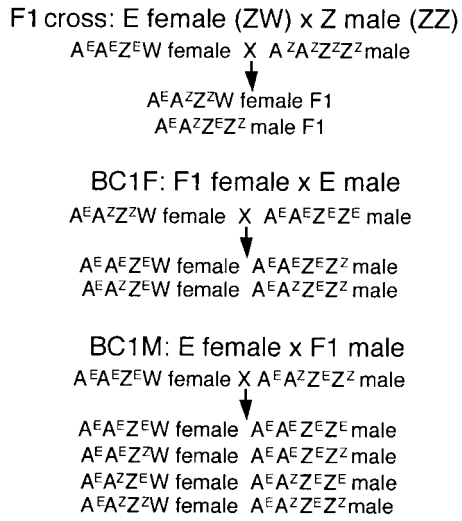


Figure 1.—"Biphasic" mating design (Heckel *et al.* 1999) that establishes linkage group relationships and orders markers within linkage groups using female-informative (BC1F) and male-informative (BC1M) backcrosses, respectively. E and Z superscripts indicate origin of autosomes (A) and sex chromosomes (Z, W) from grandparents in F_1 cross.

RESULTS

BC1F—linkage group construction and test of effect on pheromone production: Fifty-seven female BC1F progeny produced sufficient pheromone for gas chromatographic analysis. Of these, 12 females producing 99:1 E/Z and 13 females producing 65:35 E/Z were chosen as material for genetic analysis. Six BC1F males were also analyzed to identify markers linked to the sex chromosomes. A total of 216 AFLPs and 32 microsatellites segregated in BC1F and, as expected, the majority of markers originated from the paternal Z grandparent because the recurrent parent was from the E strain (Figure 1). Linkage analysis produced groups of cosegregating markers indicating a lack of recombination in female ECB. The number of markers for the 30 groups identified ranged in size from 2 to 26, and the single unlinked AFLP marker was included as group 31 because $n = 31$ for ECB (Guthrie *et al.* 1965). Only linkage group 12 showed a significant interaction between pheromone blend and chromosomal origin after correcting for multiple comparisons ($\alpha = 0.05/31$; Figure 2). Females producing the 99:1 E/Z pheromone derived their chromosome 12 from the E grandparent and females producing the 65:35 E/Z pheromone derived their chromosome 12 from the Z grandparent.

BC1M—mapping markers and traits within linkage groups: In the BC1M mapping family, 81 males showed unambiguous flight behaviors and 38 females produced measurable pheromone. Seventy-nine BC1M progeny were genotyped for AFLP markers (41 males and 38 females), and 38 progeny were genotyped for microsatellites. All males and females were genotyped for *Tpi*. A total of 213 AFLP and 45 microsatellite markers segregated in BC1M. By noting which markers in BC1M showed homology with those used in BC1F, apparently independent linkage groups could be located on the same chromosome, even when they did not sort together under the LOD threshold of 3.0. These fragmented groups coalesced under a higher LOD score of 4.0, producing a total of 31 linkage groups with 2–18 markers per group. The combined AFLP and microsatellite map covered 1697.3 cM with linkage groups ranging from 2.5 cM (group 30) to 127.3 cM (group 1) in

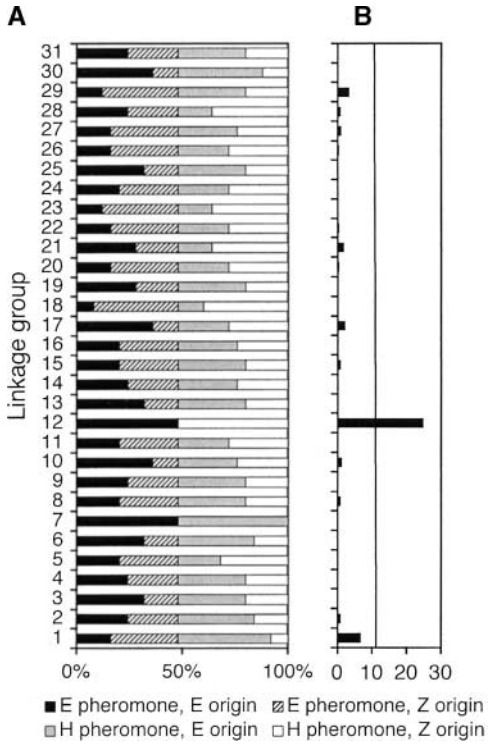


FIGURE 2.—Genomic scan for the factor responsible for pheromone blend production in female ECB. (A) For each linkage group, the proportion of females in BC1F with E (E) or hybrid (H) pheromone blend and chromosome origin. (B) Chi-square tests for the interaction of linkage group origin and pheromone blend. The vertical line indicates the corrected threshold for significance ($\alpha = 0.05/31$).

length. The average interval distance between mapped markers was 8.8 cM.

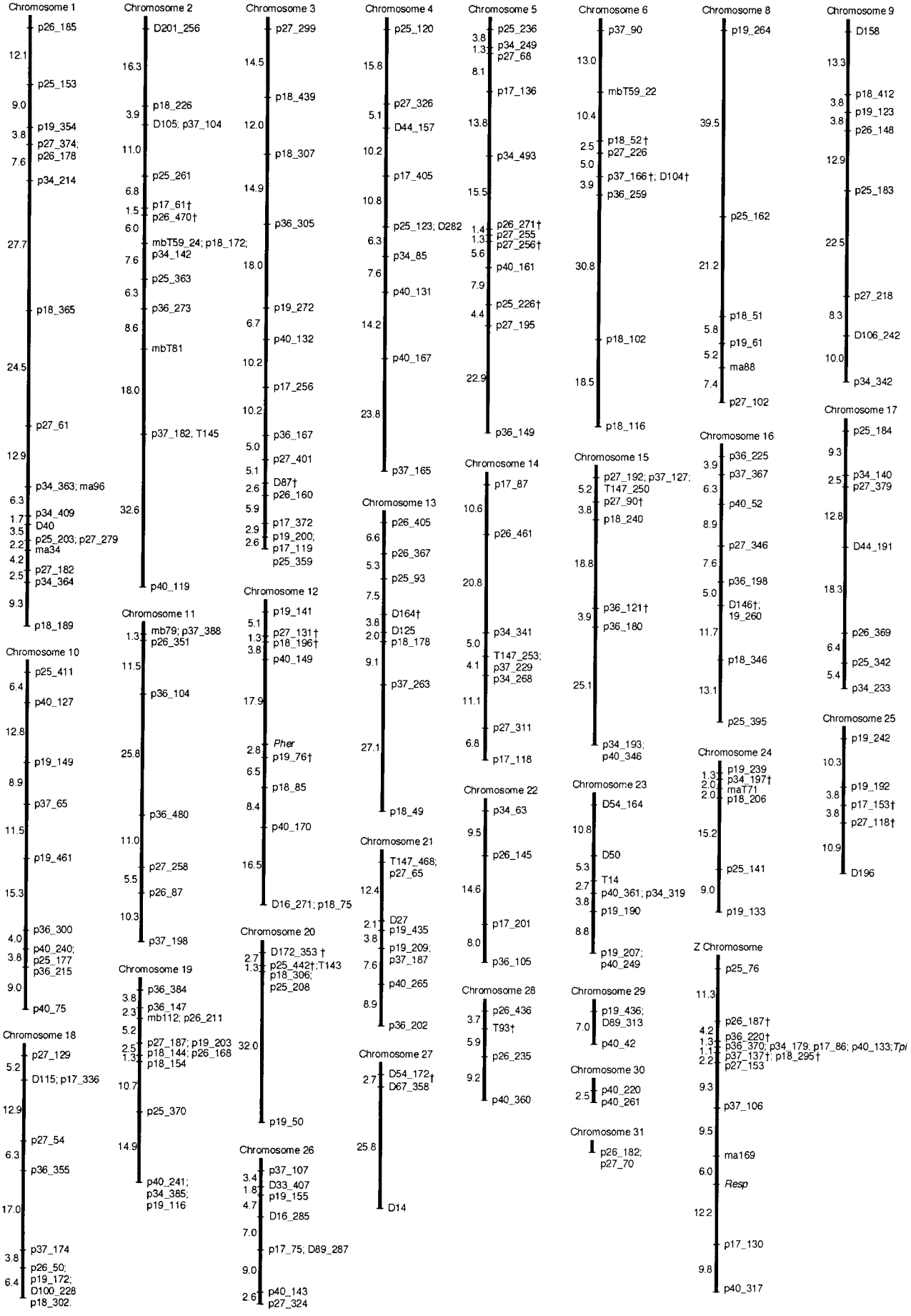
The locus for pheromone blend production (*Pher*) mapped to linkage group 12, 9.3 ± 5.5 cM from AFLP p18_85 and 17.9 ± 6.0 cM from AFLP p40_149 (Figure 3). Six additional AFLPs and one microsatellite were placed on linkage group 12, producing a 62.3-cM map. The gene for male response (*Resp*) mapped to the Z chromosome (linkage group 7), 12.2 ± 4.3 cM away from AFLP p17_130 and 6.0 ± 2.5 cM away from microsatellite ma169. Interestingly, *Tpi* mapped 28.1 ± 4.1 cM from *Resp*. The Z chromosome map consisted of 12 additional AFLP markers for a total map length of 66.9 cM. A cluster of markers on the Z chromosome grouped in the region surrounding *Tpi*, including 4 AFLP markers mapping to the same position as *Tpi* and 2 AFLPs mapping together at a second site.

Estimation of recombination rate: The distributions of both microsatellite ($\chi^2 = 1.51$, 3 d.f., $P = 0.59$) and AFLP ($\chi^2 = 2.58$, 3 d.f., $P = 0.83$) markers among linkage groups were not significantly different from random expectations. In contrast, after correcting for multiple comparisons ($\alpha = 0.05/28$) six linkage groups showed nonrandom marker distributions, including the Z chromosome. Linkage groups 3 ($\chi^2 = 20.72$, 4 d.f., $P = 0.0004$), 10 ($\chi^2 = 27.89$, 4 d.f., $P = 7 \times 10^{-6}$), and 14 ($\chi^2 = 20.9$, 4 d.f., $P = 0.0009$) showed a more even distribution of markers than expected, whereas linkage groups 19 ($\chi^2 = 25.1$, 5 d.f., $P = 2 \times 10^{-5}$), 20 ($\chi^2 = 12.92$, 2 d.f., $P = 0.0016$), and the sex chromosome ($\chi^2 = 37.45$, 5 d.f., $P = 6.9 \times 10^{-7}$), showed a distribution of markers with a significant excess of marker clustering. Other linkage groups may exhibit a random distribution of markers, but a lack of power due to low marker density is likely for some linkage groups. Using the indirect method of LYON (1976), the average recombination rate for the Z chromosome was estimated at 4.2 cM/Mb (2.1 cM/Mb sex-averaged rate) and on the basis of the kernel density estimator, recombination rates were lower near the middle of the chromosome with rates ranging from 2.3 to 5.9 cM/Mb (Figure 4). *Tpi* mapped to the area of reduced recombination, whereas male response mapped to the opposite end of the Z chromosome in an area of higher recombination.

DISCUSSION

One fundamental goal for the study of speciation is to characterize the genetic basis for trait differences that contribute to reproductive isolation among natural populations. A necessary prerequisite is knowledge of the underlying genetic architecture, but unfortunately, for all but a few species this information is lacking (see PARSONS and SHAW 2002). However, although the availability of a dense genetic map can assist in gene isolation, many reproductive barriers have a complex, polygenic basis (COYNE and ORR 1998; RITCHIE and PHILLIPS 1998). Furthermore, unless allele frequencies are known in natural populations, it is unclear whether the phenotypic variance components explained by multiple quantitative trait locus factors in a mapping population reflect an important *biological* contribution to reproductive isolation in natural populations. Because traits that play a central role in reproductive isolation in ECB are determined by single major genetic factors, phenotypic frequencies necessarily reflect genotypic frequencies in natural populations, making this insect an excellent model for studying both the genetics and the functional significance of divergence in insect communication. Here we have used crosses between the two naturally occurring pheromone strains to generate the first linkage map for ECB and to locate the genetic factors that account for variation in female pheromone production and male behavioral response, components of the communication system that are clearly involved in mate recognition and mate choice.

Our linkage map for ECB covers 1697.3 cM across 31 linkage groups with an average interval distance of 8.8 cM (Figure 3). Genome sizes in moths are generally in the 400- to 1000-Mb range (GREGORY 2001), but the closest relative to ECB for which genome size has been determined has a genome size of 500 Mb. Assuming a


Chromosome 1
p26_185
12.1
p25_153
9.0
p19_354
3.8
p27_374;
p26_178
7.6
p34_214
27.7
p18_365
24.5
p27_61
12.9
p34_363; ma96
6.3
p34_409
1.7
D40
3.5
p25_203; p27_279
2.2
ma34
4.2
p27_182
2.5
p34_364
9.3
p18_189
Chromosome 2
D201_256
16.3
p18_226
3.9
D105; p37_104
11.0
p25_261
6.8
p17_61†
1.5
p26_470†
6.0
mbT59_24; p18_172;
p34_142
7.6
p25_363
6.3
p36_273
8.6
mbT81
18.0
p37_182; T145
32.6
p40_119
Chromosome 3
p27_299
14.5
p18_439
12.0
p18_307
14.9
p36_305
18.0
p19_272
6.7
p40_132
10.2
p17_256
10.2
p36_167
5.0
p27_401
5.1
D87†
2.6
p26_160
5.9
p17_372
2.9
p19_200;
2.6
p17_119
p25_359
Chromosome 4
p25_120
15.8
p27_326
5.1
D44_157
10.2
p17_405
10.8
p25_123; D282
6.3
p34_85
7.6
p40_131
14.2
p40_167
23.8
p37_165
Chromosome 5
p25_236
3.8
p34_249
1.3
p27_68
8.1
p17_136
13.8
p34_493
15.5
p26_271†
1.4
p27_255
1.3
p27_256†
5.6
p40_161
7.9
p25_226†
4.4
p27_195
22.9
p36_149
Chromosome 6
p37_90
13.0
mbT59_22
10.4
p18_52†
2.5
p27_226
5.0
p37_166†; D104†
3.9
p36_259
30.8
p18_102
18.5
p18_116
Chromosome 8
p19_264
39.5
p25_162
21.2
p18_51
5.8
p19_61
5.2
ma88
7.4
p27_102
Chromosome 9
D158
13.3
p18_412
3.8
p19_123
3.8
p26_148
12.9
p25_183
22.5
p27_218
8.3
D106_242
10.0
p34_342
Chromosome 10
p25_411
6.4
p40_127
12.8
p19_149
8.9
p37_65
11.5
p19_461
15.3
p36_300
4.0
p40_240;
3.8
p25_177
p36_215
9.0
p40_75
Chromosome 11
mb79; p37_388
1.3
p26_351
11.5
p36_104
25.8
p36_480
11.0
p27_258
5.5
p26_87
10.3
p37_198
Chromosome 12
p19_141
5.1
p27_131†
1.3
p18_196†
3.8
p40_149
17.9
Pher
2.8
p19_76†
6.5
p18_85
8.4
p40_170
16.5
D16_271; p18_75
Chromosome 13
p26_405
6.6
p26_367
5.3
p25_93
7.5
D164†
3.8
D125
2.0
p18_178
9.1
p37_263
27.1
p18_49
Chromosome 14
p17_87
10.6
p26_461
20.8
p34_341
5.0
T147_253;
4.1
p37_229
p34_268
11.1
p27_311
6.8
p17_118
Chromosome 15
p27_192; p37_127;
5.2
T147_250
p27_90†
3.8
p18_240
18.8
p36_121†
3.9
p36_180
25.1
p34_193;
p40_346
Chromosome 16
p36_225
3.9
p37_367
6.3
p40_52
8.9
p27_346
7.6
p36_198
5.0
D146†;
19_260
11.7
p18_346
13.1
p25_395
Chromosome 17
p25_184
9.3
p34_140
2.5
p27_379
12.8
D44_191
18.3
p26_369
6.4
p25_342
5.4
p34_233
Chromosome 18
p27_129
5.2
D115; p17_336
12.9
p27_54
6.3
p36_355
17.0
p37_174
3.8
p26_50;
6.4
p19_172;
D100_228
p18_302.
Chromosome 19
p36_384
3.8
p36_147
2.3
mb112; p26_211
5.2
p27_187; p19_203
2.5
p18_144; p26_168
1.3
p18_154
10.7
p25_370
14.9
p40_241;
p34_385;
p19_116
Chromosome 20
D172_353 †
2.7
p25_442†;T143
1.3
p18_306;
p25_208
32.0
p19_50
Chromosome 21
T147_468;
p27_65
12.4
D27
2.1
p19_435
3.8
p19_209;
p37_187
7.6
p40_265
8.9
p36_202
Chromosome 22
p34_63
9.5
p26_145
14.6
p17_201
8.0
p36_105
Chromosome 23
D54_164
10.8
D50
5.3
T14
2.7
p40_361; p34_319
3.8
p19_190
8.8
p19_207;
p40_249
Chromosome 24
p19_239
1.3
p34_197†
2.0
maT71
2.0
p18_206
15.2
p25_141
9.0
p19_133
Chromosome 25
p19_242
10.3
p19_192
3.8
p17_153†
3.8
p27_118†
10.9
D196
Chromosome 26
p37_107
3.4
D33_407
1.8
p19_155
4.7
D16_285
7.0
p17_75; D89_287
9.0
p40_143
2.6
p27_324
Chromosome 27
D54_172†
2.7
D67_358
25.8
D14
Chromosome 28
p26_436
3.7
T93†
5.9
p26_235
9.2
p40_360
Chromosome 29
p19_436;
7.0
D89_313
p40_42
Chromosome 30
2.5
p40_220
p40_261
Chromosome 31
p26_182;
p27_70
Z Chromosome
p25_76
11.3
p26_187†
4.2
p36_220†
1.3
p36_370; p34_179; p17_86; p40_133; Tpi
1.1
2.2
p37_137†; p18_295†
p27_153
9.3
p37_106
9.5
ma169
6.0
Resp
12.2
p17_130
9.8
p40_317

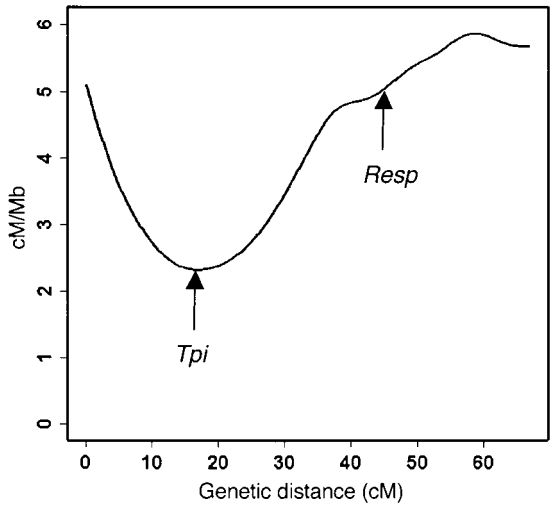


FIGURE 4.—Local recombination rates on the ECB Z chromosome estimated by taking the inverse of the cosine-kernel density function of marker density in an 8-cM window using a reflecting boundary. The map positions of *Tpi* and *Resp* are indicated.

similar genome size for ECB, the relationship between physical and recombinational map distances would be ~290 kb/cM, at the lower end of the range seen in other insects (HUNT and PAGE 1995; GREGORY 2001; PARSONS and SHAW 2002). This figure should decrease, however, as additional markers will extend the map length on those linkage groups with few markers. That we identify the same number of linkage groups as chromosomes can be attributed in part to our sequential female- and male-informative backcross mapping design (Figure 1). This strategy allowed us to recognize homologies between markers assorting as nonrecombining chromosomes in BC1F and segregating markers on recombining chromosomes in BC1M. By establishing homology, we were able to accurately sort markers in BC1M, in which high levels of recombination combined with the large number of chromosomes in ECB might have led us to identify many more linkage groups than there are chromosomes. Given the low-to-moderate marker density common for nonmodel organisms, this can be problematic even when chromosome numbers are not large (PARSONS and SHAW 2002). Thus, our ability to identify 31 linkage groups in an insect with a large haploid number underscores the utility of the biphasic approach (HECKEL *et al.* 1999) for accurate linkage map construction and argues for use of this approach in Lepidoptera and other taxa with differences in crossing over between the sexes.

From the female-informative backcross, we detected one autosomal linkage group (chromosome 12) that contributes significantly to variation in E/Z pheromone blend between the E and Z strains of ECB (Figure 2). Through the male-informative backcross, we were able to map the factor responsible for this variation and to locate AFLP markers within 10 cM (Figure 3). Previous research has shown that the Z (sex) chromosome has a significant effect on variation in male behavioral response between E and Z strains (GLOVER *et al.* 1990) and, as expected, we placed the factor that accounts for differences in male response on the Z chromosome between a microsatellite and an AFLP marker. We assign the names *Pher* and *Resp*, respectively, to the factors responsible for pheromone production and male behavioral response.

Differences in the pheromone blend produced by female ECB are likely due to changes in the specificity of the reactions in which Δ11-14-carbon-precursor acids are reduced and acetylated to produce the E and Z acetates, which are the pheromone components. Both hybrids and "pure" strain ECB females produce an ~70:30 mixture of E/Z precursor acids. Because the acetylation of the alcohol precursors is not selective (ZHU *et al.* 1996) the 99:1 E/Z, 65:35 E/Z, and 3:97 E/Z pheromone blends are likely generated by the differential specificity of alleles at a locus encoding a reductase (ROELOFS *et al.* 1987; ROELOFS and WOLF 1988). Thus, a reductase represents a candidate gene for observed variation in pheromone blend production. A candidate gene for male response is less clear, but considering that males of both strains have the peripheral sensory physiology to detect all three pheromone blends (LINN *et al.* 1999), it seems reasonable to invoke a Z-linked gene encoding a protein involved in central nervous system processing of alternative pheromone input.

The *Tpi* locus is the only marker assayed thus far that exhibits significant differences in allele frequencies between the pheromone strains, and these differences persist even where E and Z borers are sympatric (GLOVER *et al.* 1991). Initial divergence between the E and Z strains at *Tpi* might have been the result of a selective sweep at the locus itself or at a closely linked locus, but persistence of allele frequency differences in sympatry requires linkage to a factor or factors causing reduced hybrid fitness and/or assortative mating. Selective sweeps eliminate shared ancestral polymorphism and selection against hybrids eliminates introgressed genetic variation upon secondary contact (TING *et al.* 2000). In both cases, the region of reduced genetic variation is expected to be larger in regions of low recombination (*e.g.*, AQUADRO 1997). Indeed, local recombination rates have been

FIGURE 3.—Genetic linkage map for ECB showing position of factors responsible for male behavioral response (*Resp*), female pheromone blend (*Pher*), and *Tpi*. AFLP markers are labeled p*XX_XX* indicating primer combination and allele size, respectively. All other markers are microsatellites. Dagger denotes positions ordered at LOD < 2.0 (see MATERIALS AND METHODS); all other positions are ordered at LOD ≥ 2.0.

found to positively correlate with levels of genetic variation in *D. melanogaster* and in other species (Aquadro 1997; Kraft *et al.* 1998). In accord with these findings, our indirect method of estimating recombination rate using marker densities showed a 45% reduction from the average recombination rate on the Z chromosome in the area adjacent to *Tpi* (Figure 4). Although limited by its assumption of a random physical distribution of markers, this indirect approach provides a means to compare the relative differences in local recombination rates across the genome (Nachman and Churchill 1996). Indeed, *Tpi* maps to a region that has the lowest estimated recombination rate (highest marker clustering) for the entire ECB genetic map and, therefore, may be more susceptible to selection across a broader genomic region.

Prior to this study, selection at the male response locus seemed a plausible explanation for the origin and maintenance of differentiation at *Tpi* because both are Z linked. Scenarios could be proposed involving either a selective sweep of alleles for male response or incompatibilities due to hybrid males having reduced success in obtaining mates. However, the map distance of 28.1 ± 4.1 cM between *Resp* and *Tpi* (Figure 3) makes it unlikely that *Tpi* allele frequencies have been influenced via hitchhiking associated with selection at the *Resp* locus. Furthermore, the persistence of linkage disequilibrium between *Resp* and *Tpi* in sympatric populations of E and Z strains becomes more difficult to explain. One possibility is that *Resp* does have an influence on *Tpi*, but through its effects on assortative mating. Limited mating between E and Z borers would cause any initial linkage disequilibrium to decay slowly between *Resp* and *Tpi*. A second explanation is that strong premating isolation and/or substantially reduced F_1 hybrid fitness impedes all gene flow between pheromone strains; such a strong barrier would maintain linkage disequilibrium indefinitely. Yet another possibility is that a second reproductive barrier on the Z chromosome is limiting gene flow at *Tpi*, possibly in addition to the effects of *Resp*.

Postdiapause development (PDD) describes the time it takes for overwintering larvae to pupate under diapause-breaking conditions. In upstate New York, populations of ECB exhibit either one or two generations per year as a consequence of individual differences in PDD (Glover *et al.* 1992). The shorter PDD exhibited by bivoltine moths translates into an early first generation and a late second generation. Univoltine moths tend to fly in the time window between the flight periods of these two generations. At the Geneva, New York site, where gene flow has been monitored, E-strain moths are bivoltine and Z-strain moths are univoltine. Thus at this site, differences in PDD result in temporal isolation of the two pheromone strains. Furthermore, PDD has been shown through its association with *Tpi* genotype to be linked to *Tpi* and therefore must be determined by a major gene (or cluster of genes) on the Z chromosome (Glover *et al.* 1992). If selection on PDD has promoted differentiation at *Tpi* (*e.g.*, through a selective sweep or reduced hybrid fitness) and/or is responsible for the maintenance of differentiation (*e.g.*, through temporal isolation or hybrid incompatibilities), then this factor should map much closer to *Tpi* than to *Resp*. Moreover, if patterns of genetic differentiation in ECB are truly a function of linkage to potential "speciation" genes like *PDD*, *Resp*, and *Pher*, then markers that are farther away on the Z chromosome should exhibit less differentiation.

Beyond the understanding of the genetics of mating signals and responses provided by comparisons of the ECB strains, broader comparisons between ECB and other Ostrinia species should be informative about the evolution of sexual communication in the genus. The adzuki bean borer (*O. scapulalis*), a congener of ECB, expresses striking similarity in its pheromone blend variation to ECB strains (Huang *et al.* 2002) and the genes responsible for pheromone blend production and male response in the adzuki bean borer may map to the same location as *Resp* and *Pher* in ECB. This would be a significant finding because although parallel speciation, defined as the independent evolution of the "same" species, has been suggested (Schluter and Nagel 1995), cases of parallel speciation are strengthened if there is evidence for equivalent change in the same "speciation" genes. If comparisons of the ECB linkage map and a corresponding map for adzuki bean borer confirm such a relationship, support would be provided for the evolution of parallel reproductive barriers between incipient species (pheromone strains).

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